Micromethodology for the characterization of hemoglobin variants

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**MICROMETHODOLOGY FOR THE CHARACTERIZATION
OF HEMOGLOBIN VARIANTS**

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Rijksuniversiteit Limburg te Maastricht,
op gezag van de Rector Magnificus, Prof. Dr. H. Philipsen,
volgens het besluit van het College van Dekanen,
in het openbaar te verdedigen
op donderdag, 28 april 1994 om 14.00 uur

door

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geboren te Skopje op 21 mei 1963

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To my parents with all my love

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ABBREVIATIONS

AE	aminoethylated
ASO	allele specific oligonucleotide
bp	base pair(s)
BSA	bovine serum albumin
CCl ₄	carbontetrachloride
CD(s)	codon(s)
CF	cystic fibrosis
CM-	carboxymethyl-
CO ₂	carbondioxide
DABITC	4-NN-dimethylaminoazobenzene-4-isothiocyanate
dATP	deoxy adenosine triphosphate
ddATP	dideoxy adenosine triphosphate
ddCTP	dideoxy cytosine triphosphate
ddGTP	dideoxy guanosine triphosphate
ddTTP	dideoxy thymidine triphosphate
DEAE-	diethylaminoethyl-
dGTP	deoxy guanosine triphosphate
DNA	deoxyribonucleic acid
DPG	(2,3)-diphosphoglycerate
DTT	dithiothreitol
dTTP	deoxy thymidine triphosphate
EDTA	ethylene diamine tetra acetatedisodium
FSC	frameshift
Hb(s)	hemoglobin(s)
HPFH	hereditary persistence of fetal Hb
HPLC	high performance liquid chromatography
HS	hypersensitive site(s)
IEF	isoelectrofocusing
IVS	intervening sequence
kb	kilobase(s)
LCR	locus control region
MCH	mean corpuscular Hb
MCHC	mean corpuscular Hb concentration
MCV	mean corpuscular volume
MOPS	(3[N-morpholino]propanesulfonic acid)
mRNA	messenger ribonucleic acid
O ₂	oxygen
nt(s)	nucleotide(s)
PAGE	polyacrylamide gel electrophoresis
PCMB	p-chloromercuribenzoate
PCR	polymerase chain reaction
PCV	packed cell volume
PITC	phenylisothiocyanate
poly A	polyadenylation
PTC	phenylthiocarbamyl
PVP	polyvinylpyrrolidone
RBC(s)	red blood cell(s)
RET(s)	retention time(s)
SDS	sodium dodecylsulphate

SS	sickle cell anemia
SSC	sodium saline citrate buffer
SSPE	sodium saline phosphate EDTA buffer
TMAC	tetramethyl ammonium chloride
TBE	Tris borate EDTA buffer
TE	Tris EDTA buffer
TEA	Tris EDTA acetate buffer
TFA	trifluoroacetic acid
thal	thalassemia
T _m	melting temperature
TMB	5,5'-tetramethylbenzidine
UTR(s)	untranslated region(s)

CHAPTER 1

INTRODUCTION AND REVIEW OF THE LITERATURE

CHAPTER 1

INTRODUCTION

Hb has played a historic role in many scientific disciplines such as chemistry, biology, genetics, and medicine. The important functional role of Hb and the relative ease of isolating it in pure form have led to an early characterization, much earlier than that of any other human protein. The amino acid sequences of the globin subunits were among the first to be determined. The three-dimensional structure of Hb has also been determined in detail. The functional properties of the Hb molecule, the factors regulating its activity, and the structural features that underlie its functional behavior, are also well understood. The Hb molecule has served as a model for the studies of the structure, and structure-function relationships of proteins in general.

The numerous naturally occurring mutations in the globin genes have made Hb a model for studies of the molecular basis of genetic diseases and of gene expression. The mutations in the various globin genes are caused by a variety of mechanisms, including substitutions of one or more nt in the DNA, nt deletions or insertions, and various forms of genetic recombinations. Some of these mutations alter the structure of the globin, thus producing different abnormal gene products or abnormal Hbs, while others decrease the rate of synthesis of the globin chains, leading to the syndromes known as thalassemias.

Since 1949, when Hb S was found to be the cause of sickle cell anemia, the number of Hb variants has increased to more than 600. The clinical and hematological consequences have in about one-third of all cases been of sufficient severity to bring the affected individuals to medical attention; this has promoted the detection and identification of some of these abnormalities. In addition, numerous population surveys have been conducted in many areas of the world, resulting in the identification of a large number of rare and clinically silent Hb variants. They have also yielded valuable data about some of the more common Hb abnormalities.

Much progress has been made in the studies of the Hb variants, mainly because of technological advances that have provided more sensitive methods for their detection, separation, and characterization. Electrophoretic techniques have undoubtedly played the most important role, and these are still the most common methods used for the initial detection of abnormal Hbs. Chromatographic procedures that permit the separation and isolation of mutant Hbs and of individual globin chains have played an important role in the structural analyses of Hb variants. Still, both electrophoretic and classical chromatographic techniques fail to separate many abnormal Hbs. The development of HPLC has provided a new research tool for the detection of Hb variants and for the separation and isolation of abnormal globin chains and peptides. The HPLC techniques have the advantage of being fast, reproducible, and sensitive with a high resolving power. Recently, the techniques of recombinant DNA technology, widely used for delineating the molecular pathology of the thalassemia syndromes, have

been applied to identifying structurally different Hb types. The sequence analysis of amplified DNA has been shown to be useful in the characterization of the Hb variants, especially of those that are present in low amounts. In some instances, the identification at DNA level is necessary to confirm and/or explain the molecular mechanism responsible for the occurrence of some variants.

The main goal of the studies reported in this dissertation is to demonstrate the usefulness of the HPLC techniques and DNA methods for the characterization of rare Hb variants, and to contribute to our understanding of molecular mechanisms that are responsible for the occurrence of some unusual Hb abnormalities.

NORMAL AND ABNORMAL HUMAN HBS: A REVIEW OF THE NORMAL HUMAN HBS

Hb Structure and Function. The human Hb molecule is a tetramer with a molecular weight of about 64,000. It is composed of two pairs of two structurally different polypeptide chains. Each of these globin chains contains a heme group that is protoporphyrin IX complexed with an iron atom. Several Hb types are synthesized in human red cells during the different stages of development. The Hbs Gower-I ($\zeta 2\epsilon 2$), Gower-II ($\alpha 2\epsilon 2$), Portland-I ($\zeta 2\gamma 2$), Portland-II ($\zeta 2\beta 2$), and Portland-III ($\zeta 2\delta 2$) are found in the early embryonic period; Hb F is the major Hb component during fetal life and in newborns, while Hb A is major, and Hb A₂ as minor Hb component, are present in adults (1).

The amino acid sequences of all normal human globin chains have been determined (2-7). The α -like chains (α and ζ) contain 141, and the non- α or β -like chains (β , δ , γ , and ϵ) 146 amino acid residues. The ζ chain is similar to the α chain, differing in 57 of its 141 residues, while the ϵ , γ , and δ chains resemble the β chain, differing in 36, 39, and 10 positions, respectively.

Although the α and non- α chains differ considerably in their primary structure, they have similar secondary and tertiary structures (8). About 75% of the globin chain is in the shape of a right-handed α -helix (9). The α chain has eight helical segments, designated A through H. The β chain contains seven helices, closely comparable to those of the α chain, except for the D helix which is absent. The α -helix is interrupted by non-helical segments that allow folding. Proline is the usual participant of the non-helical regions since it is unable to participate in the α -helix, except as one of the first three residues from the N-terminal end (10). The globin chains are folded in such a way that amino acids with predominantly hydrophilic polar side groups are located on the exterior surface, while amino acid residues with almost exclusively non-polar, hydrophobic groups are buried within the globin subunit away from water (10). Each chain has some invariant amino acid residues that are homologous in the comparable helices of the α and β chains; for instance, the proximal histidine at helix F8 ($\alpha 87$, $\beta 92$) and the distal histidine at helix E7 ($\alpha 58$, $\beta 63$), and tyrosine at helix H2 ($\alpha 140$, $\beta 145$) (11).

The heme iron is inserted in a cleft formed by the E and F helices of the globin chain and is covalently linked to the imidazole nitrogen of the proximal F8 histidine. In addition, a large number of relatively weak hydrophobic bonds are formed between the heme ring and about 20 side chains of the globin, that stabilize the heme group and the hydrophobic interior of the heme pocket (12). Thus, the heme iron is situated in a non-polar, hydrophobic environment, that facilitates the iron's ability to combine reversibly with molecular O_2 without being oxidized.

The quaternary structure of the Hb molecule was determined by Perutz (13); it changes markedly in going from the oxy (R or relaxed) to the deoxy (T or tense) form. The subunits of Hb interact with each other by means of relatively weak, noncovalent, bonds such as van der Waals forces and hydrogen bonds. There are two types of contacts between unlike subunits that are called the $\alpha 1 \beta 1$ ($\alpha 2 \beta 2$) and $\alpha 1 \beta 2$ ($\alpha 2 \beta 1$) contacts (14). The $\alpha 1 \beta 1$ interface involves 40 contacts between residues, including nine hydrogen bonds. Since the $\alpha 1 \beta 1$ interface is extensive and rather rigid, and remains relatively fixed during oxygenation, the contacts between the $\alpha 1$ and $\beta 1$ subunits are identical in oxyHb and deoxyHb. The $\alpha 1 \beta 2$ interface however, undergoes a considerable shift in position during oxygenation. The primary sites for contact are the FG corner (i.e. the non-helical segment between the F and G helices) and the C helix of one subunit with complementary (C and FG) regions of the other subunit. Many of the $\alpha 1 \beta 2$ bonds that are present in the oxy molecular state are broken with the shift to the deoxy conformation, and a new set of bonds is formed. In deoxyHb there are about 40 contacts, including 19 hydrogen bonds. Upon oxygenation, the total number of contacts increases to 22, including 12 hydrogen bonds. The dissociation of the oxyHb under physiological conditions into identical dimers occurs at the $\alpha 1 \beta 2$ interface, while monomer formation takes place at the $\alpha 1 \beta 1$ interface, but only at pH values below 5 and above 11 (15).

DeoxyHb is stabilized by a number of inter- and intrasubunit salt bonds that are absent or very much weaker in oxyHb. The C-terminal residues of both α and β chains ($\alpha 141$ Arg and $\beta 146$ His) are particularly involved in intersubunit salt bridges (16). In contrast to the numerous contacts between unlike chains, contacts between similar chains ($\alpha \alpha 2$ and $\beta \beta 2$) are minimal (16). Like chains are separated by an internal cavity lined by polar amino acids. The two β chains are widely separated in the deoxy conformation, which makes it possible to accommodate a molecule of the allosteric effector 2,3-DPG (17). In the oxy conformation, the gap is closed when the β chains are moving together. The channel between the two chains was found to form a receptor site for certain drugs (18).

The primary functional role of Hb is the transport of O_2 from the lungs to the body tissues and that of CO_2 in the reverse direction. The O_2 binds reversibly to the heme iron of each of the four Hb subunits. As each O_2 molecule binds to the Hb molecule, the affinity of Hb for the next O_2 molecule increases. The important consequence of this cooperation (heme-heme interaction) is that a small decrease in the O_2 tension, such as that between the lung and tissue capillaries, results in the release of a sufficient amount of O_2 from the blood (15).

The cooperativity of Hb, proposed by Perutz, arises from an equilibrium between the oxy and deoxy quaternary structure (13). In the oxy conformation, the iron atoms of the heme groups lie nearly within the plane of the heme group. After O_2 is removed, the heme iron electrons are redistributed, and the iron atom is displaced from the plane of the heme ring. This shift in the position of the iron atom is transmitted through its bond to the histidine residue at position F8, thus initiating a succession of changes in the quaternary structure of the Hb molecule. The direct mechanical system of transmission from the iron atoms to the salt bridges, proposed by Perutz (13), was shown to be incorrect with the Shaanan's X-ray analysis of the structure of human oxyHb at 2.1 Å resolution (19). Molecules are more likely to jump across the energy barriers between two alternative structures (T and R), possibly by several alternative pathways (20).

The efficiency of the O_2 transport can be determined by measuring the O_2 equilibrium curve of Hb and its P_{50} value. The O_2 equilibrium curve of blood or Hb solution is characteristically sigmoid-shaped. The P_{50} represents the O_2 tension at which the Hb molecule is half-saturated with O_2 . The P_{50} for human blood at physiological pH (7.4), temperature (37°C), 2,3-DPG concentration (5 mM), and $p\text{CO}_2$ (5.3 kPa) is normally 26 ± 1 mm Hg. The P_{50} is inversely related to the O_2 affinity. Several factors can affect the O_2 affinity of Hb such as: Temperature, pH, CO_2 , 2,3-DPG binding, and chloride ions (15).

The O_2 affinity decreases with an increase in temperature. The P_{50} of normal human blood is almost doubled by an increase in temperature from 20°C to 30°C (21). This effect is physiologically important; during fever, metabolism is increased dramatically and an increase in the delivery of O_2 is required. Conversely, cold induces a decrease in metabolic requirements, and a reduced need for O_2 (22).

The O_2 affinity of Hb is reduced by increasing amounts of CO_2 , primarily due to a reduction in pH (23). Under physiological conditions the P_{50} of Hb varies inversely with the pH (alkaline Bohr effect). The alkaline Bohr effect is physiologically important; the O_2 affinity of Hb in the tissues, where the (H^+) concentration is increased, will be decreased, thus promoting the unloading of O_2 from the tissues. Similarly, when CO_2 is expelled through the lungs, there is a corresponding increase in pH and a relative increase in Hb O_2 affinity, that will favor the uptake of O_2 . Residues $\beta 146$ His and $\alpha 1$ Val contribute significantly to the alkaline Bohr effect (24,25). Other residues, such as $\beta 82$ Lys may also be important participants in the Bohr effect (24).

In addition to the CO_2 transport (as bicarbonate ions and protons) a portion of CO_2 is bound chemically to Hb through formation of carbamates with the N-termini of α and β chains (26). Under physiologic conditions, only about 10% of CO_2 produced by respiring tissues, is transported as a carbamino complex with Hb (27).

The O_2 affinity of Hb is strongly influenced by 2,3-DPG, the most abundant organic phosphate in human erythrocytes (28,29). An increase in the concentration of 2,3-DPG will increase the O_2 affinity of Hb. Under

physiologic pH and ionic strength the 2,3-DPG binds more strongly to deoxy-Hb. One molecule of 2,3-DPG binds to one molecule of deoxyHb (30). The amino acid residues involved in the binding of 2,3-DPG are: Val 1, His 2, Lys 82, and His 143 of each β chain (31). Position 143 of the γ chain is occupied by a serine residue instead of histidine, which decreases the binding of 2,3-DPG to Hb F (32). Although the levels of 2,3-DPG are similar in fetal and adult red cells, this decreased binding of 2,3-DPG to Hb F results in a higher O_2 affinity of fetal blood when compared to adult blood (33).

Chloride ions decrease the O_2 affinity of the Hb through binding to the N-terminal amino group of the α chain and β 82 Lys (34). Because the latter is an important 2,3-DPG binding site, it probably does not bind significant amounts of chloride under physiologic conditions.

Genomic Organization of Human Globin Genes. The synthesis of the human globin chains is regulated by two gene clusters: The α -globin gene cluster is located on the short arm of chromosome 16 and that of the β -globin gene on the short arm of chromosome 11 (35-41) (Fig. 1/1). The α -globin gene cluster consists of duplicated α genes ($\alpha 1$ and $\alpha 2$), one embryonic gene (ζ), three ψ genes ($\psi\alpha 1$, $\psi\alpha 2$, and $\psi\zeta$), and one gene with an, as yet, undetermined function (the $\theta 1$ gene), arranged in the following order: 5'- ζ - $\psi\zeta$ - $\psi\alpha 2$ - $\psi\alpha 1$ - $\alpha 2$ - $\alpha 1$ - $\theta 1$ -3'. The β -globin gene cluster includes six genes: One embryonic (ϵ) gene, duplicated γ ($G\gamma$ and $A\gamma$) genes, adult β and δ genes, and one ψ gene ($\psi\beta$) in this order: 5'- ϵ - $G\gamma$ - $A\gamma$ - $\psi\beta$ - δ - β -3'. It is likely that the ψ genes found in both globin gene clusters have arisen by gene duplication events, followed by mutation and subsequent inactivation. An intriguing feature of the linear organization of the globin genes on both clusters is that they are arranged on the chromosome in the same order in which they are expressed during development. An additional interesting feature of the chromosomal arrangement is that the globin gene pairs which are expressed together during development (the two α genes, the δ and β genes, the $G\gamma$ and $A\gamma$ genes) are spaced relatively close together, whereas larger distances separate the genes that are expressed at different developmental periods.

All globin genes have a similar structure consisting of three coding regions (exons 1, 2, and 3), two IVS (introns 1 and 2), a 5' promoter region, and a 3' UTR (32-47) (Fig. 1/2). The promoter region, located 100-200 bp upstream from the start site of transcription (Cap site), contains sequences necessary for an accurate and efficient initiation of transcription (48). The most proximal is the so-called TATA box, located at -30 bp. The CCAAT box is located at around -70 bp, and is duplicated in the γ -globin gene promoter. The third sequence motif, called CACCC, is duplicated in the β -globin gene and is located at -90 and -105 bp. In the δ -globin gene promoter, the CCAAT and CACCC boxes have modified structures, which may account for its low level of expression.

The coding region of the globin genes is contained in three exons. To some extent it is possible to correlate the distinct functions of Hb with each of the exons (50,51). Exon 2 encodes for the segment involved in heme binding and with $\alpha 1\beta 2$ contact formation. Amino acid residues that

participate in the $\alpha 1 \beta 1$ contact formation are mainly derived from the third exon, whereas those that contribute to the Bohr effect and to 2,3-DPG formation are more randomly distributed.

FIG. 1/1. Organization of the globin genes on chromosomes 11 and 16. The black boxes indicate functional genes. The seven normal Hb types, synthesized at particular developmental stages are also presented (from Ref. 1).

FIG. 1/2. Fine structure of human α - and β -globin genes. The black blocks represent the coding regions, the white blocks indicate the IVS, and the hatched blocks at either end represent the 5' and 3' UTR. The numbers below the diagrams correspond to the amino acid CD positions; the helical positions are given above.

In the β -like globin genes the IVS interrupt the coding sequence between CDs 30 and 31, and between CDs 104 and 105, while in the α -globin gene family they interrupt the coding region between CDs 31 and 32, and CDs 99 and 100. Although the precise CD position numbers differ between the α - and β -like globin genes, with regard to their helical positions, the interruptions occur precisely at the same positions (Fig. 1/2). The IVS are included in the precursor mRNA transcript in the nucleus, but are removed prior to expulsion of the mRNA to the cytoplasm by a process known as splicing (52). Conserved sequences, mainly the invariant GT and AG

dinucleotide sequences at the 5' and 3' ends of the intron, are essential for the proper splicing of precursor mRNA molecules (53). At the 3' end of the globin genes there is a highly conserved sequence AATAAA that is necessary for proper processing, polyadenylation, and cleavage of mRNA transcripts (52).

Developmental Expression of the Globin Genes. During development a series of changes occur, both at the major sites of erythropoiesis and in the types of Hb that are synthesized (54-56). The early embryonic period is characterized by erythropoiesis in the yolk sac and the synthesis of the embryonic Hb types, e.g. Hbs Gower-I, Gower-II, Portland-I, Portland-II, and Portland-III. From the 5th to the 13th weeks after conception, the erythropoiesis is shifted to the fetal liver, with a consequent switch to the synthesis of Hb F. During the second trimester, a switch to the final place of erythropoiesis (the bone marrow) occurs. This period is characterized with a progressive fall in γ -globin chain synthesis and a progressive rise in β - and δ -globin chain synthesis. At the same time, expression of the $G\gamma$ - and $A\gamma$ -globin genes switches from a 7 to 3 ratio observed in fetuses and newborns to a 2 to 3 ratio in most adults. The developmental expression of β -like genes is shown in Fig. 1/3.

FIG. 1/3. Developmental expression of β -like globin genes (from Ref. 1).

Regulation of expression of the five functional globin genes is a complex mechanism and is controlled by proximal (promoters, enhancers, and silencers) and distal (LCR) cis-acting DNA sequences and trans-acting factors that bind to the regulatory DNA sequences (57). The LCR is located 6 to 20 kb upstream of the ϵ -globin gene and consists of four DNase I HS. Competitive interaction of the globin genes through trans-acting factors with the LCR at different stages of development is a possible mechanism through which the switching in the synthesis of the various globin chains is controlled (58).

The Embryonic Hbs. Several embryonic Hbs are present in the early gestation period. The two embryonic Hbs, Gower-I ($\zeta_2\epsilon_2$) as major and Gower-II ($\alpha_2\epsilon_2$) as minor component, were detected as slow-moving fractions on starch gel electrophoresis (59-61). Hb Portland-I ($\zeta_2\gamma_2$) was separated from Hb A by electrophoresis at lower pH (62), while the minor Hbs Portland-II ($\zeta_2\delta_2$) and Portland-III ($\zeta_2\delta_2$) were discovered at a later time (63). The ϵ chain has been observed only in very young fetuses. It is absent in blood samples from normal babies and from newborns with β -thal major, and in young fetuses of 16-20 weeks (64). In contrast, the ζ chain is detectable in blood samples of about half of all normal newborns tested, and increased levels have been found in babies with various degrees of α chain deficiencies (65-67). Recent analyses of blood samples from adults with various forms of α -thal by both immunology and HPLC methodology have detected minute quantities of ζ chain in subjects with the more severe types of deficiencies (68).

The Fetal Hbs. Hb F was discovered in the middle of the previous century when the blood from a newborn baby was shown to be more resistant to alkali than that of an adult (69). Hb F is formed by two α and two γ chains, and is the predominant Hb during fetal life and in the neonatal period. In normal adults Hb F accounts for less than 1% of the total Hb (70).

Different forms of Hb F have been detected due to a genetic heterogeneity and to a post-translational modification. The genetic heterogeneity of Hb F involves the existence of two different γ -globin genes: G_γ with glycine at position 136 and A_γ with alanine at the same position (6). The usual $G_\gamma:A_\gamma$ ratio throughout the fetal development and at birth is about 7:3, while the small amount of Hb F in red cells from most adults contains about 40% G_γ and 60% A_γ (70). An additional important genetic γ chain heterogeneity has been observed in position 75 of the A_γ chain, where threonine can replace the normally occurring isoleucine. This A_γ^T chain or Hb F-Sardinia was first discovered on the island of Sardinia (71). Since then it has been found in nearly all populations (72,73). Furthermore, some 10-15% of Hb F in the blood has been modified through an acetylation of the amino terminal group by an enzyme-mediated, post-synthetic process (74).

Hb F is present in large quantities in newborn babies. The amount of Hb F decreases continuously during the first six months of life, because of a decrease in the synthesis of the γ chains. The rate of decline of Hb F is somewhat slower in prematurely born babies, as well as in babies with SS and some other hemoglobinopathies (75). Simultaneously with the decrease in Hb F, the ratio between the G_γ and A_γ chains changes and reaches the adult level in about three or four months. Some 20 to 30% of all babies retain their newborn $G_\gamma:A_\gamma$ ratio of 7:3 as a result of a mutation in the G_γ promoter (C→T at position -158 or G→A at position -161) (76,77).

Hb F levels are elevated in some normal healthy adults with a condition of HPPFH. Increased levels of Hb F are also present in patients with SS and with a β - or $\delta\beta$ -thal homozygosity. Elevated Hb F levels have also

been observed in a variety of acquired disorders, especially those with impaired erythropoiesis (reviewed in Ref. 15). A detailed review of these conditions falls outside the scope of this dissertation.

The Adult Hbs. The major Hb component in normal adults is Hb A. It normally makes up to 97% of the total Hb and is therefore the primary determinant of Hb-related functional properties of the blood. Hb A is formed of two α and two β chains. Approximately 5% of Hb A in normal adults is present as a glycosylated derivative known as Hb A₁, in which the amino terminal valyl residue of the β chain is in ketoamine linkage with glucose (78). Hb A₁ is the product of a non-enzymatic, post-synthetic, glycosylation of Hb A, and is elevated 2- to 3-fold in individuals with an untreated diabetes mellitus who have persistent hyperglycemia (79).

Hb A₂ represents a minor Hb fraction, accounting for 2-3% of the Hb in erythrocytes of normal adults (80). Hb A₂ is formed by two α and two δ chains. The functional properties of Hb A₂ are nearly identical to those of Hb A, but because of its low concentration in the blood, it plays no significant role in O₂ transport. Increased levels of Hb A₂ are present in adults with a heterozygosity for one of numerous β -thal mutations. Higher levels of Hb A₂ have also been found in individuals with sickle cell trait, in SS patients with a coexisting α -thal, as well as in many individuals with an unstable β chain variant. Increased levels of Hb A₂ have also been observed in some acquired disorders such as hyperthyroidism, megaloblastic anemia, and perhaps malaria. Levels of Hb A₂ are decreased in individuals with α -thal, Hb H disease, $\delta\beta$ -, and δ -thal. Low levels of Hb A₂ have also been found in patients with iron deficiency, sideroblastic anemia, and in some patients with erythroleukemia (reviewed in Ref. 15).

THE ABNORMAL HUMAN HBS

Since 1959 when sickle cell anemia was found to be due to an abnormal Hb with a single amino acid substitution (Glu→Val) at position 6 of the β chain (81), numerous Hb variants have been detected; at present more than 600 Hb variants have been found (82). An updated alphabetical list of known Hb variants is provided as an addendum to this chapter.

Molecular Basis of Hb Variants. Most abnormal Hbs result from mutations in the coding regions of the globin genes that change the sequences or the number of nts. Thus, most of the abnormal Hbs are characterized with single amino acid substitutions, two amino acid substitutions within the same globin chain, or with longer or shorter globin chains. Others are fusion or hybrid Hbs that have arisen through crossovers between misaligned chromosomes. Table 1/1 presents the total number of Hbs with different abnormalities reported in the literature.

Hbs With Single Amino Acid Substitutions. The great majority (around 90%) of Hb variants are caused by a single amino acid replacement in the globin chain, because of a single base substitution in the globin gene. At present 559 Hb variants are known to be the result of single point mutations (174 β , 305 α , 56 γ , and 24 δ chain variants). Abnormal ϵ or ζ chain

TABLE 1/1. Total Number of Variants Reported in the Literature (April 1993)

Type of Abnormality	Numbers Observed					Total
	α	β	G γ^a	A γ	δ	
Single amino acid substitutions	174	305	36	20	24	559
Two amino acid substitutions	1	14		6		21
Elongated chains	9	8				17
Shortened chains	2	16				18
Hybrid Hbs						9
TOTAL						624

^a Includes three variants that have not been completely identified.

variants with single amino acid replacements have not been discovered. The uneven distribution of the Hb variants between the different globin chains can be explained by several unrelated factors. A functionally abnormal β chain variant constitutes about half of the total Hb and will contribute significantly to the function of the red cells, which at the same time, will facilitate its identification. An abnormal α chain variant is usually present at a considerably lower quantity because of the presence of three normal globin genes in a heterozygote; this is less likely to cause a significant impairment of red cell function. The relatively small number of δ chain variants that have been identified is explainable by the small amount of Hb A₂ that is present in the red cells. Even a δ chain variant with an altered electrophoretic mobility can easily escape detection, and those with an altered function will also likely go unrecognized. The number of γ chain variants that has been described is also rather small. With the newborn screening program and introduction of highly sensitive HPLC methods, new γ chain variants are presently being identified in a larger number than ever before.

Over 90% of all single nt replacements occur at the first or second position of each CD; the second position is more frequently mutated in the β - and δ -globin genes, and the first position in the γ -globin genes. Variation of the nts in the third position of many CDs do not result in an amino acid change (1).

Among known Hb variants that have a single amino acid substitution, all except three can be explained by a single base change in the affected CD. The exceptions are: Hb Bristol [β 67(E11)Val→Asp], Hb Edmonton [β 50(D1)Thr→Lys], and Hb J-Kurosh [α 19(AB1)Ala→Asp]. It appears that the amino acid substitution in these Hb variants needs to be reevaluated. The possibilities that could account for such a change are either a polymorphic site at the nt position involved or two separate point mutations in the single CD (15).

Hbs With Two Amino Acid Substitutions. As many as 21 known Hb variants are characterized by the presence of two different amino acid substitutions within the same globin chain (Table 1/2). In most of these variants the amino acid replacement are located at widely separated sites in the globin chain. An additional interesting observation is that many of

their individual point mutations have also been found in single substitution globin variants. For example, the amino acid replacements in the β chain variant Hb C-Harlem (83) include the amino acid substitution of Hb S at $\beta 6$ and of Hb Korlu Bu at $\beta 73$, both of them present in high frequencies in populations of West Africa (84). Six of the variants with two point mutations involve the Hb S substitution. All six γ chain variants with two amino acid substitutions involve the common $A_{\gamma T}$ or $A_{\gamma 75}(E19)Ile \rightarrow Thr$ substitution. These considerations have suggested that the Hbs with double point mutations arose by one of two mechanisms: A new mutation on a gene already carrying one mutation, or a crossover between two variant genes.

TABLE 1/2. Hbs With Two Amino Acid Substitutions

Hb Name	Common Substitution	Rare or Less Common Substitution
<u>J-Singapore</u>		α 78(EF7)Asn $\rightarrow$ Asp α 79(EF8)Ala $\rightarrow$ Gly
<u>S-Antilles</u>	$\beta 6(A3)Glu \rightarrow Val$ (Hb S)	β 23(B5) Val $\rightarrow$ Ile
<u>C-Ziguinchor</u>	same	β 58(E2) Pro $\rightarrow$ Arg <u>Dhofar</u>
<u>C-Harlem; C-Georgetown</u>	same	β 73(E17)Asp $\rightarrow$ Asn <u>Korle-Bu</u>
<u>S-Providence</u>	same	β 82(EF6)Lys $\rightarrow$ Asn $\rightarrow$ Asp <u>Providence</u>
<u>S-Oman</u>	same	β 121(GH4)Glu $\rightarrow$ Lys <u>O-Arab</u>
<u>S-Travis</u>	same	β 142(H20)Ala $\rightarrow$ Val
<u>Arlington Pk</u>	$\beta 6(A3)Glu \rightarrow Lys$ (Hb C)	β 95(FG2)Lys $\rightarrow$ Glu <u>N-Baltimore</u>
<u>T-Cambodia</u>	$\beta 26(B8)Glu \rightarrow Lys$ (Hb E)	β 121(GH4)Glu $\rightarrow$ Gln <u>Hb D</u>
<u>Grenoble</u>		β 51(D2) Pro $\rightarrow$ Ser β 52(D3) Asp $\rightarrow$ Asn <u>Osu Christiansborg</u>
<u>Poissy</u>		β 56(D7) Gly $\rightarrow$ Arg <u>Hamadan</u> β 86(F2) Ala $\rightarrow$ Pro
<u>Atlanta-Coventry</u>		β 75(E19)Leu $\rightarrow$ Pro <u>Atlanta</u> β 141(H19)Leu $\rightarrow$ O <u>Coventry</u>
<u>Duino</u>		β 92(F8) His $\rightarrow$ Pro <u>Newcastle</u> β 104(G6) Arg $\rightarrow$ Ser <u>Camperdown</u>
<u>Masuda</u>		β 114(G16)Leu $\rightarrow$ Met β 119(GH2)Gly $\rightarrow$ Asp <u>Fannin-Lubbock</u>
<u>Cleveland</u>	β 121(GH4)Glu $\rightarrow$ Gln (Hb D)	β 93(F9) Cys $\rightarrow$ Arg <u>Okazaki</u>
<u>F-Xinjiang</u>	$A_{\gamma T}$ or $A_{\gamma 75}(E19)Ile \rightarrow Thr$	A_{γ} 25(B7) Gly $\rightarrow$ Arg
<u>F-Fukuyama</u>	same	A_{γ} 43(CD2)Asp $\rightarrow$ Asn
<u>F-Forest Park</u>	same	A_{γ} 73(E17)Asp $\rightarrow$ Asn
<u>F-Yamaguchi</u>	same	A_{γ} 80(EF4)Asp $\rightarrow$ Asn
<u>F-Siena</u>	same	A_{γ} 121(GH4)Glu $\rightarrow$ Lys
<u>F-Charlotte</u>	same	A_{γ} 136(H14)Ala $\rightarrow$ Gly

Hbs With Elongated Polypeptide Chains. Thus far, 17 Hb variants with elongated polypeptide chains have been described (Table 1/3). It is likely that they have arisen by one of the following mechanisms: Single point mutation in the chain termination CD, frameshift mutation, preservation of initiation methionine, or insertion of three or multiple of three bases.

TABLE 1/3. Hbs With Elongated Polypeptide Chains

Residues	Amino Acid Sequence Changes	Hb Name
<u>COOH Elongations</u>		
	141 142 143 172	
α 141(H19)	Arg- GLN -ALA-----Glu-COOH	<u>Constant Spring</u>
α 141(H19)	Arg- LYS -Ala-----Glu-COOH	<u>Icaria</u>
α 141(H19)	Arg- SER -Ala-----Glu-COOH	<u>Koya Dora</u>
α 141(H19)	Arg- GLU -Ala-----Glu-COOH	<u>Seal Rock</u>
<u>Frameshifts</u>		
	139 140 141 146	
α 139(HC1)	ASN -Thr-Val-----Arg-COOH	<u>Wayne</u>
	143 144 145 156	
β 143(H21)	PRO -Ser-Ile-----Tyr-COOH	<u>Saverne</u>
	145 146 147 157	
β 145(HC2)	SER -Ile-Thr-----Tyr-COOH	<u>Cranston</u>
	146 147 148 157	
β 146(HC3)	His- THR -Lys-----Tyr-COOH	<u>Tak</u>
<u>NH₂ Extensions</u>		
	-1 +1 +2	
α 1(NA1)	MET - GLU -Leu-Ser-	<u>Thionville</u>
	-1 +1 +2	
β 1(NA1)	MET -Val- PRO -Leu-	<u>Long Island-Marseille</u>
	-1 +1 +2	
β 1(NA1)	MET - GLU -His-Leu-	<u>Doha</u>
	-1 +1 +2	
β 1(NA1)	MET - MET -His-Leu-	<u>South Florida</u>
<u>Insertions</u>		
	37 38	
α 37(C2)- α 38(C3)	Pro- GLU -Thr	<u>Catonsville</u>
	116 117	
α 116(GH4)- α 117(GH5)	Glu- HIS - LEU - PRO - ALA - GLY -Phe	<u>Zaire</u>
	118 119	
α 118(H1)- α 119(H2)	Thr- GLU - PHE - THR -Pro	<u>Grady (Dakar)</u>
	95 96	
β 95(FG2)- β 96(FG3)	Lys- LEU - HIS - LYS - ASP - LYS -Leu	<u>Koriyama</u>
<u>Insertions/Deletions</u>		
	72 76	
β 72(E16)- β 76(E20)	Ser- ALA - ARG - LYS - GLU -Ala	<u>Montreal</u>

An UAA termination CD normally follows the CD for arginine at 141 in the α -globin mRNA, and serves as the signal for chain completion. There are several examples of point mutations that involve this termination CD, replacing the stop signal with CDs for various amino acids. The resulting globin chains are elongated at their C-terminus by 31 residues. All four

known Hbs (Constant Spring, Icaria, Koya Dora, and Seal Rock) have arisen by a point mutation in the chain termination CD of the $\alpha 2$ -globin gene (85-88).

Another group of variants with an extension at the C-terminus include four variants that are products of genes carrying a frameshift at the third exon. These variants are Hb Wayne (frameshift at CD $\alpha 139$), Hb Saverne (at CD $\beta 143$), Hb Cranston (at CD $\beta 145$), and Hb Tak (at CD $\beta 146$) (89-92). The only α chain variant, Hb Wayne, is the result of a one base deletion (i.e. A) at CD 139 of the $\alpha 2$ -globin gene (89,93). Hb Saverne has also arisen from a one base deletion, and its β chain is 156 amino acids long (90). The two other β chain variants are the result of two base insertions, that occurs at CD 145 in Hb Cranston (91) and at CD 146 in Hb Tak (2); their sequences beyond the sites of mutation are identical, both being extended by 11 additional amino acids.

Four known variants are elongated at their amino terminus; one is an α chain variant, Hb Thionville (94), and three are β chain variants: Hb Long Island-Marseille (95), Hb Doha (96), and Hb South Florida (97). All these variants carry a mutation at CD 1 or 2, and have a methionyl residue preceding the normal valyl residue at position 1 of the α and β chains. Methionine is the first residue to be incorporated, but it is normally cleaved during translation of the nascent polypeptide chain. The substitutions at the first two positions of the polypeptide chain interfere with the enzymatic cleavage step that normally removes the amino terminal methionine (97).

Four abnormal variants have been described that are elongated at sites other than at the carboxy or amino termini. Three are α variants: Hb Catonsville (98), Hb Zaire (99), and Hb Grady (Dakar) (100), and one is a β chain variant, Hb Koriyama (101). Hb Grady was the first variant of this group to be discovered; it has an insertion of three residues between positions 118 and 119 (100). The mechanism responsible for these mutations is an insertion of three or multiple of three nts, that does not impair the reading frame of the mRNA, and leads to the insertion of one or more amino acids.

Hbs With Shortened Polypeptide Chains. Fifteen variants (two α and 13 β) have a shorter globin chain due to deletion of one to five amino acid residues (Table 1/4). Thirteen of these are considered to be the result of small deletions in the coding region of the appropriate globin gene, while two are due to a point mutation causing premature translation termination (nonsense mutation). The α chain variant Hb Natal has arisen by a base pair substitution leading to a stop CD at the penultimate CD of the $\alpha 2$ -globin gene (102), and Hb McKees Rocks by an analogous substitution in the β -globin gene (103).

Two additional shortened β chain variants, Hbs Birmingham and Galicia are the result of deletions of nine and three nts from the coding sequences of the β -globin gene. These specific deletions result in the insertion of one amino acid residue in addition to the deletion of four and two amino acids, respectively (104).

TABLE 1/4. Hbs With Shortened Polypeptide Chains

Residues	Deletions/Insertions	Hb Name
<u>Deletions</u>		
$\alpha 6(A4)$	Asp->0	<u>Boyle Heights</u>
$\beta 6(A3)$ or $\beta 7(A4)$	Glu->0	<u>Leiden</u>
$\beta 17(A14)$ - $\beta 18(A15)$	Lys-Val->0	<u>Lyon</u>
$\beta 23(B5)$	Val->0	<u>Freiburg</u>
$\beta 33(B15)$ and $\beta 34(B16)$	Val->0	<u>Korea</u>
$\beta 41(C7)$ or $\beta 42(CD1)$	Phe->0	<u>Bruxelles</u>
$\beta 43(CD2)$ - $\beta 45(CD4)$	Phe-Glu-Ser->0	<u>Niteroi</u>
or	or	
$\beta 42(CD1)$ - $\beta 44(CD3)$	Glu-Ser-Phe->0	<u>Tochigi</u>
$\beta 56(D7)$ - $\beta 59(E3)$	Gly-Asn-Pro-Lys->0	
$\beta 57(E1)$ - $\beta 58(E2)$	Asn-Pro->0	<u>Ehime</u>
$\beta 74(E18)$ - $\beta 75(E19)$	Gly-Leu->0	<u>St. Antoine</u>
$\beta 75(E19)$	Leu->0	<u>Vicksburg</u>
$\beta 87(F3)$	Thr->0	<u>Tours</u>
$\beta 91(F7)$ - $\beta 95(FG1)$	Leu-His-Cys-Asp-Lys->0	<u>Gun Hill</u>
$\beta 141(H19)$	Leu->0	<u>Coventry</u>
<u>Deletions/Insertions</u>		
$\beta 96(FG3)$ - $\beta 99(G1)$	96 97 98 99 Leu---LEU---Asp-	<u>Galicia</u>
$\beta 140(H18)$ - $\beta 145(HC2)$	140 141 142 143 144 145 Ala-----GLN-----Tyr	<u>Birmingham</u>
<u>Nonsense Mutations</u>		
$\alpha 140(HC2)$	Tyr->Term (Tyr-Arg->0)	<u>Natal</u>
$\beta 145(HC2)$	Tyr->Term (Tyr-Arg->0)	<u>McKees Rocks</u>

Fusion Hbs. Fusion or hybrid Hbs are variants that have arisen through nonhomologous crossover events between two misaligned chromosomes; nine such Hbs have been described (Table 1/5).

The $\delta\beta$ -Lepore gene is a combination of the 5' end of the δ gene and the 3' end of the β gene (105). Three different types of Hb Lepore have been described, in which the transition from δ -> β sequence occurs at different points. Most prevalent is Hb Lepore-Boston, with δ chain sequence through amino acid position 87 and a β chain sequence starting at position 116 (106). It has been described in different populations. Two other Lepore Hbs, Lepore-Hollandia (107) and Lepore-Baltimore (108) have $\delta\beta$ chains with a crossover at $\delta 22/\beta 50$ and $\delta 50/\beta 86$, respectively. All Lepore Hbs are synthesized at a low level and its carriers exhibit thalassemia-like hematological features (105).

The crossover event responsible for the occurrence of the Lepore chromosome gives rise to another abnormal chromosome, the "anti-Lepore" chromosome, that carries normal δ and β genes as well as a hybrid $\beta\delta$ gene. Four different anti-Lepore Hbs have been characterized: Hbs Miyada (109), P-Nilotic (110), P-Congo (111), and Lincoln Park (112). Hb Lincoln Park has

TABLE 1/5. Fusion Hbs^a

No. in helix		A6	A9	B4	D1	F2	F3	G18	G19	H2	H4	
No. in chain	1	9	12	22	50	86	87	116	117	124	126	146
δ chain	Val	Thr	Asn	Ala	Ser	Ser	Gln	Arg	Asn	Gln	Met	His
β chain	Val	Ser	Thr	Glu	Thr	Ala	Thr	His	His	Pro	Val	His
Lepore-Hollandia	_____δ-----β_____											
Lepore-Baltimore	_____δ-----β_____											
Lepore-Washington-Boston	_____δ-----β_____											
Parchman	_____δ-----β_____β-----δ_____											
Miyada	_____β-----δ_____											
P-Congo	_____β-----δ_____											
P-Nilotic	_____β-----δ_____											
Lincoln Park	_____β-----δ_____δ ¹³⁷ Val-→O											
No. in helix				EF4	EF5	F2	F3					
No. in chain	1			80	81	86	87					146
A _Y chain	Gly			Asp	Leu	Ala	Gln					His
β chain	Val			Asn	Leu	Ala	Thr					His
Kenya	_____A _Y -----β_____											

^a From Ref. 82.

the same structural characteristics as Hb P-Nilotic but in addition, it has a deletion of a valine residue at position $\delta 137$. Because of the presence of a normal β gene in cis, patients with anti-Lepore Hbs are clinically normal (105).

Hb Parchman is a more complex gene product, having δ chain sequences in both its amino and carboxyl terminal portions, and a β sequence in the middle. It probably arose by a double crossover event (113).

A hybrid gene that involves a γ -globin gene has also been described; Hb Kenya is a fusion product of the $A\gamma$ - and β -globin genes (114). The normal inter- γ - β -globin gene segment (about 22.5 kb) has been deleted from the Hb Kenya chromosome. An anti-Kenya gene has not been identified.

Clinical Classification of Hb Variants. Many of the amino acid substitutions, even some of those that produce abnormal physical properties in the variant Hb, are clinically silent and were only detected during population screenings. Other substitutions give rise to a variety of clinical symptoms in the affected individuals. In this section the abnormal Hbs are classified according to their main clinical symptoms.

The Hb S Syndromes. Homozygosity for Hb S (SS) and compound heterozygosity for Hb S and β -thal or some abnormal Hb, form the syndrome of sickle cell disease. Diseases involving Hb S occur widely throughout tropical Africa, the Mediterranean, and the Middle East (115,116). There is evidence that the condition has reached its extraordinarily high frequencies because carriers are protected against Plasmodium falciparum malaria (117).

The first description of a patient with SS was provided by Herrick in 1910 (118). In 1949, Pauling and associates showed that it was due to an electrophoretically abnormal Hb (119), while in 1959, Ingram detected a Glu→Val replacement at position 6 of the β chain (81).

The pathophysiology of the sickle syndrome is related to the decreased solubility of Hb S and its tendency to form long stable polymers in the deoxygenated state (120). The polymerization is reversible and Hb S molecules return to the normal soluble state upon oxygenation. However, after several cycles of sickling and unsickling, some of the erythrocytes become irreversibly sickled and sequestered by the spleen (121). The rigidity of the RBC membrane promotes occlusion of the microcirculation with subsequent regional ischemia and necrosis. Thus, the serious sickling disorders are characterized by anemia, painful crises, and damage to organs such as bone, kidney, and the central nervous system.

Some Hb variants such as Hbs O-Arab and D-Los Angeles, form gels with Hb S at relatively low concentrations, and can contribute significantly to the severity of sickle cell disease in individuals with one of these variants in combination with Hb S (122,123). In contrast, a few other Hbs such as Korle Bu, Hasharon, and Stanleyville-II, have been shown to have inhibitory effects on Hb S gelation (124-126). Most importantly, Hb F is known to modify the polymerization kinetics of Hb S, and to decrease the rate of deoxy Hb S gelation, thus explaining the amelioratory effect of Hb F on the clinical severity of sickle cell disease (127).

The Unstable Hbs (Hemolytic Anemias). A large group of Hb variants show instability caused by their altered molecular structures. As a result, these Hbs tend to precipitate within the erythrocytes, with the formation of insoluble protein inclusions (Heinz bodies). Patients have a disorder of varying clinical severity which is called "congenital Heinz body hemolytic anemia" (15) and have a life-long history of anemia and jaundice with episodes of aggravation often coincident with a recurrent infection (128).

The first case with "apparent idiopathic Heinz body anemia" was reported in 1952 by Cathie (129). Following this report, a number of similar cases have been described (130-133). Most of them were later found to be due to an unstable Hb variant. Hb Zurich or $\beta 63(E7)His \rightarrow Arg$ was the first variant to be structurally characterized (134). Cathie's patient was later shown to have Hb Bristol [$\beta 67(E11)Val \rightarrow Asp$] (135).

The instability of the Hb variant results from several different mechanisms (Table 1/6). Amino acid substitutions within the heme pocket may impair the binding of the heme group, or disrupt the hydrophobic heme pocket (15). Hb variants with substitution at the proximal or distal histidine cannot bind heme, which is the apparent cause for their instability. In several variants, such as Hbs Bristol (135) and Shepherds Bush (136), the polar residue replaces a nonpolar residue in the interior of the subunit. Such substitution will result in a change in tertiary structure, allowing water to penetrate into the normally hydrophobic heme pocket.

TABLE 1/6. Molecular Mechanisms Responsible For Some Unstable Hb Variants

Molecular Mechanism	Hb Variant	Amino Acid
Amino acid substitution in the vicinity of the heme pocket		
Substitutions at the proximal histidine (F8)	Iwata <u>St. Etienne</u>	α 87(F8) His $\rightarrow$ Arg β 92(F8) His $\rightarrow$ Glu
Substitutions at the distal histidine (E7)	Zurich <u>Bicetre</u>	β 63(E7) His $\rightarrow$ Arg β 63(E7) His $\rightarrow$ Pro
Introduction of a nonpolar amino acid into the heme pocket	Bristol <u>Shepherds Bush</u>	β 67(E11)Val $\rightarrow$ Asp β 74(E18)Gly $\rightarrow$ Asp
Disruption of the secondary structure	Sun Prairie <u>Southampton</u>	α 130(H13)Ala $\rightarrow$ Pro β 106(G8) Leu $\rightarrow$ Pro
Substitutions in the 11 contact region	<u>Philly</u>	β 35(C1) Tyr $\rightarrow$ Phe
Amino acid deletions	Boyle Heights <u>Tours</u>	α 6(A4) Asp $\rightarrow$ O β 87(F3) Thr $\rightarrow$ O
Elongation of the globin chain	Saverne <u>Cranston</u>	β 143(H21) (FSC) β 145(HC2) (FSC)

An important mechanism for instability of the Hb variants is also the disruption of the secondary structure through introduction of a proline residue that is unable to participated in an α -helix, except as one of the

initial three residues. Four α and 22 β chain variants with this type of substitution have been detected. Ten of the 22 β chain variants include substitution of proline for leucine. In addition, seven have an Ala- $\rightarrow$ Pro replacement, six a His- $\rightarrow$ Pro, one a Thr- $\rightarrow$ Pro, and one a Gln- $\rightarrow$ Pro substitution (1). Hb Valletta [β 87(F3)Thr- $\rightarrow$ Pro] is a stable variant, since the introduction of a proline residue in the third position of the F helical segment does not disrupt the secondary structure (137).

Molecular instability is also an accompanying feature of Hb variants that have amino acid substitutions at the α 1 β 1 interface, thus allowing dimers to dissociate into monomers; Hb Philly [β 35(C1)Tyr- $\rightarrow$ Phe] is an example of this type of unstable variant (138).

Deletions of amino acid residues alter the primary and secondary structure, as well as the conformation of the Hb molecule, and most of these variants are unstable. Finally, globin chain elongation can result in instability due to hydrophobic properties of the extended chain, i.e. Hbs Cranston and Saverne (139).

Abnormal Hbs With High O₂ Affinity (Erythrocytosis). One group of Hb variants has amino acid substitutions that increase the affinity of Hb for O₂. Some of these variants cause erythrocytosis in the carriers. The first such variant detected was Hb Chesapeake which was found in an 81-year-old man, who was in essentially good health but had a markedly elevated Hb level (140). Most of the patients with a high affinity Hb type and with erythrocytosis are asymptomatic, except for the occasional presence of a ruddy complexion. Most commonly, erythrocytosis is diagnosed during a routine hematological examination. However, an individual with Hb Malmo, who had experienced symptoms of severe cardiac dysfunction, has been reported; he has benefited from phlebotomy and the transfusion of normal blood (141).

The major reason for an increased O₂ affinity is a structural alteration that affects the equilibrium between the T and R quaternary conformations of the Hb tetramer. Amino acid substitutions that decrease the stability of the T structure and promote the R state will increase the affinity of the Hb molecule. Most of the high affinity Hbs have mutations in the binding sites of 2,3-DPG, in the carboxy terminus of the β chain, and at the α 1 β 2 interface, all being important regions for regulating the function of the Hb molecule (1).

An example is Hb Kempsey, one of the particularly well-studied high O₂ variants with an amino acid replacement at the α 1 β 2 interface (142,143). The substitution, β 99(G1)Asp- $\rightarrow$ Asn, inhibits the formation of the most important hydrogen bond between α 42 Tyr and β 99 Asp, that stabilizes the T structure.

Hb Hiroshima is a high affinity variant with a mutation at the C-terminal end of the β chain; the histidine residue is replaced by aspartic acid at position β 146 (144). All variants that affect the β 146 His also have a reduced Bohr effect, thus confirming the identification of this residue as a major source of the alkaline Bohr effect (20).

Hb Helsinki [β 82(EF6)Lys→Met] and Hb Syracuse [β 143(H21)His→Pro] are examples of high affinity variants that have substitutions at sites responsible for binding of 2,3-DPG (145,146).

The Hb M Variants (Congenital Cyanosis). Because of their distinctive physical appearance, individuals affected by one of the Hb M variants can be readily detected. They have cyanosis but are otherwise totally asymptomatic (15). The German physicians Horlein and Weber provided the first convincing evidence of a familial hemoglobinopathy in 1948 (147). They described a family with carriers of a congenital cyanotic syndrome and proved that the genetic defect resides in the globin and not in the heme. The structural analysis of M Hbs was first completed in 1961 when three Hb M variants were characterized: Hb M-Boston [α 58(E7)His→Tyr], Hb M-Saskatoon [β 63(E7)His→Tyr], and Hb M-Milwaukee-I [β 67(E11)Val→Glu] (148). All of the Hb M variants have amino acid replacements at positions that are in close proximity to the heme groups, thus stabilizing the heme iron atoms in the ferric (F^{+3}) state. The iron in the abnormal subunit is therefore incapable of binding O_2 . Most of the Hb M variants also have low affinity for O_2 . In four of the five M Hbs, involving either α or β chains, the distal or the proximal histidine is replaced by tyrosine (148-150). In the fifth, Hb Milwaukee-I, Val 67 is replaced by glutamic acid; the longer side chain of this amino acid can reach and perturb the heme iron (151). The two known fetal Hb M variants are Hb F-M-Osaka [$G_{\gamma}63$ (E7)His→Tyr] and Hb F-M-Fort Ripley [$G_{\gamma}92$ (F8)His→Tyr] (152,153).

Abnormal Hbs With Low O_2 Affinity. A number of Hb variants are characterized by a lower than normal affinity for O_2 . Some of the unstable Hb variants and some of the M Hbs also have decreased O_2 affinity. Individuals with Hb variants that have significantly low O_2 affinity exhibit a slate gray color involving the skin and mucous membranes (15). Hb Kansas [β 102(G4)Asn→Thr] was the first of these variants to be identified and is the most thoroughly studied (154,155). The amino acid substitution at β 102 in Hb Kansas prevents the formation of $\alpha\beta$ 2 hydrogen bonds between α 94 Asp and β 102 Asn that normally stabilizes the R quaternary structure. This change shifts the R-T equilibrium in favor of the low O_2 affinity T quaternary form. Low affinity for O_2 and cyanosis in the carriers have been encountered in two other Hb variants with a substitution at β 102, i.e. Hb Beth Israel (→Ser) (156) and Hb Saint Mande (→Tyr) (157).

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ADDENDUM

Alphabetical Hb Variant List (Adapted from: Hemoglobin, 17:179, 1993)

Variant Name	Residue/Chain/Mutation	Abnormal Property
A ₂ -Adria	51 delta Pro->Arg	
A ₂ -Babinga	136 delta Gly->Asp	
A ₂ -Canada	99 delta Asp->Asn	+ O ₂ affinity
A ₂ -Coburg	116 delta Arg->His	
A ₂ -Fitzroy	142 delta Ala->Asp	
A ₂ -Flatbush	22 delta Ala->Glu	
A ₂ -Grovetown	75 delta Leu->Val	
A ₂ -Honai	90 delta Glu->Val	
A ₂ -Indonesia	69 delta Gly->Arg	
A ₂ -Manzanares	121 delta Glu->Val	unstable
A ₂ -Melbourne	43 delta Glu->Lys	
A ₂ -Niigata	1 delta Val->Ala	
A ₂ -NYU	12 delta Asn->Lys	
A ₂ -Parkville	47 delta Asp->Val	
A ₂ -Pelendri	141 delta Leu->Pro	thalassemic
A ₂ -Roosevelt	20 delta Val->Glu	
A ₂ -Sphakia	2 delta His->Arg	
A ₂ -Troodos	116 delta Arg->Cys	thalassemic
A ₂ -Victoria	24 delta Gly->Asp	
A ₂ -Wrens	98 delta Val->Met	unstable
A ₂ -Yialousa	27 delta Ala->Ser	
A ₂ -Yokoshima	25 delta Gly->Asp	
A ₂ -Zagreb	125 delta Gln->Glu	unstable
A ₂ ' (B ₂)	16 delta Gly->Arg	
Aalborg	74 beta Gly->Arg	unstable
Abraham Lincoln	32 beta Leu->Pro	unstable
Abruzzo	143 beta His->Arg	+ O ₂ affinity
Adana	59 alpha Gly->Asp	unstable
Agenogi	90 beta Glu->Lys	+ O ₂ affinity
Aichi	50 alpha His->Arg	sl. unstable
Aida	64 alpha Asp->Asn	
Alabama	39 beta Gln->Lys	
Al-Ain Abu Dhabi	18 alpha Gly->Asp	
Alamo	19 beta Asn->Asp	
Albany-Suma	11 alpha Lys->Asn	
Alberta	101 beta Glu->Gly	+ O ₂ affinity
Altdorf	135 beta Ala->Pro	unstable; + O ₂ affinity
Anantharaj	11 alpha Lys->Glu	
Andrew-Minneapolis	144 beta Lys->Asn	+ O ₂ affinity
Ankara	10 beta Ala->Asp	
Ann Arbor	80 alpha Leu->Arg	unstable
Arlington Park	6 beta Glu->Lys;	
	95 beta Lys->Glu	(N-Baltimore)
Arya	47 alpha Asp->Asn	sl. unstable

Variant Name	Residue/Chain/Mutation	Abnormal Property
Asabara	74 alpha Asp->His	
Atago	85 alpha Asp->Tyr	
Athens-GA	40 beta Arg->Lys	+ O ₂ affinity
Atlanta	75 beta Leu->Pro	unstable
Atlanta-Coventry	75 beta Leu->Pro; 141 beta Leu->O	unstable (Coventry)
Attleboro	138 alpha Ser->Pro	+ O ₂ affinity
Austin	40 beta Arg->Ser	+ O ₂ affinity; + dissociation
Avicenna	47 beta Asp->Ala	
Aztec	76 alpha Met->Thr	
Bab-Saadoun	48 beta Leu->Pro	sl. unstable
Baden	18 beta Val->Met	sl. unstable
Barcelona	94 beta Asp->His	
Bari	45 alpha His->Gln	
Baylor	81 beta Leu->Arg	unstable; + O ₂ affinity
Beckman	135 beta Ala->Glu	unstable; + O ₂ affinity
Beijing	16 alpha Lys->Asn	
Beilinson	47 alpha Asp->Gly	
Beirut	126 beta Val->Ala	
Belfast	15 beta Trp->Arg	unstable; + O ₂ affinity
Belliard	56 alpha Lys->Asn	
Beograd	121 beta Glu->Val	
Beth Israel	102 beta Asn->Ser	+ O ₂ affinity
Bethesda	145 beta Tyr->His	+ O ₂ affinity
Bibba	136 alpha Leu->Pro	unstable; + dissociation
Bicetre	63 beta His->Pro	unstable; auto-oxidizing
Birmingham	β -141-144 Deleted (Leu-Ala-His-Lys)->Gln	unstable Inserted
Bologna	61 beta Lys->Met	+ O ₂ affinity
Borås	88 beta Leu->Arg	unstable
Bougardirey-Mali	119 beta Gly->Val	sl. unstable
Boumerdes	37 alpha Pro->Arg	
Boyle Heights	α -6 Deletion Asp->O	unstable
Brest	127 beta Gln->Lys	unstable
Brigham	100 beta Pro->Leu	+ O ₂ affinity
Brisbane	68 beta Leu->His	
Bristol	67 beta Val->Asp	unstable
British Columbia	101 beta Glu->Lys	+ O ₂ affinity
Brockton	138 beta Ala->Pro	unstable
Bruxelles	β -41 or β -42 Deletion Phe->O	unstable; + O ₂ affinity
Bryn Mawr	85 beta Phe->Ser	unstable; + O ₂ affinity
București	42 beta Phe->Leu	unstable; + O ₂ affinity
Buenos Aires	85 beta Phe->Ser	unstable; + O ₂ affinity
Buginese-X	116 alpha Glu->Lys	
Bunbury	94 beta Asp->Asn	

Variant Name	Residue/Chain/Mutation	Abnormal Property
Burke	107 beta Gly->Arg	+ O ₂ affinity
Bushwick	74 beta Gly->Val	unstable
B ₂ (A ₂ ')	16 delta Gly->Arg	
C	6 beta Glu->Lys	
C-Georgetown	6 beta Glu->Val;	sickling
	73 beta Asp->Asn	(Korle Bu)
C-Harlem	6 beta Glu->Val;	sickling
	73 beta Asp->Asn	(Korle Bu)
C-Ziguinchor	6 beta Glu->Val;	sickling
	58 beta Pro->Arg	(Dhofar)
Cagliari	60 beta Val->Gly	unstable
Calais	76 beta Ala->Pro	+ O ₂ affinity
Camden	131 beta Gln->Glu	
Camperdown	104 beta Arg->Ser	sl. unstable
Caribbean	91 beta Leu->Arg	unstable; + O ₂ affinity
Casper	106 beta Leu->Pro	unstable; + O ₂ affinity
Castilla	32 beta Leu->Arg	unstable
Catonsville	α -37 Insertion	unstable; + O ₂ affinity
	37(+1)38	
Chad	23 alpha Glu->Lys	
Chandigarh	94 beta Asp->Gly	
Chapel Hill	74 alpha Asp->Gly	
Chaya	43 beta Glu->Gln	
Chemilly	99 beta Asp->Val	+ O ₂ affinity
Chesapeake	92 alpha Arg->Leu	+ O ₂ affinity
Chesterfield	28 beta Leu->Arg	unstable; thalassemic
Cheverly	45 beta Phe->Ser	
Chiapas	114 alpha Pro->Arg	
Chiba	42 beta Phe->Ser	unstable; + O ₂ affinity
Chicago	136 alpha Leu->Met	
Chico	66 beta Lys->Thr	+ O ₂ affinity
Chongqing	2 alpha Leu->Arg	unstable; + O ₂ affinity
Christchurch	71 beta Phe->Ser	unstable
City of Hope	69 beta Gly->Ser	
Cleveland	93 beta Cys->Arg;	unstable; + O ₂ affinity
	121 beta Glu->Gln	(Okazaki)
Cochin-Port Royal	146 beta His->Arg	
Cocody	21 beta Asp->Asn	
Coimbra	99 beta Asp->Glu	+ O ₂ affinity; polycythemic
Collingwood	60 beta Val->Ala	unstable
Columbia Missouri	88 alpha Ala->Val	+ O ₂ affinity
Complutense	127 beta Gln->Glu	
Connecticut	21 beta Asp->Gly	+ O ₂ affinity
Constant Spring	α -141 Extension	
	141(+31)=172	
Contaldo	103 alpha His->Arg	unstable

Variant Name	Residue/Chain/Mutation	Abnormal Property
Cordele	47 alpha Asp->Ala	unstable
Coventry	β -141 <u>Deletion</u> Leu->0	unstable
Cowtown	146 beta His->Leu	+ O ₂ affinity
Cranston	α -145 Frameshift 145(+12) ->157	unstable; + O ₂ affinity
Crete	129 beta Ala->Pro	
Creteil	89 beta Ser->Asn	+ O ₂ affinity
D-Baltimore	68 alpha Asn->Lys	
D-Bushman	16 beta Gly->Arg	
D-Camperdown	121 beta Glu->Val	
D-Chicago	121 beta Glu->Gln	+ O ₂ affinity
D-Granada	22 beta Glu->Val	
D-Ibadan	87 beta Thr->Lys	
D-Iran	22 beta Glu->Gln	
D-Los Angeles	121 beta Glu->Gln	+ O ₂ affinity
D-Neath	121 beta Glu->Ala	
D-North Carolina	121 beta Glu->Gln	+ O ₂ affinity
D-Ouled Rabah	19 beta Asn->Lys	
D-Portugal	121 beta Glu->Gln	+ O ₂ affinity
D-Punjab	121 beta Glu->Gln	+ O ₂ affinity
D-St. Louis	68 alpha Asn->Lys	
D-Washington	68 alpha Asn->Lys	
Dagestan	60 alpha Lys->Glu	
Dakar	∇ -118 <u>Insertion</u> 118(+3)119	unstable; + O ₂ affinity
Dallas	97 alpha Asn->Lys	+ O ₂ affinity
Daneshgah-Tehran	72 alpha His->Arg	
Davenport	78 alpha Asn->His	
Deaconess	131 beta Gln->Lys	unstable
Deer Lodge	2 beta His->Arg	
Denmark Hill	95 alpha Pro->Ala	+ O ₂ affinity
Detroit	95 beta Lys->Asn	
Dhofar	58 beta Pro->Arg	
Dhonburi	126 beta Val->Gly	unstable
Djelfa	98 beta Val->Ala -1 +2 +2	unstable; + O ₂ affinity
Doha	$\leftarrow$ -1 <u>Extension</u> Met-Glu-His-Leu-	
Drenthe	27 beta Ala->Asp	unstable
Duan	75 alpha Asp->Ala	
Duarte	62 beta Ala->Pro	unstable; + O ₂ affinity
Duino	92 beta His->Pro; 104 beta Arg->Ser	unstable (Newcastle)
Dunn	6 alpha Asp->Asn	
Durham-N.C.	114 beta Leu->Pro	unstable; thalassemic

Variant Name	Residue/Chain/Mutation	Abnormal Property
E	26 beta Glu->Lys	
E-Saskatoon	22 beta Glu->Lys	unstable
Edmonton	50 beta Thr->Lys	
Egypt	121 beta Glu->Lys	
Ehime	β -57-58 Deletion Asn-Pro->O	
Ethiopia	140 alpha Tyr->His	$\uparrow$ O ₂ affinity
Etobicoke	84 alpha Ser->Arg	$\uparrow$ O ₂ affinity
Evans	62 alpha Val->Met	unstable
Evanston	14 alpha Trp->Arg	
Extremadura	133 beta Val->Leu	sl. unstable
F-Albaicin	8 G-gamma Lys->Glu or Gln	
F-Alexandra	12 gamma Thr->Lys	
F-Auckland	7 G-gamma Asp->Asn	
F-Austell	40 G-gamma Arg->Lys	
F-Baskent	128 A-gamma Ala->Thr	
F-Beech Island	53 A-gamma Ala->Asp	
F-Bonaire-GA	39 A-gamma Gln->Arg	
F-Brooklyn	66 G-gamma Lys->Gln	
F-Calluna	12 A-gamma Thr->Arg	
F-Caltech	120 G-gamma Lys->Gln	
F-Carlton	121 G-gamma Glu->Lys	
F-Catalonia	15 G-gamma Trp->Arg	
F-Charlotte	136 A-gamma-T Ala->Gly	
F-Clarke	65 G-gamma Lys->Asn	
F-Cobb	37 A-gamma Trp->Arg	
F-Columbus-GA	94 G-gamma Asp->Asn	
F-Cosenza	25 G-gamma Gly->Glu	
F-Dammam	79 A-gamma Asp->Asn	
F-Dickinson	97 A-gamma His->Arg	
F-Forest Park	73 A-gamma-T Asp->Asn	
F-Fuchu	21 G-gamma Glu->Gln	
F-Fukuyama	43 A-gamma-T Asp->Asn	
F-Granada	22 G-gamma Asp->Val	
F-Heather	12 G-gamma Thr->Arg	
F-Hull	121 A-gamma Glu->Lys	
F-Iwata	72 A-gamma Gly->Arg	
F-Izumi	6 A-gamma Glu->Gly	
F-Jamaica	61 A-gamma Lys->Glu	
F-Jiangsu	134 A-gamma Val->Met	
F-Kennestone	77 G-gamma His->Arg	
F-Kingston	55 G-gamma Met->Arg	
F-Kotobuki	6 A-gamma Glu->Gly	
F-Kuala Lumpur	22 A-gamma Asp->Gly	
F-La Grange	101 G-gamma Glu->Lys	
F-Lodz	44 G-gamma Ser->Arg	
F-Malaysia	1 G-gamma Gly->Cys	

Variant Name	Residue/Chain/Mutation		Abnormal Property
F-Malta-I	117	G-gamma His->Arg	
F-Marietta	80	G-gamma Asp->Asn	
F-Meinhama	5	G-gamma Glu->Gly	
F-Melbourne	16	G-gamma Gly->Arg	
F-Minoo	72	G-gamma Gly->Arg	
F-Oakland	26	G-gamma Glu->Lys	
F-Onoda	146	G-gamma His->Tyr	+O ₂ affinity
F-Pendergrass	36	A-gamma Pro->Arg	
F-Poole	130	G-gamma Trp->Gly	unstable
F-Pordenone	6	A-gamma Glu->Gln	
F-Port Royal	125	G-gamma Glu->Ala	
F-Sardinia (A T)	75	A-gamma Ile->Thr	
F-Shanghai	66	G-gamma Lys->Arg	
F-Siena	121	A-gamma-T Glu->Lys	
F-Texas-I	5	A-gamma Glu->Lys	
F-Texas-II	6	gamma Glu->Lys	
F-Tokyo	34	G-gamma Val->Ile	
F-Ube	108	gamma Asn->Lys	
F-Urumqi	22	G-gamma Asp->Gly	
F-Victoria Jubilee	80	A-gamma Asp->Tyr	
F-Woodstock	40	A-gamma Arg->Lys	
F-Xinjiang	25	A-gamma-T Gly->Arg	unstable
F-Xin-Su	73	A-gamma Asp->His	
F-Yamaguchi	80	A-gamma-T Asp->Asn	
F-M-Fort Ripley	92	G-gamma His->Tyr	cyanosis
F-M-Osaka	63	G-gamma His->Tyr	metHb
Fannin-Lubbock	119	beta Gly->Asp	sl. unstable
Ferndown	6	alpha Asp->Val	+O ₂ affinity
Finlandia	36	beta Pro->Thr	+O ₂ affinity
Fort de France	45	alpha His->Arg	+O ₂ affinity
Fort Gordon	145	beta Tyr->Asp	+O ₂ affinity
Fort Worth	27	alpha Glu->Gly	
Freiburg	β-23	Deletion Val->O	+O ₂ affinity
Fukuoka	2	beta His->Tyr	
Fukutomi	126	alpha Asp->Val	
Fukuyama	77	beta His->Tyr	
G-Accra	73	beta Asp->Asn	+O ₂ affinity
G-Audhali	23	alpha Glu->Val	
G-Azakouli	68	alpha Asn->Lys	
G-Bristol	68	alpha Asn->Lys	
G-Chinese	30	alpha Glu->Gln	
G-Copenhagen	47	beta Asp->Asn	
G-Coushatta	22	beta Glu->Ala	
G-Ferrara	57	beta Asn->Lys	unstable
G-Galveston	43	beta Glu->Ala	
G-Georgia	95	alpha Pro->Leu	+O ₂ affinity; +dissociation

Variant Name	Residue/Chain/Mutation	Abnormal Property
G-Hong Kong	30 alpha Glu->Gln	
G-Honolulu	30 alpha Glu->Gln	
G-Hsi-Tsou	79 beta Asp->Gly	+O ₂ affinity
G-Knoxville-I	68 alpha Asn->Lys	
G-Makassar	6 beta Glu->Ala	
G-Norfolk	85 alpha Asp->Asn	+O ₂ affinity?
G-Pest	74 alpha Asp->Asn	
G-Philadelphia	68 alpha Asn->Lys	
G-Port Arthur	43 beta Glu->Ala	
G-San Jose	7 beta Glu->Gly	
G-Saskatoon	22 beta Glu->Ala	
G-Singapore	30 alpha Glu->Gln	
G-Szuhu	80 beta Asn->Lys	
G-Taegu	22 beta Glu->Ala	
G-Taichung	74 alpha Asp->His	
G-Taipei	22 beta Glu->Gly	
G-Taiwan Ami	25 beta Gly->Arg	
G-Texas	43 beta Glu->Ala	
G-Waimanalo	64 alpha Asp->Asn	
Gainesville	46 beta Gly->Arg	
Galicia	β -97-98 Deleted (His-Val)->Leu Inserted	unstable
Garden State	82 alpha Ala->Asp	
Gavello	47 beta Asp->Gly	
Geelong	139 beta Asn->Asp	unstable
Genova	28 beta Leu->Pro	unstable; +O ₂ affinity
Gifu	80 beta Asn->Lys	
Gothenburg	58 alpha His->Tyr	+O ₂ affinity
Grady	α -118 Insertion 118(+3)119	unstable; +O ₂ affinity
Grange Blanche	27 beta Ala->Val	+O ₂ affinity
Graz	2 beta His->Leu	
Great Lakes	68 beta Leu->His	+O ₂ affinity
Grenoble	51 beta Pro->Ser; 52 beta Asp->Asn	(Osu Christiansborg)
Guangzhou-Hangzhou	64 alpha Asp->Gly	
GuiZhou	77 alpha Pro->Arg	
Gun Hill	β -91-95 Deletion Leu-His-Cys-Asp-Lys->0	unstable; +O ₂ affinity; +dissociation
Hafnia	115 beta His->Gln	
Hamadan	56 beta Gly->Arg	
Hamilton	11 beta Val->Ile	
Hammersmith	42 beta Phe->Ser	unstable; +O ₂ affinity
Hanamaki	139 alpha Lys->Glu	
Handa	90 alpha Lys->Met	+O ₂ affinity
Handsworth	18 alpha Gly->Arg	

Variant Name	Residue/Chain/Mutation	Abnormal Property
Harbin	16 alpha Lys->Met	unstable; $\uparrow O_2$ affinity
Hasharon	47 alpha Asp->His	unstable
Hazebrouck	38 beta Thr->Pro	unstable; $\uparrow O_2$ affinity
Heathrow	103 beta Phe->Leu	$\uparrow O_2$ affinity
Hekinan	27 alpha Glu->Asp	
Helsinki	82 beta Lys->Met	$\uparrow O_2$ affinity
Henri Mondor	26 beta Glu->Val	sl. unstable
Hijiyama	120 beta Lys->Glu	
Hikari	61 beta Lys->Asn	
Hikoshima	54 alpha Gln->Arg	
Himeji	140 beta Ala->Asp	unstable; $\uparrow O_2$ affinity
Hirosaki	43 alpha Phe->Leu	
Hirose	37 beta Trp->Ser	$\uparrow O_2$ affinity
Hiroshima	146 beta His->Asp	$\uparrow O_2$ affinity
Hobart	20 alpha His->Arg	
Hofu	126 beta Val->Glu	
Hope	136 beta Gly->Asp	unstable
Hopkins-I	95 beta Lys->Glu	
Hopkins-II	112 alpha His->Asp	unstable; $\uparrow O_2$ affinity
Hörlein-Weber	63 beta His->Tyr	ferriHb; $\uparrow O_2$ affinity
Hoshida	43 beta Glu->Gln	
Hotel-Dieu	99 beta Asp->Gly	$\uparrow O_2$ affinity
Hsin Chu	22 beta Glu->Ala	
Hyogo	28 beta Leu->Pro	unstable; $\uparrow O_2$ affinity
I	16 alpha Lys->Glu	
I-Burlington	16 alpha Lys->Glu	
I-High Wycombe	59 beta Lys->Glu	
I-Interlaken	15 alpha Gly->Asp	
I-Philadelphia	16 alpha Lys->Glu	
I-Skamania	16 alpha Lys->Glu	
I-Texas	16 alpha Lys->Glu	
I-Toulouse	66 beta Lys->Glu	unstable; ferriHb
Icaria	α -141 Extension	
	141(+31)=172	
Indianapolis	112 beta Cys->Arg	very unstable
Ingelheim	99 beta Asp->Glu	$\uparrow O_2$ affinity; polycythemic
Inkster	85 alpha Asp->Val	
Iowa	119 beta Gly->Ala	
Isehara	92 beta His->Asn->Asp	unstable
Istanbul	92 beta His->Gln	unstable; $\uparrow O_2$ affinity; $\uparrow$ dissociation
Iwata	87 alpha His->Arg	
J	54 alpha Gln->Glu	
J-Abidjan	51 alpha Gly->Asp	
J-Aljezur	12 alpha Ala->Asp	

Variant Name	Residue/Chain/Mutation	Abnormal Property
J-Altgeld Gardens	92 beta His->Asp	unstable
J-Amiens	17 beta Lys->Asn	
J-Anatolia	61 alpha Lys->Thr	
J-Antakya	65 beta Lys->Met	
J-Auckland	25 beta Gly->Asp	
J-Baltimore	16 beta Gly->Asp	
J-Bangkok	56 beta Gly->Asp	
J-Bari	64 beta Gly->Asp	unstable
J-Birmingham	120 alpha Ala->Glu	
J-Broussais	90 alpha Lys->Asn	
J-Buda	61 alpha Lys->Asn	
J-Cairo	65 beta Lys->Gln	
J-Calabria	64 beta Gly->Asp	unstable
J-Camaguey	141 alpha Arg->Gly	
J-Cambridge	69 beta Gly->Asp	
J-Cape Town	92 alpha Arg->Gln	↑O ₂ affinity
J-Chicago	76 beta Ala->Asp	
J-Cordoba	95 beta Lys->Met	
J-Cosenza	63 beta Gly->Asp	unstable
J-Cubujuqui	141 alpha Arg->Ser	
J-Dalao	57 beta Asn->Asp	
J-Georgia	16 beta Gly->Asp	
J-Guantanamo	128 beta Ala->Asp	unstable
J-Habana	71 alpha Ala->Glu	
J-Honolulu	59 beta Lys->Thr	
J-Iran	77 beta His->Asp	
J-Ireland	16 beta Gly->Asp	
J-Kaohsiung	59 beta Lys->Thr	
J-Korat	56 beta Gly->Asp	
J-Kurosh	19 alpha Ala->Asp	
J-Lens	13 beta Ala->Asp	
J-Lome	59 beta Lys->Asn	
J-Luhe	8 beta Lys->Gln	
J-Manado	56 beta Gly->Asp	
J-Medellin	22 alpha Gly->Asp	
J-Meerut	120 alpha Ala->Glu	
J-Meinung	56 beta Gly->Asp	
J-Norfolk	57 alpha Gly->Asp	
J-Nyanza	21 alpha Ala->Asp	
J-Oxford	15 alpha Gly->Asp	
J-Paris-I	12 alpha Ala->Asp	
J-Paris-II	54 alpha Gln->Glu	
J-Pontoise	63 alpha Asp->Ala	
J-Rajappan	90 alpha Lys->Thr	
J-Rambam	69 beta Gly->Asp	
J-Rovigo	53 alpha Ala->Asp	unstable
J-Sardegna	50 alpha His->Asp	
J-Sicilia	65 beta Lys->Asn	

Variant Name	Residue/Chain/Mutation	Abnormal Property
J-Singa	78 alpha Asn->Asp	
J-Singapore	78 alpha Asn->Asp; 79 alpha Ala->Gly	
J-Taichung	129 beta Ala->Asp	
J-Tongariki	115 alpha Ala->Asp	
J-Toronto	5 alpha Ala->Asp	
J-Trinidad	16 beta Gly->Asp	
J-Wenchang-Wuming	11 alpha Lys->Gln	
Jackson	127 alpha Lys->Asn	
Jacksonville	54 beta Val->Asp	unstable
Jenkins	95 beta Lys->Glu	
Jianghua	120 beta Lys->Ile	
Johnstown	109 beta Val->Leu	+O ₂ affinity
K-Cameroon	129 beta Ala->Glu or Asp	
K-Ibadan	46 beta Gly->Glu	
K-Woolwich	132 beta Lys->Gln	
Kagoshima	57 alpha Gly->Asp	
Kairouan	30 beta Arg->Thr	
Kanagawa	40 alpha Lys->Met	+O ₂ affinity
Kansas	102 beta Asn->Thr	+O ₂ affinity; +dissociation
Kaohsiung	113 beta Val->Glu	
Karachi	5 alpha Ala->Pro	
Karatsu	120 beta Lys->Asn	
Kariya	40 alpha Lys->Glu	
Karlskoga	21 beta Asp->His	
Kawachi	44 alpha Pro->Arg	
Kempsey	99 beta Asp->Asn	+O ₂ affinity
Kenitra	69 beta Gly->Arg	
Kenwood	95 beta Lys->Glu	
Kenya	<u>Fusion Aγ-81/B-86</u>	
Khartoum	124 beta Pro->Arg	unstable
Knossos	27 beta Ala->Ser	
Kobe	32 beta Leu->Pro	unstable
Kodaira	146 beta His->Gln	+O ₂ affinity
Kofu	84 beta Thr->Ile	
Kokura	47 alpha Asp->Gly	unstable; +O ₂ affinity
Köln	98 beta Val->Met	unstable; +O ₂ affinity
Korea	<u>B-33-34 Deletion</u> Val->O	unstable; thalassemic
Koriyama	<u>B-95 Insertion</u> 95(+5)96	unstable
Korle-Bu	73 beta Asp->Asn	+O ₂ affinity
Koya Dora	<u>α-141 Extension</u> 141(+16)=157	
Kurashiki	74 alpha Asp->His	

Variant Name	Residue/Chain/Mutation	Abnormal Property
L-Ferrara	47 alpha Asp->His	unstable
L-Gaslini	47 alpha Asp->Gly	
L-Persian Gulf	57 alpha Gly->Arg	
La Desirade	129 beta Ala->Val	unstable
La Lamentin	20 alpha His->Gln	
La Roche-Sur-Yon	81 beta Leu->His	unstable; +O ₂ affinity
Las Palmas	49 beta Ser->Phe	sl. unstable
Legnano	141 alpha Arg->Leu	+O ₂ affinity
Leiden	β -6 or β -7 Deletion Glu->O	unstable; sl. +O ₂ affinity
Leipzig	63 beta His->Tyr	ferriHb; +O ₂ affinity
Lepore-Baltimore	Fusion δ -50/ β -86	
Lepore-Hollandia	Fusion δ -22/ β -50	+O ₂ affinity
Lepore-Washington-Boston	Fusion δ -87/ β -116	+O ₂ affinity
Leslie	131 beta Gln->Lys	unstable
Lille	74 alpha Asp->Ala	
Lincoln Park	Fusion δ -22/ β -50; δ -137 Val->O	
Linköping	36 beta Pro->Thr	
Little Rock	143 beta His->Gln	+O ₂ affinity
Loire	88 alpha Ala->Ser -1 +1 +2	+O ₂ affinity
Long Island-Marseille	β -1 Extension Met-Val-Pro-Leu-	
Louisville	42 beta Phe->Leu	unstable; +O ₂ affinity
Lufkin	29 beta Gly->Asp	
Luxembourg	24 alpha Tyr->His	unstable
Lyon	β -17-18 Deletion Lys-Val->O	sl. unstable; +O ₂ affinity
M-Akita	92 beta His->Tyr	ferriHb
M-Arhus	63 beta His->Tyr	ferriHb; +O ₂ affinity
M-Boston	58 alpha His->Tyr	+O ₂ affinity
M-Chicago	63 beta His->Tyr	ferriHb; +O ₂ affinity
M-Emory	63 beta His->Tyr	ferriHb; +O ₂ affinity
M-Erlangen	63 beta His->Tyr	ferriHb; +O ₂ affinity
M-Hida	63 beta His->Tyr	ferriHb; +O ₂ affinity
M-Hyde Park	92 beta His->Tyr	ferriHb
M-Iwate	87 alpha His->Tyr	ferriHb; +O ₂ affinity
M-Kankakee	87 alpha His->Tyr	ferriHb; +O ₂ affinity
M-Kiskunhalas	58 alpha His->Tyr	+O ₂ affinity
M-Kurume	63 beta His->Tyr	ferriHb; +O ₂ affinity
M-Milwaukee-I	67 beta Val->Glu	ferriHb; +O ₂ affinity
M-Oldenburg	87 alpha His->Tyr	ferriHb; +O ₂ affinity
M-Osaka	58 alpha His->Tyr	+O ₂ affinity
M-Radom	63 beta His->Tyr	ferriHb; +O ₂ affinity
M-Saskatoon	63 beta His->Tyr	ferriHb; +O ₂ affinity
M-Sendai	87 alpha His->Tyr	ferriHb; +O ₂ affinity

Variant Name	Residue/Chain/Mutation	Abnormal Property
Machida	6 beta Glu->Gln	
Madrid	115 beta Ala->Pro	unstable
Mahidol	74 alpha Asp->His	
Malay	19 beta Asn->Ser	
Malmö	97 beta His->Gln	+ O ₂ affinity
Manitoba	102 alpha Ser->Arg	sl. unstable
Maputo	47 beta Asp->Tyr	
Masuda	114 beta Leu->Met; 119 beta Gly->Asp	sl. unstable (Fannin-Lubbock)
Matera	55 beta Met->Lys	unstable
Matsue-Okii	75 alpha Asp->Asn	
McKees Rocks	145 beta Tyr->Term	++ O ₂ affinity
Meilahti	36 beta Pro->Thr	unstable; + O ₂ affinity
Memphis	23 alpha Glu->Gln	
Mequon	41 beta Phe->Tyr	
Mexico	54 alpha Gln->Glu	
Michigan-I	47 alpha Asp->Gly	
Michigan-II	47 alpha Asp->Gly	
Milledgeville	44 alpha Pro->Leu	+ O ₂ affinity
Minneapolis-Laos	118 beta Phe->Tyr	
Mississippi	44 beta Ser->Cys	
Mito	144 beta Lys->Glu	+ O ₂ affinity
Miyada	Fusion β -12/8-22	
Miyano	41 alpha Thr->Ser	+ O ₂ affinity
Miyashiro	23 beta Val->Gly	unstable; + O ₂ affinity
Mizuho	68 beta Leu->Pro	unstable
Mizunami	83 beta Gly->Asp	sl. + O ₂ affinity
Mizushi	75 alpha Asp->Gly	
Moabit	86 alpha Leu->Arg	unstable; + O ₂ affinity
Mobile	73 beta Asp->Val	+ O ₂ affinity
Monroe	30 beta Arg->Thr	
Montefiore	126 alpha Asp->Tyr	+ O ₂ affinity
Montgomery	48 alpha Leu->Arg	
Montreal	β -72-76 Deleted (Ala-Arg-Cys-Gln) Inserted	unstable
Moscva	24 beta Gly->Asp	unstable; + O ₂ affinity
Motown	131 beta Gln->Glu	
Mozhaisk	92 beta His->Arg	unstable; + O ₂ affinity
Mugino	47 alpha Asp->Gly	
Munakata	90 alpha Lys->Met	+ O ₂ affinity
Muscat	32 beta Leu->Val	sl. unstable
Muskegon	83 beta Gly->Arg	
N-Baltimore	95 beta Lys->Glu	
N-Cosenza	15 alpha Gly->Asp	
N-Memphis	95 beta Lys->Glu	
N-New Haven	16 beta Gly->Asp	

Variant Name	Residue/Chain/Mutation		Abnormal Property
N-Seattle	61	beta Lys->Glu	
N-Timone	8	beta Lys->Glu	
Nagasaki	17	beta Lys->Glu	
Nagoya	97	beta His->Pro	unstable; +O ₂ affinity
Nancy	145	beta Tyr->Asp	+O ₂ affinity
Natal	α -140-141	<u>Deletion</u> Tyr-Arg->O	+O ₂ affinity
Neapolis	126	beta Val->Gly	unstable; thalassemic
Necker Enfants-Malades	20	alpha His->Tyr	
Newcastle	92	beta His->Pro	
New Mexico	100	beta Pro->Arg	
New York	113	beta Val->Glu	unstable; +O ₂ affinity
Nigeria	81	alpha Ser->Cys	
Nikosia	17	beta Lys->Gln	
Nishik-I	57	alpha Gly->Asp	
Nishik-II	57	alpha Gly->Asp	
Nishik-III	57	alpha Gly->Asp	
Niteroi	β -43-45	<u>Deletion</u> (Phe-Glu-Ser->O) or β -42-44 (Phe-Glu-Ser->O)	unstable; +O ₂ affinity
Noki	76	alpha Met->Lys	
North Chicago	36	beta Pro->Ser	+O ₂ affinity
North Shore	134	beta Val->Glu	unstable
North Shore-Caracas	134	beta Val->Glu	unstable
Nottingham	98	beta Val->Gly	unstable; +O ₂ affinity
Nouakchott	114	alpha Pro->Leu	+hydrophobicity
Novi Sad	63	beta His->Tyr	ferriHb; +O ₂ affinity
Nunobiki	141	alpha Arg->Cys	+O ₂ affinity
O-Arab	121	beta Glu->Lys	
O-Indonesia	116	alpha Glu->Lys	
O-Padova	30	alpha Glu->Lys	
Oak Ridge	121	beta Glu->Gln	+O ₂ affinity
Ocho Rios	52	alpha Asp->Ala	
Ogi	34	alpha Leu->Arg	+O ₂ affinity
Ohio	142	beta Ala->Asp	+O ₂ affinity
Okaloosa	48	beta Leu->Arg	unstable; +O ₂ affinity
Okayama	2	beta His->Gln	+O ₂ affinity
Okazaki	93	beta Cys->Arg	unstable; +O ₂ affinity
Oleander	116	alpha Glu->Gln	
Oliviere	116	alpha Glu->Lys	
Olmsted	141	beta Leu->Arg	
Olomouc	86	beta Ala->Asp	+O ₂ affinity
Olympia	20	beta Val->Met	+O ₂ affinity
Osler	145	beta Tyr->Asp	+O ₂ affinity
Osu Christiansborg	52	beta Asp->Asn	
Ottawa	15	alpha Gly->Arg	
Owari	121	alpha Val->Met	
Ozieri	71	alpha Ala->Val	

Variant Name	Residue/Chain/Mutation	Abnormal Property
P-Congo	Fusion β -22/6-116	
P-Galveston	117 beta His→Arg	
P-Nilotic	Fusion β -22/6-50	+ O ₂ affinity
Palmerston North	23 beta Val→Phe	+ O ₂ affinity
Parchman	Fusion δ -12/8-22; β -50/6-87	
Pasadena	75 beta Leu→Arg	unstable; + O ₂ affinity
Pavie	135 alpha Val→Glu	
Perspolis	64 alpha Asp→Tyr	
Perth	32 beta Leu→Pro	unstable
Petah Tikva	110 alpha Ala→Asp	unstable
Peterborough	111 beta Val→Phe	unstable; + O ₂ affinity
Pierre-Benite	90 beta Glu→Asp	+ O ₂ affinity
Philly	35 beta Tyr→Phe	unstable; + O ₂ affinity
Pitie-Salpetriere	34 beta Val→Phe	+ O ₂ affinity
Poissy	56 beta Gly→Arg;	(Hamadan)
	86 beta Ala→Pro	unstable
Pontoise	63 alpha Ala→Asp	
Port Huron	56 alpha Lys→Arg	
Port Phillip	91 alpha Leu→Pro	unstable
Porto Alegre	9 beta Ser→Cys	+ O ₂ affinity; polymerization; + heme-heme
Potomac	101 beta Glu→Asp	+ O ₂ affinity
Prato	31 alpha Arg→Ser	
Presbyterian	108 beta Asn→Lys	+ O ₂ affinity
Providence	82 beta Lys→Asn→Asp	+ O ₂ affinity
Pyrgos	83 beta Gly→Asp	
Q-India	64 alpha Asp→His	
Q-Iran	75 alpha Asp→His	
Q-Thailand	74 alpha Asp→His	
Quebec-Chori	87 beta Thr→Ile	
Queens	34 alpha Leu→Arg	+ O ₂ affinity
Questembert	131 alpha Ser→Pro	highly unstable
Quin Hai	78 beta Leu→Arg	
Quong Sze	125 alpha Leu→Pro	
Radcliffe	99 beta Asp→Ala	+ O ₂ affinity
Rahere	82 beta Lys→Thr	+ O ₂ affinity
Rainier	145 beta Tyr→Cys	+ O ₂ affinity; alk. resistant
Raleigh	1 beta Val→Ac-Ala	+ O ₂ affinity; + dissociation
Rampa	95 alpha Pro→Ser	+ O ₂ affinity; + dissociation
Rancho Mirage	143 beta His→Asp	
Randwick	15 beta Trp→Gly	unstable
Redondo	92 beta His→Asn→Asp	unstable
Regina	96 beta Leu→Val	+ O ₂ affinity
Reims	23 alpha Glu→Gly	sl. unstable

Variant Name	Residue/Chain/Mutation	Abnormal Property
Richmond	102 beta Asn->Lys	asymmetric hybrids
Rio Grande	8 beta Lys->Thr	
Riverdale-Bronx	24 beta Gly->Arg	unstable
Riyadh	120 beta Lys->Asn	
Roseau-Pointe a Pitre	90 beta Glu->Gly	unstable; + O ₂ affinity
Rothschild	37 beta Trp->Arg	
Rouen	140 alpha Tyr->His	+ O ₂ affinity
Rush	101 beta Glu->Gln	unstable
Russ	51 alpha Gly->Arg	
S	6 beta Glu->Val	sickling
S-Antilles	6 beta Glu->Val;	sickling
	23 beta Val->Ile	
S-Oman	6 beta Glu->Val;	sickling
	121 beta Glu->Lys	(O-Arab)
S-Providence	6 beta Glu->Val;	sickling
	82 beta Lys->Asn->Asp	(Providence)
S-Travis	6 beta Glu->Val;	sickling; + O ₂ affinity
	142 beta Ala->Val	
Sabine	91 beta Leu->Pro	unstable
Saint-Jacques	140 beta Ala->Thr	+ O ₂ affinity
Saint Mande	102 beta Asn->Tyr	+ O ₂ affinity
Saitama	117 beta His->Pro	unstable
Saki	14 beta Leu->Pro	unstable
San Diego	109 beta Val->Met	+ O ₂ affinity
San Francisco (Pacific)	98 beta Val->Met	unstable; + O ₂ affinity
Santa Ana	88 beta Leu->Pro	unstable
Sarrebourg	131 beta Gln->Arg	unstable
Sassari	126 alpha Asp->His	
Savannah	24 beta Gly->Val	unstable
Savaria	49 alpha Ser->Arg	
Saverne	β -143 Frameshift	unstable; + O ₂ affinity
	143(+13) ->156	
Sawara	6 alpha Asp->Ala	+ O ₂ affinity
Seal Rock	α -141 Extension	
	141(+31)=172	
Sealy	47 alpha Asp->His	unstable
Seattle	70 beta Ala->Asp	+ O ₂ affinity
Sendagi	42 beta Phe->Val	unstable; + O ₂ affinity
Serbia	112 alpha His->Arg	
Setif	94 alpha Asp->Tyr	unstable; + O ₂ affinity
Shaare Zedek	56 alpha Lys->Glu	
Shanghai	131 beta Gln->Pro	unstable
Shelby	131 beta Gln->Lys	unstable
Shenyang	26 alpha Ala->Glu	
Shepherds Bush	74 beta Gly->Asp	unstable; + O ₂ affinity
Sherwood Forest	104 beta Arg->Thr	

Variant Name	Residue/Chain/Mutation	Abnormal Property
Shimonoseki	54 alpha Gln->Arg	
Shuangfeng	27 alpha Glu->Lys	
Siam	15 alpha Gly->Arg	
Sinai	47 alpha Asp->His	
Singapore	141 alpha Arg->Pro	
Siriraj	7 beta Glu->Lys	
Sogn	14 beta Leu->Pro	
Southampton	106 beta Leu->Pro	unstable; ↑ O ₂ affinity
	-1 +1 +2	
South Florida	β-1 Extension Met-Met-His-Leu-	
South Milwaukee	105 beta Leu->Phe	↑ O ₂ affinity
Spanish Town	27 alpha Glu->Val	
St. Antoine	β -74-75 Deletion	unstable
	Gly-Leu->O	
St. Claude	127 alpha Lys->Thr	
St. Etienne	92 beta His->Gln	unstable; ↑ O ₂ affinity; ↑ dissociation
St. Francis	121 beta Glu->Gly	
St. Louis	28 beta Leu->Gln	unstable; ↑ O ₂ affinity; ferriHb
St. Lukes	95 alpha Pro->Arg	↑ dissociation
Stanleyville-I	68 alpha Asn->Lys	
Stanleyville-II	78 alpha Asn->Lys	
Stanmore	111 beta Val->Ala	unstable
Strasbourg	23 beta Val->Asp	
Strumica	112 alpha His->Arg	
Suan-Dok	109 alpha Leu->Arg	unstable
Summer Hill	52 beta Asp->His	
Sunnybrook	36 beta Pro->Arg	
Sun Prairie	130 alpha Ala->Pro	unstable
Sunshine Seth	94 alpha Asp->His	
Suresnes	141 alpha Arg->His	↑ O ₂ affinity
Swan River	6 alpha Asp->Gly	
Sydney	67 beta Val->Ala	unstable
Syracuse	143 beta His->Pro	↑ O ₂ affinity
T-Cambodia	26 beta Glu->Lys;	
	121 beta Glu->Gln	(D)
Tacoma	30 beta Arg->Ser	unstable; ↑ Bohr & heme-heme
Tagawa-I	90 alpha Lys->Asn	
Tagawa-II	47 alpha Asp->Gly	
Tak	β-146 Frameshift	sl. unstable; ↑ O ₂ affinity
	146(+11) ->157	
Takamatsu	120 beta Lys->Gln	
Ta-11	83 beta Gly->Cys	
Tampa	79 beta Asp->Tyr	
Tarrant	126 alpha Asp->Asn	↑ O ₂ affinity
Tashikuergan	19 alpha Ala->Glu	

Variant Name	Residue/Chain/Mutation	Abnormal Property
Terre Haute	106 beta Leu->Arg	very unstable
Thailand	56 alpha Lys->Thr -1 +1 +2	
Thionville	α -1 Extension Met-Glu-Leu-Ser	
Tianshui	39 beta Gln->Arg	
Tigraye	79 beta Asp->His	+O ₂ affinity
Tilburg	73 beta Asp->Gly	+O ₂ affinity
Titusville	94 alpha Asp->Asn	+O ₂ affinity; +dissociation
Tochigi	β -56-59 Deletion Gly-Asn-Pro-Lys->O	unstable; O ₂ affinity (?)
Tokoname	139 alpha Lys->Thr	+O ₂ affinity
Tokuchi	131 beta Gln->Glu	
Tonosho	110 alpha Ala->Thr	sl. unstable; +O ₂ affinity; +dissociation
Torino	43 alpha Phe->Val	unstable; +O ₂ affinity
Tottori	59 alpha Gly->Val	unstable
Tours	β -87 Deletion Thr->O	unstable; +O ₂ affinity
Toyama	136 alpha Leu->Arg	unstable
Toyake	142 beta Ala->Pro	unstable; +O ₂ affinity
Tübingen	106 beta Leu->Gln	unstable; +O ₂ affinity
Tunis	124 beta Pro->Ser	
Turriff	99 alpha Lys->Glu	
Twin Peaks	113 alpha Leu->His	
Ty Gard	124 beta Pro->Gln	+O ₂ affinity
Ube-1	98 beta Val->Met	unstable; +O ₂ affinity
Ube-2	68 alpha Asn->Asp	
Ube-4	116 alpha Glu->Ala	
Umi	47 alpha Asp->Gly	
Uppsala	54 alpha Gln->Glu	
Vaasa	39 beta Gln->Glu	
Valetta	87 beta Thr->Pro	
Vancouver	73 beta Asp->Tyr	+O ₂ affinity
Vanderbilt	89 beta Ser->Arg	
Vicksburg	β -75 Deletion Leu->O	stable
Villejuif	123 beta Thr->Ile	
Volga	27 beta Ala->Asp	unstable
Waco	40 beta Arg->Lys	+O ₂ affinity
Warsaw	42 beta Phe->Val	unstable; +O ₂ affinity
Warwickshire	5 beta Pro->Arg	
Wayne	α -139 Frameshift 139(+7) ->146	+O ₂ affinity

Variant Name	Residue/Chain/Mutation	Abnormal Property
Westmead	122 alpha His->Gln	
Wien	130 beta Tyr->Asp	unstable
Willamette	51 beta Pro->Arg	↑ O ₂ affinity
Winnipeg	75 alpha Asp->Tyr	
Wood	97 beta His->Leu	↑ O ₂ affinity
Woodville	6 alpha Asp->Tyr	↑ O ₂ affinity
Yahata	112 beta Cys->Arg	
Yakima	99 beta Asp->His	↑ O ₂ affinity
Yamagata	132 beta Lys->Asn	sl. ↑ O ₂ affinity
Yatsushiro	60 beta Val->Leu	
Yokohama	31 beta Leu->Pro	unstable
York	146 beta His->Pro	↑ O ₂ affinity
Yoshizuka	108 beta Asn->Asp	↑ O ₂ affinity
Ypsilanti	99 beta Asp->Tyr	↑ O ₂ affinity
Yuda	130 alpha Ala->Asp	↑ O ₂ affinity
Yukuhashi	58 beta Pro->Arg	
Yukuhashi-II	47 alpha Asp->Gly	
Yuza	21 beta Asp->Tyr	
Zaire	α-116 Insertion 116(+5)117	
Zambia	60 alpha Lys->Asn	
Zengcheng	114 beta Leu->Met	
Zürich	63 beta His->Arg	unstable; ↑ O ₂ affinity

CHAPTER 2

M A T E R I A L S A N D M E T H O D S

CHAPTER 2

1. Materials and Methods. This chapter provides a description of the methodologies used for the detection and characterization of the Hb variants; details of the particular applications are given in Chapters 3, 4, 5, 6, and 7.

2. Collection of Blood Samples. Several individuals with abnormal Hb and some of their family members were included in this research project. They originated from different countries (United States, China, India, Canada, and the former Yugoslavia). Some of these patients were detected during newborn screening programs, while others were referred for detailed investigation because of their anemia.

Blood samples were collected in vacutainers with EDTA as anticoagulant (Becton Dickinson Vacutainer System, Rutherford, N.J., USA). The samples were kept cold (4°C) at all times. Those obtained overseas were transported in wet ice by fast air service to Augusta, GA, USA.

3. Hematological Evaluation

3/a Hematological Parameters. Hematological values were obtained with a fully automated cell counter (Sysmex CC-620, Toa Medical Electronics Co., Kobe, Japan). The RBC indices, MCV (in fl) and MCH (in pg), were calculated from the values of the total Hb concentration (g/dl), PCV (in l/l), and RBC counts ($\times 10^{12}/l$).

3/b Reticulocyte Count. The brilliant cresyl blue stain method was used to estimate the reticulocyte count. Equal parts of stain (3 g sodium citrate, 0.9 g sodium chloride, 1 g brilliant cresyl blue dissolved in 100 ml distilled water) and blood were mixed in a diluting pipet. The mixture was rotated for 20 minutes, after which a smear was made. Next, the reticulocytes were counted with a microscope.

3/c Peripheral Blood Smears. A moderately thin blood smear was prepared. An air-dried smear was stained with Wright-Giemsa preparation (2 g Wright's stain, 500 mg Giemsa, 970 ml methanol, and 30 ml glycerol) for 1 minute, and an equal volume of 6.7 mM phosphate buffer was added. After 5 minutes, the slide was washed and air-dried. The cells were examined to evaluate the possible presence of an anisocytosis, poikilocytosis, hypochromia, microcytosis, and of target cells.

3/d Detection of Inclusion Bodies (Heinz bodies). A standard solution of brilliant cresyl blue in physiological saline (1 g/dl) was mixed with fresh blood in a ratio of 1:1, and the mixture incubated for 2 hours at 37°C. A moderately thin blood smear was made and examined under a microscope with an oil immersion lens (100X). Heinz bodies are expected to be present in the blood samples of carriers of an unstable Hb variant (with or without splenectomy).

4. Hb Identification and Quantitation

4/a Preparation of the Hemolysate. Red blood cells were washed three times with isotonic saline and hemolyzed with 1.5-times volumes of water and 0.4-times the volume of CCl_4 for 15 minutes at room temperature with occasional stirring. When an unstable Hb was present the addition of CCl_4 was avoided. The mixture was then centrifuged at 2,500 g for 20 minutes and the clear supernatant solution removed with a Pasteur pipet.

4/b IEF. Hb variants were initially detected by IEF using commercially available agarose gels and buffers (Isolab, Inc., Akron, OH, USA). The analysis is sensitive enough to separate Hb variants with pIs which differ by 0.02 or greater (1). Usually, 6 μl of hemolysate were applied to a gel and electrophoresed for 2 hours at 30 W constant power using an LKB Multiphor II Horizontal Unit (Pharmacia LKB, Uppsala, Sweden). A pH gradient in the agarose gel is formed during electrophoresis and the Hb variants migrate through the gel until their individual pIs equal the corresponding pH on the gel. Next the gel was fixed in 10% trichloroacetic acid for 10 minutes, and the bands were visualized with a heme-specific dye (o-dianisidine) and H_2O_2 . Band identification was by comparing the distances of migration to control containing the known Hb types of A, F, S, C, and Bart's.

4/c Cation Exchange HPLC. Cation exchange HPLC on a polyCAT A column (4.6 x 200 mm, with a particle size of 5 μm ; PolyLC, Columbia, MD, USA) was used for separation and quantitation of the normal and abnormal Hbs (2,3). The analysis was done on a Waters HPLC system (Waters Chromatography Division, Milford, MA, USA) (2,3). The column was initially purged for 5 minutes with 100% developer B (35 mM Bis-Tris, 1.5 mM KCN, 150 mM Na-acetate, 16.85 mM NH_4 -acetate, pH 7.0) and equilibrated for 10 minutes with 25% developer B and 75% developer A (35 mM Bis-Tris, 1.5 mM KCN, 3 mM NH_4 -acetate, pH 6.47). Some 50-200 μg of Hb (10-15 μl hemolysate) were applied to the column and the chromatogram was developed with a gradient of 25 to 85% developer B in 85 minutes with a flow-rate of 0.8 ml/min. The absorbance of the effluent was recorded at 415 nm with a chart speed of 0.25 cm/min. Numerous Hb variants can be separated by this procedure. Fig. 2/1 illustrates the separation of Hbs F, A, A₂, S, and C by this procedure, and gives the positions of the abnormal Hbs studied in this research project.

4/d Reversed Phase HPLC. A large-pore C₄ column (Vydac, Hesperia, CA, USA) and two water/acetonitrile/TFA developers were used to separate and quantitate the normal and abnormal globin chains (4,5). The separation of globin chains by this method depends primarily on differences in their hydrophobicities. As a general rule, the more polar (less hydrophobic) chains elute earlier in the chromatogram. The presence of TFA in the developers results in a very low pH (2.0), that causes dissociation of Hb in heme and globin chains. The column was initially purged with 100% developer A (60% acetonitrile, 0.1% TFA) and equilibrated for 10 minutes with 50% developer A and 50% developer B (20% acetonitrile, 0.1% TFA). Some 50-100 μg of Hb was applied to the column and the chromatogram was developed with a gradient of 50 to 60% developer A in 60 minutes at a flow-rate of 1.0 ml/min. The absorbance of the effluent was continuously recorded

at 220 nm with a chart speed of 0.25 cm/min. Fig. 2/2 illustrates the separation of globin chains in a newborn sample and gives the positions of the abnormal chains studied.

FIG. 2/1. Separation of Hbs F, A, A₂, S, and C in an artificial mixture, by cation exchange HPLC. The positions of the abnormal Hbs detected during this study are also given.

4/e Quantitation of Hb A₂ by Microchromatography. In several instances the Hb A₂ level was determined by anion exchange DEAE-cellulose chromatography using Tris-HCl developers (6). The resin (DE-52; Whatman, Ltd., Maidstone, Kent, UK) was equilibrated by repeated washings with 0.05 M Tris-HCl, pH 8.5, 0.1% KCN. A small column (0.5-0.8 x 6 cm) was prepared and about 100 µl of diluted hemolysate (1 drop of an 8-12 g/dl hemolysate diluted with 6 drops of distilled water and 1 drop of 2% KCN) were slowly applied. After the Hb was adsorbed onto the ion exchanger, 0.05 M Tris-HCl, pH 8.3, was gently applied. The effluent was collected in a test tube that was calibrated at 5 ml. Hb A₂, which was visible on the column as a distinct band about 2 to 5 mm wide, was eluted in about 30 minutes. The remaining Hb was eluted with the 0.05 M Tris-HCl, pH 6.5, and collected in a test tube, calibrated at 20 ml. After the volumes of the effluent fractions had been adjusted to 5 and 20 ml, respectively, the absorbance values of the two fractions were measured at 415 nm. Hb A₂ was calculated as a % of the total Hb.

4/f Quantitation of Hb F by Alkali Denaturation. In some instances, Hb F was quantitated by an alkali denaturation procedure that is based on higher resistance of Hb F to denaturation in alkali (7). The procedure was as follows: 0.2 to 0.3 ml hemolysate containing 5 to 8 g/dl Hb were pipetted into 5 ml of Drabkin's solution (1.0 g sodium bicarbonate, 0.052 g potassium cyanate, and 0.198 g potassium ferri-cyanate in 1,000 ml dis-

FIG. 2/2. Separation of the common globin chains (β^A , α^A , A_{γ}^T , G_{γ} , and A_{γ}^I) present in a newborn sample by reversed phase HPLC. The positions of the abnormal chains detected during this study are shown. Abbreviations: B, (F-)Brooklyn; C, (F-)Catalonia; D, Davenport; I, Iowa; J, (F-)Jiangsu; M, Montreal; S, S; SP, Sun Prairie; V, Volga; Y, Yokohama; Z, Zengcheng.

tilled water). The solution was mixed well and 2 ml fractions were transferred into two test tubes (one was used for the test, and one served as a control). At timed intervals, 0.2 ml 1.2 N NaOH were added to the test tube; the solution was carefully mixed, and the denaturing reaction was allowed to proceed for exactly 2 minutes. The reaction was stopped and the denatured Hb was precipitated by adding 2.0 ml saturated $(\text{NH}_4)_2\text{SO}_4$. At the same time, 0.2 ml distilled water and 2.0 ml saturated $(\text{NH}_4)_2\text{SO}_4$ were added to the control tube. Both tubes were allowed to stand for 5 minutes, whereafter the precipitate was removed by filtration through Whatman #1 filter paper. The % Hb F was calculated as the % Hb remaining in the solution.

5. Detection of Unstable Hbs. The presence of the unstable Hb variant was demonstrated by the heat stability, isopropanol, and PCMB tests.

5/a Heat Stability Test. In this test the heat stability of the Hb variant is compared with that of a normal control. The test was performed as follows: 1 ml of a freshly prepared hemolysate containing about 40 mg Hb was mixed with 40 ml of 0.1 M phosphate buffer, pH 7.4. Five ml fractions of the diluted Hb solution was pipetted into eight test tubes.

The tubes were incubated in a water bath at a constant temperature of 62°C for a period of 0, 2, 4, 6, 8, 10, 15, and 20 minutes. After incubation, the tubes were placed on ice for five minutes and the precipitate removed by centrifugation at 2,500 g for 10 minutes. The OD's of the supernatant were determined at 540 nm. The percentage of denatured Hb in each tube was calculated from the original amount of Hb present (tube 0), and that remaining in solution. A normal control was always simultaneously analyzed and the results obtained for the sample of interest were compared with those of the normal control.

5/b Isopropanol Test. The isopropanol test uses a less polar buffer solution that weakens the hydrophobic bonding of the globin molecule, and allows the test to be done at 37°C (8). The test was as follows: 0.2 ml of 10% hemolysate was transferred to 2 ml of the isopropanol buffer solution (17% isopropanol in 0.1 M Tris-HCl, pH 7.4) contained in a small stoppered tube. The solutions were mixed by inversion and placed in a water bath at 37°C for 20 minutes. An unstable Hb forms a precipitate after 5 minutes and is flocculent after 20 minutes, whereas a normal control hemolysate remains clear.

The preparative isopropanol test was used for the isolation of Hb Volga (Chapter 5b). Larger volumes were used to allow the isolation of a sufficient quantity of the unstable Hb. The incubation at 37°C was stopped at 20 minutes, and the precipitate was isolated by centrifugation. The precipitated Hb was redissolved in ice-cold 1% HCl and its globin was isolated by its addition to ice-cold acetone.

5/c PCMB Test. The PCMB test was first used for the isolation of native α and β chains (9). When normal Hb reacts with PCMB the protein dissociates into α and β subunits, but no precipitation occurs (10). However, an unstable Hb not only dissociates more readily when exposed to PCMB, but precipitation of the abnormal chain is also observed. Starch gel electrophoresis is used to evaluate the degree of the dissociation and of the precipitation of the unstable subunit. The technique is useful not so much for screening and detection of unstable Hbs, as for the confirmation and isolation of the abnormal α - and β -globin subunit.

We have noticed that Hb Yokohama readily precipitates with high concentrations of salt while performing the PCMB test. The procedure used for isolation of Hb Yokohama is given in Chapter 5a.

6. Structural Analyses

6/a Preparative DEAE-Cellulose Chromatography. Preparative anion exchange DEAE-cellulose chromatography with an NaCl gradient was used for the isolation of Hb variants that have shown an abnormal electrophoretic behavior. The resin was equilibrated by repeated washings with a stock buffer (0.2 M glycine, 15 mM KCN buffer, pH 7.5). A large column (2-3 x 60-80 cm) was prepared and some 200 mg Hb in the hemolysate were applied. The chromatogram was developed using buffers with varying quantities of NaCl, namely 0.005, 0.01, 0.02, 0.03, and 0.06 M, in the stock buffer. In some instances, the NaCl gradient was applied to the column by introducing developers with 0.03 M NaCl from a supply bottle into the closed mixing

flask containing developer with 0.005 M NaCl. The Hb fractions were either eluted directly from the column or segments of resin containing the desired Hb fractions were cut and transferred to a second chromatographic column and eluted with a stripping buffer (0.01% KCN, 0.2 M NaCl, 0.2 M glycine). Large quantities of diluted Hb solution that were obtained were often concentrated under pressure in an Amicon filtration system (Amicon International Corporation, Danvers, MA, USA) using a Diaflo ultrafiltration membrane PM 10.

6/b CM-Cellulose Chromatography. CM-cellulose chromatography with a sodium phosphate gradient (11) was used to separate α from non- α chains in total hemolysates or in samples of individual Hbs that have previously been isolated by preparative DEAE-cellulose chromatography. CM-cellulose (CM-52) was washed three times with deionized water, and 10-20 ml of developer A (0.006 M Na_2HPO_4 , 0.05 M β -mercaptoethanol, pH 6.5) were added to the resin. The mixture was stirred and poured into a sintered glass column (1 x 15 cm) to produce a final bed column of 10 cm in height. The column was equilibrated with developer A for 1 hour at a flow-rate of 30 ml/hour. About 2 ml of Hb solution, containing 50-100 mg Hb, was poured with stirring into a suitable centrifuge tube, containing 45 ml of acid acetone (acetone with 1% 12 N HCl), previously chilled to -20°C . The precipitating globin was recovered by centrifugation and washed once with ice-cold acetone. The globin (40-90 mg) was immediately dissolved in a minimum volume of developer A and applied to the column. Elution was started with developer A for 1 hour, after which a gradient system was introduced that had a linear Na^+ gradient obtained by mixing 400 ml of developer A with 400 ml of developer B (0.044 M Na_2HPO_4 , 0.05 M β -mercaptoethanol, pH 6.5), both in open cylinders. Sometimes, in order to improve the separation of some globin chains, different phosphate molarities were used. The effluent was collected with a fraction collector and the protein content of the fractions was analyzed spectrophotometrically at 280 nm. The contents of the tubes, containing an appropriate chain, were collected and urea was removed from the protein solutions by dialysis against water with Spectropor Membrane dialysis tubing having a molecular weight cut-off of approximately 3,500 (Spectrum Medical Industries, Inc., Los Angeles, CA, USA). The protein was recovered by lyophilization.

6/c Preparative Reversed Phase HPLC. Preparative reversed phase HPLC (12) was widely used in this study, especially for the isolation of the abnormal γ chains. A preparative C_{18} HPLC column (2.15 x 30 cm, Bio-Sil, TSK-ODS; Bio-Rad Laboratories, Richmond, CA, USA) and two water-acetonitrile-TFA developers were used. The column is capable of loading at least 20 mg Hb. Developer A contained 60% acetonitrile, 0.1% TFA, and developer B 20% acetonitrile, 0.1% TFA. The column was initially purged with 100% developer A, and equilibrated with the initial percentage of developers from the gradient that has to be applied. Some 5-20 mg Hb in hemolysate were applied to the column and the chromatogram was developed with a gradient that allowed the best separation of the abnormal chain in the shortest possible time. Fig. 2/3 illustrates the isolation of the $\Lambda\gamma$ -F-Jiangsu chain, obtained with a gradient of 62 to 69% developer A in 135 minutes at a flow-rate of 5 ml/min. The globin chains were detected at a wavelength of 220 nm; the abnormal chain was collected in a beaker and recovered by lyophilization.

FIG. 2/3. A preparative reversed phase HPL chromatogram on a C₁₈ column. Separation of globin chains in a total hemolysate of a newborn sample with the A_γ-F-Jiangsu variant.

6/d Aminoethylation of Cysteiny Residues. The tryptic resistant core peptides were studied after aminoethylation of the cysteiny residues. The principle of this procedure is that protein sulphydryl groups react with ethylenimine to form $-S-CH_2-CH_2-NH_2-$ groups. The structure of AE-cysteine is similar enough to the structure of lysine that peptide bonds involving this derivative will also serve as a substrate for trypsin (13-15). Some 5-10 mg of protein was dissolved in 8 M urea with 0.2% EDTA and transferred to a 4 ml screw cap vial. Thirty μ l EDTA solution (50 mg EDTA/ml), 300 μ l Tris-HCl buffer (5.23 g Tris/30 ml, pH 8.6) and 10 μ l β -mercaptoethanol were added to the protein solution. The vial was completely filled with the 8 M urea and 0.2% EDTA solution, capped under nitrogen, and kept at room temperature overnight, with constant stirring. Next day, 10 μ l of ethylenimine was added to the solution; the vial was again capped under nitrogen and allowed to remain at room temperature for 2 to 3 hours, with constant stirring. The modified protein was freed of urea and other small molecules by chromatography of the content of the vial through a Sephadex G-25 column in 0.2 N acetic acid. The protein-containing fractions were pooled and lyophilized.

6/e Tryptic Digestion of Polypeptide Chains. Tryptic digests were made from isolated chains, and in some instances, previously aminoethylated chains. Some 1-5 mg of globin chain was dissolved in 5-10 ml of deionized water. After addition of 4 mg NH_4HCO_3 , the pH was adjusted to 8.8-9.0 with 0.5% NaOH. Trypsin, dissolved in 0.001 M HCl, was added three times during the digestion, namely at 0, 30, and 120 minutes, in a ratio of 1:100 trypsin to protein. The digestion was performed at room temperature, with constant stirring, for 6 hours. The digestion was stopped by lowering the pH to 2.0 with 1 M HCl. The water was removed by lyophilization.

6/f HPLC Separation of Tryptic Peptides. The peptides, obtained after tryptic digestion, were separated on a large-pore C₁₈ column (Vydac) using a Waters HPLC system (16). The developers used were as follows: Developer A 0.01 M ammonium acetate, pH 6.0; developer B 50% acetonitrile,

50% 0.01 M ammonium acetate, pH 6.0. The column was initially purged with 100% developer B, and equilibrated for 10 minutes with 100% developer A. The sample, 0.5-2.5 mg, was dissolved in 500 μ l 10% acetic acid and applied to the column. The chromatogram was developed with 100% developer A for 10 minutes, followed by a linear gradient from 0 to 60% developer B for 120 minutes at a flow-rate of 1 ml/min. The absorbance of the effluent was continuously recorded at 220 nm with a chart speed of 40 cm/hour. The peptides were collected in test tubes and dried. Fig. 2/4 illustrates the separation of peptides in a tryptic digest of the α , AE-B, and γ chains. The positions of the abnormal peptides detected in the abnormal Hbs studied are also given.

In order to obtain pure peptides, rechromatography of particular peptides was sometimes necessary. This was done on the same column by using water-acetonitrile-TFA developers (17). Developer A was 0.1% TFA in water, and developer B was 50% acetonitrile, 0.1% TFA. The gradient applied for the development of the chromatogram depended upon the retention time in the initial chromatogram of the peptide that has to be purified.

6/g Amino Acid Analyses. The amino acid composition of the isolated tryptic peptides was determined with a Pico-Tag (Waters) amino acid analyzer in the laboratory in Augusta, GA, USA, and with a Beckman 6300 amino acid analyzer (Beckman Instruments, Inc., Palo Alto, CA, USA) in the laboratory in Skopje, Macedonia.

The Pico-Tag method was performed in three steps, according to the procedure recommended of the manufacturer: i) hydrolysis of the peptide samples, ii) pre-column derivatization of the samples, and iii) analysis by reversed phase HPLC.

i. The dried peptide samples were redissolved in 100 μ l water-acetonitrile solution (1:1 by volume), and transferred into the sample tubes. The sample tubes were placed into the reaction vial and dried under vacuum on the Work Station. Two hundred μ l of phenol-HCl solution (6 N HCl with 1% phenol) were pipetted onto the bottom of the reaction vial. After three alternate cycles of vacuum-nitrogen purging on the Work Station, the reaction vial was sealed under vacuum and placed in the oven at 105-110°C for 20-24 hours. Following hydrolysis, the samples were evaporated and 10 μ l of redrying solution (ethanol-water-triethylamine, 2:2:1 by volume) were added to each of the sample tubes. The samples were redried on the Work Station.

ii. Next, the samples were derivatized with PITC to produce PTC amino acids. Twenty μ l of freshly prepared derivatization reagent (ethanol-triethylamine-water-PITC, 7:1:1:1 by volume) were added to each redried sample. The samples were kept at room temperature for 20 minutes, and dried under vacuum on the Work Station.

iii. The PTC amino acids were separated on a reversed phase HPLC C₁₈ column that was kept in a column heater at 38°C. The developers used were as follows: Developer A 6% acetonitrile in 0.14 M sodium acetate trihydrate, 0.05% TEA, pH 6.2; developer B 60% acetonitrile, 40% water.

FIG. 2/4. Separation of tryptic peptides by reversed phase HPLC. Shown are soluble tryptic peptides of the α , AE- β , and A_γ chains. The positions of peptides $G_\gamma T-15$ and $A_\gamma T-9$ are given (peaks with dashed lines). The positions of the abnormal peptides detected in this study are also indicated. Abbreviations: B, (F-)Brooklyn; C, (F-)Catalonia; D, Davenport; I, Iowa; J, (F-)Jiangsu; M, Montreal; S, S; SP, Sun Prairie; V, Volga; Y, Yokohama; Z, Zengcheng.

The column was initially purged with 100% developer B, and equilibrated with 100% developer A. The samples were dissolved in 40 μ l of sample diluent solution (5% acetonitrile in 5 mM Na_2HPO_4 , pH 7.4) and 20 μ l were

applied to the column. The chromatogram was developed with a gradient of 0 to 50% developer B in 25 minutes with a flow-rate of 1.0 ml/min. The amino acids were monitored at a wavelength of 254 nm and the amino acid concentrations were calculated automatically by comparison with the standard sample that was applied with every five samples.

Another method used for the determination of the amino acid composition of hydrolyzed peptides was based on the reaction of the amino acids with the ninhydrin reagent. The separation of the amino acids was done on a sodium high performance column with Na-A, Na-B, and Na-D high performance buffers, supplied by the manufacturer (6300 amino acid analyzer; Beckman). The chromatogram was developed according to the protocol recommended (System 6300/7300 Application Notes A6300-AN-002B). The amino acids were recorded at 540 nm, and the amino acid concentrations were calculated with an integrator (SP 4290, Spectra Physics, San Jose, CA, USA).

6/h Sequence Analyses by Microsequencing Procedures. The amino acid sequence of some peptides was done manually with a microsequencing double coupling procedure described by Chang et al (18). Each degradation was performed in six steps:

i. Coupling: The dried peptide was dissolved in 80 μ l of 50% pyridine, 50% water, transferred to the centrifuge tube, and 40 μ l DABITC solution (10 nmol DABITC/ μ l pyridine) were added. The tube was flushed with nitrogen, covered with parafilm, and placed in a heating block at 50°C for 45 minutes. Next, 10 μ l PITC were added and the second coupling reaction was allowed to proceed at 50°C for 30 minutes.

ii. Extraction: The excess reagents and by-products were removed by mixing with two portions of 400 μ l n-heptane:ethylacetate solution (2.5:1 by volume) on a vortex mixer, followed by centrifugation. The organic phase was removed and discarded, while the water phase was dried under vacuum.

iii. Cleavage: The dried material was dissolved in 50 μ l anhydrous TFA, flushed with nitrogen; the tube was covered with parafilm and heated in a heat block at 50°C for 15 minutes. The sample was then dried in a vacuum desiccator.

iv. Extraction: The dried material was dissolved in 30 μ l distilled water, after which the cleaved amino acid was extracted by mixing with two portions of 50 μ l butylacetate on a vortex. The mixture was centrifuged and the butylacetate extract, that contained the cleaved amino acid, was transferred to a clean tube. The rest of the peptide that remained in the water phase was evaporated and subjected to the next degradation cycle.

v. Conversion: The butylacetate extract was dried and redissolved in 30 μ l 50% TFA, 50% water. Conversion of thiazolinones of amino acids into thiohydantoin was carried out at 50°C for 50 minutes. The sample was dried and redissolved in 10-20 μ l ethanol.

vi. Identification: The identification of DABTH-amino acids was done on micropolyamide sheets (3 x 3 cm; Schleicher and Schuell, Keene, NH, USA) with a two-dimensional separation. The synthetic marker was co-chromatographed with each sample to facilitate identification. The marker was prepared as follows: 500 μ l 50% pyridine, 60 μ l diethylamine, 30 μ l ethylamine, and 565 μ g DABITC were mixed, heated at 55°C for 1 hour, dried under vacuum and redissolved in ethanol. About 1-2 μ l of the marker was applied to the polyamide sheet using a microcapillary tube. The spot was allowed to dry, whereafter the sample was applied at exactly the same spot. The separation was done in small containers with the following solvents: Solvent A 33% acetic acid, 67% water, was used for the first dimensional separation; solvent B toluene-n-hexane-acetic acid (2:1:1 by volume) was used for the second dimensional separation. The sheets were dried and the color developed by exposing to HCl vapor.

7. DNA Analyses

7/a Isolation of Human Genomic DNA From Leukocytes. DNA was isolated from the white blood cells by the method of Poncz et al (19). About 5-10 ml whole blood was centrifuged at 1,500 g for 10 minutes; the plasma was discarded; most of the white blood cells were transferred to another tube and used for DNA isolation, while the remaining red blood cells were saved for Hb analyses. The leukocytes were washed twice with 30-30 ml 1X reticulocyte saline (104 mM NaCl, 4 mM KCl, 6.8 mM MgCl₂) and centrifuged at 2,500 g for 10 minutes at 4°C. The red blood cells were hemolyzed with 30 ml of freshly prepared lysing solution [131 mM NH₄Cl, 0.9 mM (NH₄)₂CO₃, pH 6.5] at room temperature for 20 minutes with occasional shaking. The white blood cells were pelleted by centrifugation at 2,500 g for 10 minutes at 4°C. The supernatant was removed and the lysing procedure was repeated once more. The pellet, consisting mainly of white blood cells, was resuspended in 10 ml STE buffer (0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5, 1 mM EDTA) and 1 ml of 10% SDS, whereafter 0.1 ml of proteinase K (10 mg/ml in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA) was added; the mixture was incubated at 37°C overnight, without shaking. The mixture became viscous upon incubation. The proteins were removed by phenol-chloroform-isoamylalcohol extractions as follows: An equal volume of phenol saturated with 20 mM Tris-HCl, pH 8.0, containing 0.1% hydroxyquinoline as an antioxidant, was added to the viscous solution, and the mixture was gently shaken for 10 minutes at room temperature, cooled on ice for 10 minutes, and centrifuged at 2,500 g for 10 minutes at 4°C. The upper aqueous phase was transferred to a clean tube and the phenol extraction was repeated once more. Next, an equal volume of chloroform-isoamylalcohol (19:1 by volume) was added; the mixture was gently shaken for 10 minutes and centrifuged at 2,500 g for 10 minutes at 4°C. The upper aqueous phase was transferred to a fresh tube and the DNA was precipitated with 5 volumes of ice-cold absolute ethanol. The DNA precipitate was transferred to a clean 1.5 ml Eppendorf tube, washed twice with 70% ethanol, and dried under vacuum. The pellet was dissolved in 1 ml TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) by incubation at 37°C overnight. The DNA concentration was determined from its optical density at 260 nm (1 OD = 50 μ g/ml DNA). The DNA was stored at 4°C.

7/b PCR Amplification. PCR is based upon the *in vitro* amplification of a specific DNA fragment flanked by two synthetic oligonucleotide primers, complementary to the opposite strands of the desired fragment (20). Repeated cycles of denaturation, annealing, and extension result in an exponential accumulation of the target fragment (10^6 -fold). The use of a heat-stable DNA polymerase and a programmable thermocycler automates the procedure (21). The PCR method was used in order to obtain large amounts of specific double-stranded DNA fragments, used in the dot-blot hybridization, and for restriction enzyme digestion or to generate large amounts of specific single-stranded DNA fragments, used in DNA sequencing analysis.

The oligonucleotides, serving as amplification primers, as well as the sequencing primers used in the DNA sequencing analysis and specific probes used in the dot-blot analysis, were synthesized by a solid-phase oligonucleotide synthesis method (22) on a 380 B DNA synthesizer (Applied BioSystems, Inc., Foster City, CA, USA) in the laboratory in Augusta, GA, and on a Plus 1 DNA synthesizer (Beckman) in the laboratory in Skopje, Macedonia.

The PCR was performed in three phases using an Automated Thermocycler (Perkin Elmer Cetus, Norwalk, CT, USA). The reaction mixture contained 1 μ g genomic DNA, 20 mM MOPS, pH 7.8, 50 mM NaCl, 2.5 mM $MgCl_2$, 200 μ M of each dNTP, and 100 pmol of each of the oligonucleotide primers, in a total volume of 100 μ l. For the generation of single-stranded DNA templates, an unbalanced ratio of 100 pmol to 1 pmol was used. In the first phase, the genomic DNA was denatured at 95°C for 5 minutes. The temperature was decreased to 80°C and 2.5 U of Taq polymerase was added to each sample. Samples were overlaid with 2-3 drops of mineral oil to prevent evaporation. In the second phase, the samples were subjected to 35 repeated cycles of denaturation at 95°C for 1 minute, annealing of the primers at 56°C for 1 minute, and extension of the annealed primers at 72°C for 2 minutes. The temperature of annealing depends upon the sequences of the primers, while the time of the primer extension depends upon the length of the region that has to be amplified. In the third phase, the samples were incubated for 10 minutes at 72°C, so that a complete extension of all DNA strands synthesized in the previous step was obtained. The amplification product was checked by applying 5 μ l of each reaction on a 1.5% agarose gel in the presence of ethidium bromide (10 μ g/ml) along with a Hind III-digested λ DNA as a size marker. The samples were kept at 4°C for further analyses.

7/c DNA Sequencing. DNA sequencing was used in this study for the determination of the primary sequence of the coding region of the abnormal β -Montreal and β -Yokohama genes. The analysis was performed on a single-stranded, PCR-amplified, DNA template in three phases: i) purification of the single-stranded DNA template, ii) sequencing reaction, and iii) denaturing PAGE.

i. Purification of the Single-Stranded DNA Template: The single-stranded amplified DNA was purified by precipitation with 1 volume of 2.5 M ammonium acetate and 2 volumes of ice-cold ethanol at room temperature for 15 minutes. The mixture was centrifuged for 15 minutes at 4°C; the

resulting pellet washed four to five times with 70% ethanol, dried under vacuum, and dissolved in 10 μ l water. The recovery and DNA concentration were estimated on a 1.5% agarose gel by comparison with 0.3 μ g M-13 control single-strand DNA.

ii. Sequencing Reaction: The sequencing reaction was done with a dideoxy chain termination method (23), using a Sequenase Version 2.0 DNA sequencing kit [United States Biochemical (USB), Cleveland, OH, USA]. The reaction was performed in three steps (annealing, labeling, and termination), following the protocol recommended by the manufacturer.

The following reagents were used to anneal the primer to the template: 50 μ l of sequencing primer, 2 μ l of 5X reaction buffer (200 mM Tris-HCl, pH 7.0, 100 mM MgCl₂, 250 mM NaCl), 0.3 μ g of the purified single-stranded DNA template in a total volume of 10 μ l, adjusted with sterile water. The mixture was incubated at 65°C for 3 minutes and allowed to cool slowly to room temperature over a period of about 30 minutes.

The labeling reaction was achieved by adding the following reagents to the annealed template: 1.0 μ l 0.1 M DTT, 2.0 μ l diluted labeling mix (7.5 μ M dATP, 7.5 μ M dTTP), 0.5 μ l α -³⁵S-dATP (800 Ci/mM, 10.0 mCi/ml; DuPont, Boston, MA, USA), and 2.0 μ l of diluted sequenase enzyme. The sequenase enzyme (13 U/ μ l) was diluted 1:8 in enzyme dilution buffer (10 mM Tris-HCl, pH 7.5, 5 mM DTT, 0.5 mg/ml BSA). The reaction mixture was incubated at room temperature for 3 minutes.

Termination reaction: 2.5 μ l of each termination mixture (ddATP, ddCTP, ddGTP, ddTTP) were pipetted into four tubes labeled A, C, G, and T. Each termination mixture consists of 50 μ M NaCl, 80 μ M of each dATP, dCTP, dGTP, and dTTP, and 8 μ M of the corresponding ddNTP. The tubes were pre-warmed at 37°C for 1 minute. After the labeling reaction was completed, 3.5 μ l of the reaction mixture was added to each of the labeled tubes. The tubes were incubated at 37°C for 5 minutes, after which the reaction was stopped by adding 4 μ l of Stop solution (95% formamide, 20 mM EDTA, 0.05% Bromphenol Blue, 0.05% XyleneCyanol FF). The samples were kept on ice until loaded onto the gel.

iii. Denaturing Gel Electrophoresis: A 0.4 mm thick 8% polyacrylamide gel in 7 M urea-TBE buffer gel was prepared 2 to 20 hours prior to use. The gel was run on a vertical electrophoresis apparatus for DNA sequencing (Model S2; Bethesda Research Laboratories, Gibco-BRL, Gaithersburg, MD, USA) using a high voltage constant power supply (2297-Microdrives; LKB-Broma, Uppsala, Sweden). Prior to loading the samples, the gel was pre-electrophoresed for 30 minutes at 70 W constant power. The DNA samples were denatured at 75-80°C for 3 minutes and 3 μ l of each reaction were applied to the gel. The gel was run at 75 W constant power (1,400-1,600 V; 50-60 mA) for 2-4 hours. Next, the gel was soaked in a 10% acetic acid-12% methanol solution for 30 minutes, transferred to (Whatman) 3 MM paper, and dried under vacuum at 80°C on a gel dryer (Slab Dryer, Model 483; Bio-Rad) for about 40 minutes. The dried gel was exposed directly to Kodak X-ray film (Eastman Kodak Company, Rochester, N.Y., USA) for 10 to 16 hours.

7/d Dot-Blot Hybridization Analyses. Dot-blot hybridization was used for the verification of the nt changes found in Hb Montreal and for the localization of the mutation in Hb Sun Prairie on the α -globin genes. The analyses were performed in three steps: i) preparation of dot-blot membranes, ii) 5' end-labeling of the normal and mutant oligonucleotides and their purification, and iii) dot-blot hybridization.

i. Preparation of Dot-Blot Membranes: The double-stranded PCR amplified DNA was denatured by adding 20 μ l 4 M NaOH, 0.25 M EDTA in a total volume of 200 μ l, adjusted with water. The mixture was kept at room temperature for 30 minutes. The denatured DNA was blotted in duplicate onto a nylon membrane (Zeta Probe Nylon Membrane; Bio-Rad) under vacuum using a spotting device (Bio-Dot; Bio-Rad). The DNA was fixed to the membrane by baking in a vacuum oven at 80°C for 2 hours.

ii. 5' End-Labeling of the Oligonucleotides and Their Purification: Two allele-specific oligonucleotide probes (normal and mutant) were labeled at the 5' end with γ -³²P-ATP (7,000 Ci/mM, 160 mCi/ml; ICN Biochemicals, Inc., Irvine, CA, USA). The labeling was done in an Eppendorf tube by mixing 50 pmol of the oligonucleotide, 5 μ l of the 10 x T₄ polynucleotide kinase buffer (0.5 M Tris-HCl, pH 7.6, 100 mM MgCl₂, 100 mM β -mercaptoethanol), 50 μ Ci γ -³²P-ATP, and 3-6 U of cloned T₄ polynucleotide kinase (30 U/ μ l; USB) in a total volume of 50 μ l, adjusted with water. The reaction mixture was incubated at 37°C for 1 hour.

The unincorporated radioactive nts were removed from the probes by using commercially available Nensorb (NEN) columns (NEN Products, DuPont) under the conditions recommended by the manufacturer. The column was washed with 2 ml 100% methanol and equilibrated with 2 ml reagent A (0.1 M Tris-HCl, 10 mM triethylamine, 1.0 mM disodium EDTA, pH 7.7). The labeled DNA mixture was diluted with 400 μ l reagent A and applied directly to the column. Next, the unincorporated radioactive nts were removed by washing the column with 2 ml reagent A. The 5' end-labeled oligonucleotide was eluted from the column with 500 μ l reagent B (50% methanol) and collected in an Eppendorf tube. The activity of the probes as CPM was determined in a scintillation counter (Beckman).

iii. Dot-Blot Hybridization: The duplicate membranes were prehybridized for 30 minutes at 55°C in separate, sealed plastic bags, with 10 ml of a prehybridization solution containing 5 x SSPE, 5 x Denhardt's, 0.5% SDS (20 x SSPE = 3.6 mM NaCl, 10 mM NaH₂PO₄, 20 mM Na₂EDTA, pH 7.4; 50 x Denhardt's = 1% Ficoll, 1% PVP, 1% BSA). The probe (50-100 x 10⁶ cpm) was added to the particular membrane and the hybridization was conducted for 1 to 2 hours in a circulating water bath. The temperature of the hybridization depends upon the T_m of the probe [$T_m = 4 \times (G + C) + 2 \times (A + T)$]. The membranes were washed twice with 2 x SSPE, 0.1% SDS solution at room temperature for 10 minutes. The next washing was done with TMAC washing solution (3.0 M TMAC, 50 mM Tris-HCl, pH 8.0, 2.0 mM EDTA, 0.1% SDS) for 20 minutes at 42°C, followed by another wash at 58°C for 30 minutes. The use of TMAC in a washing solution equilibrates the A-T and G-C bonding strength differences, thus allowing the use of the same temperature for stringent washes for probes of the same size (24). Following washings,

the membranes were dried, wrapped in plastic film, and exposed to Kodak x-ray film (Eastman Kodak) for 2 to 12 hours.

7/e HLA DQ α Typing. AmpliTypeTM HLA DQ α Forensic DNA Amplification and Typing Kit (Perkin Elmer Cetus) was used for the detection and identification of human leukocyte antigen (HLA) DQ α alleles. The test was primarily used for paternity studies. The kit distinguishes six alleles (HLA DQ α 1.1, 1.2, 1.3, 2, 3, and 4) that define 21 different genotypes. The procedure was performed in three steps as recommended by the manufacturer: i) PCR amplification, ii) hybridization, and iii) color development.

i. PCR Amplification: The reaction mixtures contained 0.5 μ g (0.1 to 20 μ l) DNA, 50 μ l PCR buffer containing HLA DQ α sequence-specific biotinylated primers, AmpliTaq DNA polymerase, dATP, dCTP, dGTP, and dTTP, and 50 μ l 0.8 mM MgCl₂. The samples were overlaid with mineral oil and subjected to 35 cycles of denaturation (95°C for 1 minute), annealing (60°C for 30 seconds), and extension (72°C for 30 seconds) on a Thermocycler (Perkin Elmer Cetus). The denaturation step in the first cycle and the extension step in the last cycle are prolonged for 4 and 7 minutes, respectively.

ii. Hybridization: Nine sequence specific DNA probes, which distinguish six HLA DQ α alleles, are spotted onto a DNA probe strip that is part of the kit. DNA probe strips are placed in separate wells of the typing tray and 3.3 ml hybridization-enzyme conjugate solution [3.3 ml hybridization solution (5 x SSPE, 0.5% SDS) and 27 μ l enzyme conjugate solution (horseradish peroxidase-streptavidin conjugate) per strip] are pipetted into each well. The PCR-amplified DNA is denatured at 95°C for 3 to 10 minutes, and immediately added to the contents of each well. The tray is covered by a clean plastic lid, and the amplified DNA is hybridized to the probes by incubation in a shaking water bath at 55°C for 20 minutes. Following hybridization, the contents of each well are aspirated and the strips rinsed with 10 ml washing solution (2.5 x SSPE, 0.1% SDS). The stringent wash is done with washing solution (10 ml per strip) in a shaking water bath at 55°C for 10 minutes, followed by another wash at room temperature for 5 minutes.

iii. Color Development: 10 ml 0.1 M sodium citrate buffer, pH 5.0, is pipetted into each well, and the tray is placed on an orbital shaker at room temperature for 5 minutes. The color development solution is prepared 10 minutes before use by mixing 10 ml sodium citrate buffer, pH 5.0, 10 ml 3% hydrogen peroxide, and 0.5 ml chromogen solution for each strip (chromogen solution = 30 mg of 3'3', 5,5'-TMB dissolved in 15 ml reagent grade 100% ethanol). The citrate buffer is aspirated from the wells and 10 ml of newly prepared color development solution is added to each well, the tray is covered with aluminum foil, and the color allowed to develop at room temperature for 20 to 30 minutes. The color development is stopped by washing the strips in deionized water. Fig. 2/5 shows the HLA DQ α typing results for two families with a child carrying an unstable Hb. The results confirmed a de novo mutation in both probands. For details see Chapters 5a and 5b.

FIG. 2/5. HLA DQ α typing results for two families with a child having an unstable Hb variant: Hb Yokohama [β 31(B13)Leu→Pro] (A), and Hb Volga [β 27(B9)Ala→Asp] (B). F = father; M = mother, P = proband.

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CHAPTER 3a

Hb DAVENPORT or $\alpha_{278}(\text{EF7})\text{Asn} \rightarrow \text{His}\beta_2$

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CHAPTER 3

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α CHAIN VARIANTS

IDENTIFIED BY HPLC

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CHAPTER 3a

HB DAVENPORT OR $\alpha_278(\text{EF7})\text{ASN}\rightarrow\text{HIS}\beta_2$

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ABSTRACT

Hb Davenport is a new, stable α chain variant, that was detected in two members of a Caucasian family living in Iowa. Hematological data were within normal limits. It is characterized by an Asn $\rightarrow$ His replacement at position α_78 . Mild acidic hydrolysis of the large $\alpha\text{T}-9$ peptide, separation of the resulting fragments by reversed HPLC, and sequence analysis of a few of these peptides greatly facilitated the identification of this substitution.

INTRODUCTION

Of the 156 amino acid substitutions in the α chain of Hb listed in Ref. 1, 38 have been observed in the peptide α T-9. This large tryptic peptide contains 29 amino acid residues, occupies positions 62 through 90 of the α chain and is characterized, among others, by high alanine and aspartic acid contents. Three Hb variants have been detected in which one of the seven alanine residues in this peptide is replaced by another amino acid, while some 20 variants have been described in which one of the six aspartic acid or asparagine residues is replaced by another amino acid (see Ref. 1 for details). The characterization of these variants is often complicated, and a distinction between substitutions such as Asp $\rightarrow$ His, Asp $\rightarrow$ Asn or Asn $\rightarrow$ Asp at positions 64, 68, 74, 75, 78, and 85 is not easily made. A few years ago we introduced in our laboratory a relatively mild hydrolysis procedure (described below) in which the α T-9 peptide is hydrolyzed at the N- and C-terminal ends of the aspartyl residues but not of the asparagine residues; the resulting smaller fragments are then separated by reversed phase HPLC and analyzed. With this method we have identified an Asn $\rightarrow$ His replacement at position α 78 in a Hb variant found in a family from Iowa.

MATERIALS AND METHODS

Blood samples were collected in vacutainers with EDTA as anticoagulant and studied at the local institution and at the laboratories in Augusta, GA. Informed consent was obtained.

Hematological data were collected with automated cell counters. Red cell lysates were studied by IEF (2), citrate agar electrophoresis (3) by cation exchange HPLC (4,5), and by reversed phase HPLC (6,7). The abnormal α chain (α^X) was separated from the normal α and β chains by CM-cellulose chromatography (8), and digested with trypsin for 6 hours at room temperature and at pH 8.5. The resulting peptides were separated by reversed phase HPLC (9) and their amino acid compositions were determined by a Waters PicoTag amino acid analyzer system.

Peptide α T-9 was isolated from about 2 mg of α^X chain (0.1-0.15 μ Mole), dissolved in 2.0 ml 2.2% formic acid (HCOOH) and hydrolyzed in a sealed tube under a mild vacuum for 5 hours at 110°C in a heating block (10). The resulting fragments were recovered by freeze-drying and separated by reversed phase HPLC with the developing system described in Ref. 11. The amino acid compositions of the isolated fragments were determined with the same amino acid analyzer, while sequence analysis of the amino termini of the two peptides was as described in Ref. 12.

RESULTS

The propositus, an infant girl, was tested for the presence of an abnormal Hb through the facilities of the University of Iowa Hygienic Laboratory. She and her father had a Hb variant which moved between Hb A and

Hb F in IEF and eluted slightly faster than Hb A₂ in cation exchange HPLC. The family is of German descent but has been established in Iowa for several generations. Hematological data for the father were most complete and were normal. The concentration of the variant was estimated at 20% (Hb X + Hb A₂ was 22.3%) and that of the slower eluting abnormal Hb A₂ (i.e. $\alpha_2^{\text{X}}\beta_2$) at 0.4%. The α^{X} chain eluted slightly ahead of α^{A} on reversed phase HPLC.

Fig. 3a/1 (top) illustrates the separation of the soluble peptides from a tryptic digest of the α^{X} chain. All peptides except for the core peptide $\alpha\text{T-12}$, were recovered and identified. The $\alpha\text{T-9}$ peptide was recovered as $\alpha\text{T-9}$, as $\alpha\text{T-8,9}$, as $\alpha\text{T-9,10}$, and even as $\alpha\text{T-8,9,10}$. The amino acid compositions of these four peptides (Table 3a/1) readily identified the fragments and detected an Asp (or Asn)->His replacement.

FIG. 3a/1. Top: Separation of the soluble peptides in a tryptic digest of the isolated α^{X} chain by reversed phase HPLC (9). **Bottom:** Separation of the fragments after mild acidic digestion of the $\alpha\text{T-9}^{\text{X}}$ peptide by reversed phase HPLC (11). Amino acid composition data identified the locations of these fragments in the intact α chain (listed at the right).

Peptide $\alpha\text{T-9}^{\text{X}}$ was also isolated from a second chromatogram and hydrolyzed with 2.2% HCOOH as described. The separation of the fragments by reversed phase HPLC with the developers described by Schroeder (11) is

TABLE 3a/1. Amino Acid Compositions of the α T-9^X and the Fragments Obtained After Mild Hydrolysis With 2.2% HCOOH^a

Amino Acid	T-9 ^X	T-8,9 ^X	T-9 ^X ,10	T-8,9 ^X ,10	Fragments ^b			
					3	4	5	6
Aspartic Acid	<u>4.88(6)</u>	<u>4.99(6)</u>	<u>5.00(6)</u>	<u>5.01(6)</u>	2.00	1.28	<u>0</u>	<u>0.72</u>
Serine	1.71(2)	1.77(2)	1.77(2)	2.03(2)			1.89	1.88
Histidine	<u>3.72(3)</u>	<u>3.87(3)</u>	<u>3.62(3)</u>	<u>3.94(3)</u>	0.98	0.98	<u>1.00</u>	<u>3.01</u>
Arginine	0	0	1.02(1)	1.03(1)				
Threonine	0.88(1)	0.87(1)	0.91(1)	1.00(1)	0.89	0.90		
Alanine	7.00(7)	7.07(7)	7.02(7)	7.04(7)	2.80	2.90	2.06	2.80
Proline	1.03(1)	0.97(1)	1.20(1)	0.94(1)			0.98	0.92
Valine	2.91(3)	3.00(3)	3.00(3)	3.10(3)	2.00	2.05		
Methionine	0.86(1)	0.89(1)	0.88(1)	0.86(1)			0.67	0.75
Leucine	4.01(4)	4.01(4)	4.94(5)	5.04(5)	1.00	1.01	2.13	2.98
Lysine	1.00(1)	2.00(2)	1.11(1)	1.99(2)				1.00
No. of Residues	29	30	31	32	10	9	9	15
Positions in α^X	62-90	61-90	62-92	61-92	65-74	65-73	76-84	76-90

^a In moles/mole.

^b See Fig. 3a/1 (bottom).

shown in Fig. 3a/1 (bottom section), while the amino acid compositions of four fragments are listed in Table 3a/1. Sequence analysis of the amino termini of the peptides in zones 5 and 6 located an Asn→His replacement in the third position of both.

DISCUSSION

The mild hydrolysis of peptide α T-9, followed by the complete separation of the resulting fragments by reversed phase HPLC, greatly facilitated the identification of the substitution in this α chain variant. The positions in the α chain of the five fragments isolated by this method, are shown in the following scheme. The circled numbers refer to the peaks in Fig. 3a/1 (bottom) and to the amino acid compositions listed in Table 3a/1.

These results readily identified an Asx→His replacement in fragments ⓕ and ⓖ, while the sequence analyses characterized it as an Asn→His substitution in the third position of both peptides; i.e. position α 78. This mutation (presumably AAC→CAC in CD 78) has not been observed before. The new variant was named Hb Davenport after the city of residence of the family.

Hb Davenport is the 39th variant with a substitution of an amino acid residue in the α T-9 peptide. Other variants in which the Asn residue is replaced are Hb Stanleyville-II (α 78 Asn→Lys) (13) and Hb J-Singa (α 78 Asn→Asp) (14). Asn α 78(EF7) is not involved in any major contact with the heme group or in contacts between chains, and its replacement by another amino acid is not likely to affect Hb stability. Hb Davenport carriers are not clinically affected by the presence of this variant.

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CHAPTER 3b

HB SUN PRAIRIE OR $\alpha_2130(H13)ALA \rightarrow PRO\beta_2$;
SECOND OBSERVATION IN AN INDIAN ADULT

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Soon after the study of the patient M.R. with an apparent Hb Sun Prairie homozygosity was completed and recorded (see Ref. 1) we observed the same α chain variant in a young 20-year-old Indian male with clinical and hematological features of a chronic hemolytic anemia. His Hb level was 10.8 g/dl and PCV value was 0.29 l/l; a distinct reticulocytosis was present (12%), while the blood smear was characterized by anisopoikilocytosis, microcytosis with hypochromia, and target cells. Heat stability tests were essentially negative. A modest icterus was noted and gallstones were detected by X-ray of his abdomen. The availability of a red cell lysate and isolated DNA permitted only limited studies, but these provided additional data important for the characterization of this new variant.

The Hb abnormality was undetectable by electrophoresis at alkaline pH and by IEF; the methods have been described before (2,3). In a PolyCAT cation exchange HPL chromatogram (4,5), a shoulder appeared on the Hb A zone, and the Hb A₂ zone appeared complex, suggesting the presence of additional Hb components (Fig. 3b/1, top). The abnormal α chain was readily detected by reversed phase HPLC (6,7) eluting well ahead of the normal α chain (Fig. 3b/1, bottom). The α^X chain was isolated by preparative reversed phase HPLC (8) and studied as described (1). The tryptic peptide separation was similar to that shown in Fig. 3 of Ref. 1, except that a slower gradient allowed a complete separation of the $\alpha T-13^X$ and $\alpha T-5 + \alpha T-6$ peptides. The amino acid composition of $\alpha T-13^X$ was: Ser 2.90(3); Thr 1.84(2); Ala 0(1); Pro 0.99(0); Val 2.00(2); Leu 2.02(2); Phe 1.10(1); Lys 0.99(1), with values for the normal $\alpha T-13$ between parentheses; this leaves no doubt that the only alanine residue in this peptide, $\alpha 130$, is replaced by proline.

FIG. 3b/1. Top: Separation of the Hb fractions present in the whole red cell lysate of patient H.C. by PolyCAT cation exchange HPLC (4,5). Hb X_2 is eluted slightly later than Hb A_2 ; separation is incomplete. Bottom: Separation of the globin chains present in a total red cell lysate of patient H.C. by reversed phase HPLC (6,7).

Because of the apparent heterogeneity of the Hb A and Hb A_2 zones we decided to isolate these for further analysis. This was accomplished by preparative DEAE-cellulose chromatography (9), which allows the rapid separation of these Hbs (Fig. 3b/2, top left). Hb A_2 and two zones of Hb A were eluted from this material and their chain composition was studied by reversed phase HPLC. The results are given in the three chromatograms which make up the remainder of Fig. 3b/2. The Hb A_2 zone contained no β chain, but only δ , α , and 28% α^X . The two Hb A zones contained a considerably smaller quantity of α^X , and the values of 10.8-12.3% were nearly the same as that found in the whole red cell lysate (Fig. 3b/1, bottom). The slightly higher level of α^X in fraction 2 of Hb A suggests a partial resolution of Hb A and Hb X.

FIG. 3b/2. **Top left:** Diagram illustrating the separation of Hb A (plus Hb X) and Hb A₂ (plus Hb X₂) on a DEAE-cellulose column (9). Fractions numbered 1, 2, and 3 were isolated and concentrated, and analyses by reversed phase HPLC (6,7) are presented.

This study of a second patient with the Hb Sun Prairie variant provided two surprising pieces of information. First, the level of the α^X chain (and thus of Hb Sun Prairie) in this Indian adult appeared to be higher than that of the child with an apparent homozygosity for this variant. Second, the relative quantity $\alpha_2^X\delta_2$ in the Hb A₂ zone (28%) is about 2.5 times that of $\alpha_2^X\beta_2$ in the Hb A zone (11-12%). This may be explained by: 1) A difference in stability with Hb X₂ ($\alpha_2^X\delta_2$) having a near normal stability, or 2) a greater affinity of the α^X chain for δ chains than for β chains.

Because of these new findings we decided to analyze a fresh blood sample from the child (M.R.) in whom Hb Sun Prairie was first discovered. The sample was collected in EDTA and shipped in wet ice by overnight courier to Augusta, GA where it was analyzed the next day and at various periods thereafter. The quantitative data are listed in Table 3b/1. The total quantity of α^X in the freshly collected sample was found to be 10.3% which is the same value as that observed in the hemolysate of the second patient H.C. (Table 3b/1). Aging of the sample resulted in a decrease of the α^X levels in the total hemolysate and in all Hb fractions. This suggests that the stability of the abnormal Hb A₂ ($\alpha_2^X\delta_2$) is similar to that for

the abnormal Hb A ($\alpha_2^X\beta_2$), and that the high $\alpha_2^X\delta_2$ level in the Hb A₂ fraction is probably due to a greater affinity of the α^X chain for δ chains than for the β chain.

TABLE 3b/1. Relative Quantities of the α^X Chain in Total Red Cell Lysates and Hb Fractions From DEAE-Cellulose Columns^a

Patient --->	H.C.		M.R.	
Condition of Sample	10 Days in Transit	Fresh	10 Days at 4°C	3 Mos. at -70°C
Whole Lysate	10.4	10.3	8.9	3.8
A ₁ (zone 1) ^b	10.8	8.8	2.0	0.2
A ₀ (zone 2) ^b	12.3	11.9	6.5	0.4
A ₂ (zone 3) ^b	28.0	24.0	21.2; 19.4	9.5

^a Data in % α^X of total $[100 \cdot \alpha^X / (\alpha + \alpha^X)]$.

^b See Fig. 3b/2 for the origin of these zones.

Another major concern was the similar quantities of the α^X chain (ca. 10-11%) in the red cell lysates obtained from the two patients, one (M.R.) being homozygous and the other (H.C.) perhaps heterozygous. In order to further evaluate the Hb Sun Prairie gene dosage in patient M.C., DNA samples from each individual were amplified and hybridized with synthetic oligonucleotides specific for the G→C mutation in CD 130 of the α_2 -globin gene, as described in Ref. 1. The results are illustrated in Fig. 3b/3. Patient H.C. is indeed heterozygous and the G→C mutant site is at CD 130 of one of the two α_2 -globin genes. The amplified DNA of the α_2 -globin gene of patient M.R. hybridized with the mutant probe only, again indicating a homozygosity for this mutation. The similar quantities of α -Sun Prairie present in these two patients are most unusual unless patient M.R. is not homozygous. Attempts to detect a normal α_2 -globin gene in patient M.R. have not been successful; selection of additional primers for amplification did not change the hybridization data, while sequence analysis of the 3' segment (including the third exon) of the amplified DNA of the α_2 -globin gene did not reveal any mutant site which could prevent hybridization with the normal probe. Only the CCT sequence (for proline) was detected for CD 130. Thus, this problem remains unsolved and family studies are needed to further detail the abnormality in the child M.R., who, unfortunately, is adopted.

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MUTANT

NORMAL

FIG. 3b/3. Hybridization of amplified DNA of the two patients and four normal controls with probes specific for the G→C mutation at CD 130. Primers, probes, and methods were described previously (1). The six samples at the left in each hybridization experiment contain DNA amplified from α 1-globin genes, and those at the right contain DNA amplified from α 2-globin genes.

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CHAPTER 4

Hb Iowa OR $\alpha 282119(\text{GH2})\text{Trp} \rightarrow \text{Arg}$

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CHAPTER 4

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β CHAIN VARIANTS IDENTIFIED BY HPLC

4a	Hb Iowa or $\alpha 282119(\text{GH2})\text{Trp} \rightarrow \text{Arg}$	87
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CHAPTER 4a

HB IOWA OR $\alpha_2\beta_{119}(\text{GH2})\text{GLY}\rightarrow\text{ALA}$

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(Adapted from: Hemoglobin, 14:423-429, 1990)

ABSTRACT

Hb Iowa with a Gly $\rightarrow$ Ala mutation at position $\beta_{119}(\text{GH2})$ was observed in a Black infant and her mother. The baby was also heterozygous for Hb S; Hb Iowa was confused with Hb F at birth because its electrophoretic mobility was similar to that of Hb F₁. The β chain of Hb Iowa could be readily separated from the β^A , α , and γ chains by PAGE and by reversed phase HPLC. Structural characterization was through amino acid analyses of peptides isolated from a tryptic digest of the aminoethylated β -Iowa chain. The Gly $\rightarrow$ Ala replacement in Hb Iowa does not affect its stability and oxygen carrying properties; hematological data for mother and child were within normal ranges.

INTRODUCTION

The Iowa State-wide screening and care program for patients with significant hemoglobinopathies was established in May of 1987 and has identified several subjects with different forms of sickle cell disease. Neonatal testing has been initiated, and dry, whole blood filter paper specimens collected for the Newborn Metabolic Screen, constitute the initial samples which are analyzed by IEF using the eluted hemolysate. Abnormalities are confirmed from anticoagulated blood specimens obtained by venipuncture. Through this activity a Black female infant was suspected of having SS but upon repeat testing was identified as a compound heterozygote for Hb S and a variant with a mobility in IEF between Hb A and Hb F. This report describes the identification of this variant and some of its properties.

MATERIALS AND METHODS

Blood samples from the propositus and her mother were collected in vacutainers with EDTA as anticoagulant, and studied at the local institutions and at the laboratories in Augusta, GA. Informed consent was obtained.

Hematological data were collected with automated cell counters and with standard laboratory procedures. Electrophoresis was with cellulose acetate at alkaline pH and citrate agar at pH 6.1 (1), and with IEF (2) using agar plates which are commercially available from Isolab, Inc. (Akron, OH, USA). Quantitation of the various Hb fractions was by cation exchange HPLC using a PolyCAT column (3,4). The mobilities of the individual chains were evaluated by reversed phase HPLC using the large-pore Vydac C₄ column (5,6) and by PAGE (7). Oxygen affinity measurements were made with a Hemox Analyzer (Southampton, PA, USA). A solubility test to confirm the presence of Hb F followed an established procedure (1).

A larger quantity of Hb X was isolated from the red cell lysate of the propositus by DEAE-cellulose chromatography (1); the mobility of Hb X in this system is the same as that of Hb A. The α and β chains were separated by CM-cellulose chromatography (8) and the abnormal β^X chain was aminoethylated (9). Next, the AE- β^X chain was digested with trypsin for 6 hours at room temperature and at pH 8.7. The resulting tryptic peptides were separated by reversed phase HPLC (10) and their amino acid compositions were determined with a Waters Pico Tag amino acid analyzer.

RESULTS

The Family. When last studied, the propositus was a 9-month-old Black baby. Analysis of her red cell lysate by IEF revealed the presence of Hb S and Hb X which had a mobility slightly slower than that of Hb A. Her mother had the same variant in combination with Hb A. Hematological data, listed in Table 4a/1, were within normal limits. A Heinz body preparation on the mother's blood was negative. A solubility test on the hemolysate of the child confirmed the presence of an Hb S trait.

TABLE 4a/1. Hematological Data

Subject	Sex-Age	Hb g/dl	PCV l/l	RBC 10 ¹² /l	MCV fl	MCH pg	MCHC g/dl
T.W.	F-9 mos.	11.2	0.350	3.88	90	28.9	32.0
V.W.	F-adult	12.1	0.385	4.39	88	27.6	31.4

Properties of the Variant. Heat stability and oxygen affinity analyses were made on the red cell lysate of the mother, which contained about the same quantities of Hb A and Hb X, and that of a normal control; no differences were observed. Fig. 4a/1 illustrates the separation of the Hb components by cation exchange HPLC. Hb X eluted at the same position as Hb A. The level of Hb S in the propositus was slightly higher than that of Hb X. Both the propositus and her mother had normal levels of Hb A₂, and the level of 7.2% Hb F in the lysate of the propositus is not unusual for a child of that age.

FIG. 4a/1. Separation by cation exchange HPLC (3,4) of the Hbs in red cell lysates of the propositus (T.W.) and her mother (V.W.). The gradients used for the elution of the Hbs were slightly different in the two experiments.

The separation of the globin chains was evaluated by PAGE and reversed phase HPLC. With both techniques it was possible to detect normal α chains and abnormal β chains. The β^A and β^X chains were readily detected in the red cell lysate of the mother because the mobility of β^X in PAGE was slightly less than that of β^A . The separation of the β^S and β^X chain in the child's lysate was even more evident because the mobility of β^S in this system was slightly greater than that of β^A . Fig. 4a/2 illustrates

the mobilities of the three β chains in reversed phase HPL chromatography. The β^X chain eluted just ahead of the normal β chain but a complete separation of these two chains could not be obtained.

FIG. 4a/2. Separation by reversed phase HPLC (5,6) of α and β chains from the Hbs in red cell lysates of the propositus (T.W.) and her mother (V.W.).

Identification of the Substitution in the β^X Chain. All peptides from a tryptic digest of the AE- β^X chain were recovered from the reversed phase HPL chromatogram, and none had an abnormal chromatographic mobility in this system (data not shown). Amino acid analyses readily identified the various peptides. All had the expected compositions except peptide T-12B which showed the replacement of a glycine residue by an alanine residue. The composition of this octapeptide was: Gly 0(1); His 2.21(2); Ala 1.96(1); Val 1.00(1); Leu 0.92(1); Phe 0.94(1); Lys 0.96(1); values for the normal T-12B peptide are listed between parentheses; data are given in mole/mole peptide. The Gly→Ala substitution occurs at position 7 in this peptide which corresponds to position 119 of the intact β chain. This new variant was named Hb Iowa.

COMMENTS

Glycine at position 119, which is the second position of the inter-helical region between helices G and H, is involved in the $\alpha 181$ contact mainly through residue 111 of the α chain (11). Substitution of glycine at $\beta 119$ by aspartic acid has been observed in Hb Fannin-Lubbock (12,13) and by valine in Hb Bougardirey-Mali (14). Both variants had near normal functional and physicochemical properties; only a slight instability was observed. Similar data were obtained for Hb Iowa; the Gly→Ala replacement appears to have no effect at all, as even the heat stability of this variant was normal. Thus, the replacement of this glycine residue by various

amino acids (Asp, Val, Ala) is not detrimental to the function of the Hb molecule, despite its involvement in molecular contacts between the $\alpha 1$ and $\beta 1$ chains.

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CHAPTER 4b

HB ZENGCHENG OR $\alpha_2\beta_{2114}(\text{G16})\text{LEU} \rightarrow \text{MET}$

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The use of refined methodology, such as IEF (1), cation exchange HPLC (2,3), and reversed phase HPLC (4,5), as procedures for testing for the possible presence of Hb abnormalities has recently led to the detection of several new variants. This was again the case with Hb Zengcheng which was observed when a large batch of cord blood samples from Chinese newborns was tested by IEF and reversed phase HPLC.

Cord blood sample #833 was collected from a normal Chinese baby originating in the city of Zengcheng in Guangdong Province, P.R. China. Examination of the Hb by IEF (1) showed the presence of a slightly fast-moving β chain variant, of which the β^X and β^A chains were readily separated by reversed phase HPLC (5) (Fig. 4b/1). A quantity of the β^X chain was isolated by preparative HPLC using a C₁₈ column (6). This β^X (estimated at ~2 mg) was aminoethylated (7), digested with trypsin for 6 hours at room temperature and at pH 8.2, and the resulting peptides were separated by a reversed phase HPLC procedure (8). All expected peptides were recovered but only peptide T-12B had a different chromatographic mobility and eluted slightly faster than the T-12B peptide of the normal β^A chain. Its amino acid composition was: Gly 1.07(1); His 1.62(2); Ala 1.14(1); Met 0.63(0); Leu 0(1); Phe 0.85(1); Lys 0.92(1); values for T-12B of the normal β^A chain are listed between parentheses. Sequence analysis with a manual micro procedure (9) gave a Val-Met-Ala- amino terminal sequence, confirming a Leu→Met substitution at position 114 of the β chain. This mutation has not been observed before, and the variant is named Hb Zengcheng or $\alpha_2\beta_{2114}(\text{G16})\text{Leu} \rightarrow \text{Met}$.

FIG. 4b/1. Separation of the β^X , β^A , α , and γ chains, present in a cord blood red cell lysate of the Hb Zengcheng heterozygote, by reversed phase HPLC. The quantities of the β^X and β^A chains were estimated at 3.8% and 3.0%, respectively, of the total protein. The ratio between G_γ and A_γ was normal.

The leucine residue at position $\beta 114$ is internally located and is not involved in any major contacts with heme or globin subunits. Its replacement by another hydrophobic residue (methionine) is not expected to alter its physicochemical or functional properties. Family studies and a second analysis of the Hb Zengcheng heterozygote were not possible.

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CHAPTER 5

β CHAIN VARIANTS IDENTIFIED BY HPLC AND DNA ANALYSES

5a	Hb Yokohama [β 31(B13)Leu->Pro] Detected as a
<u>De Novo</u> Mutation in a Yugoslavian Boy	99
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CHAPTER 5a

HB YOKOHAMA [$\beta 31(\text{B}13)\text{LEU} \rightarrow \text{PRO}$] DETECTED AS A DE NOVO MUTATION IN A YUGOSLAVIAN BOY

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ABSTRACT

HB Yokohama [$\beta 31(\text{B}13)\text{Leu} \rightarrow \text{Pro}$] was observed in a young Yugoslavian boy as a de novo mutation. The child exhibited severe transfusion-dependent hemolytic anemia. The variant was detected and quantitated at 10.5% by reversed phase HPLC. Structural characterization of the abnormal β chain was done by HPLC analysis, on material obtained by high salt precipitation. The mutation was confirmed by sequencing of the amplified DNA.

INTRODUCTION

Hb Yokohama [β 31(B13)Leu->Pro] was first described in 1981 by Nakatsuji et al (1). The variant was found in a 33-year-old Japanese woman with a history of chronic hemolytic anemia and in her son who manifested milder symptoms. Here we describe a second observation of this variant in a 10-year-old Yugoslavian boy suffering from hemolytic anemia.

MATERIALS AND METHODS

Hematological data were obtained with an automated cell counter and with routine hematological procedures. Red cell lysates were studied by starch gel electrophoresis at alkaline pH (2), and with PAGE in the presence of urea, acid, and Triton X-100 (3). The percentages of Hb A₂ and Hb F were determined by microcolumn chromatography (4) and alkali denaturation procedures (5), respectively. The abnormal β chain was quantitated by reversed phase HPLC (6). The stability of the abnormal Hb was determined by heat stability and PCMB tests (7). The abnormal Hb was isolated by precipitation with sodium chloride at 50°C for 60 minutes (1 vol. 5 gr % hemolysate, 2 vol. 0.1 M Tris buffer, pH 6.0, 1 vol. 4 M NaCl). The abnormal β chain was separated from the α chain by CM-52 column chromatography (8). The isolated β^X chain was digested with trypsin for 5 hours at room temperature and at pH 8.6. The resulting peptides were separated by reversed phase HPLC (9). One zone was rechromatographed by a similar procedure (10). The amino acid compositions of the peptides were determined with a Beckman 6300 amino acid analyzer.

DNA was isolated from the leukocytes by the method of Poncz et al (11). The 710 bp fragment of the β -globin gene was amplified (12) and directly sequenced (13).

RESULTS

The propositus is a 10-year-old Yugoslavian boy living in Nis. His anemia was noted when he was 7 months of age. A few months later, he was hospitalized because of severe anemia and subicterus. At that time he was diagnosed as having a hereditary spherocytosis and was transfused for the first time. Anisocytosis, poikilocytosis, and microspherocytosis were noted on the peripheral blood smear. Liver and spleen were palpable 4 and 5 cm below the costal margin, respectively. Thereafter, he was hospitalized and transfused several times due to acute hemolytic crisis. He was splenectomized at the age of 2.5 years. Ever since then he has received several blood transfusions during acute infectious episodes. The first time he was sent to our institution was in 1988. Hematological data for the propositus, obtained before and after splenectomy, are given in Table 5a/1. Peripheral blood smear revealed anisocytosis, poikilocytosis, target cells, and basophilic stippling. Heinz bodies were readily detectable after incubation of the blood at 37°C for 2 hours. The heat stability test was positive suggesting the presence of 14-16% unstable Hb. The PCMB test was also positive; the precipitate consisted of β chains with a normal

mobility. No abnormal Hb was detected by starch gel electrophoresis at alkaline pH, PAG electrophoresis, and anion and cation exchange chromatography. An abnormal β chain (10.5%) was detected by a reversed phase HPLC procedure (Fig. 5a/1). The Hb A₂ value of 4.9% was higher than normal; Hb F was also increased (4.5%). The parents of the boy were normal without any Hb abnormalities.

TABLE 5a/1. Hematological Data of the Propositus^a

	Before Splenectomy 1/83-4/83	After Splenectomy 4/88-1/90
Hb (g/dl)	4.8 - 7.2	8.9 - 10.9
PCV (l/l)	n.d.	0.286- 0.330
RBC ($10^{12}/l$)	2.8 - 3.1	2.9 - 3.6
MCV (fl)	n.d.	99.4 - 106.6
MCH (pg)	17.7 - 25.4	26.4 - 34.2
Reticulocytes (%)	6.2 - 9.7	5.5
Hb A ₂ (%) ^b	n.d.	4.6 - 4.9
Hb F (%) ^c	n.d.	3.0 - 4.5
Hb X (%) ^d	n.d.	10.5

^a Values obtained before transfusion.

^b By microcolumn chromatography.

^c By alkali denaturation.

^d By reversed phase HPLC.

n.d. = not determined.

FIG. 5a/1. Separation of the β^X , β^A , and α^A chains, present in a red cell lysate of the propositus, by reversed phase HPLC.

Structural analyses were done by a reversed phase HPLC procedure. The tryptic peptides of the β^X chain are shown in Fig. 5a/2. Almost all peptides were recovered with normal mobilities and normal amino acid compositions. Peptide β T-3 was recovered as a peak much smaller than normally observed, while peptide β T-4 was nearly absent. The small zone, present at the position of the normal β T-4 peptide, probably originated from the small amount of normal β chain that was present in the precipitate. An abnormal peptide eluted together with peptide β T-8,9. This aberrant peptide was identified as β^X T-3,4 because the substitution of proline for leucine produced a Lys-Pro linkage that trypsin does not cleave. Rechromatography was used to separate the β T-8,9 and β^X T-3,4 peptides. The amino acid composition of the abnormal peptide was: Asp 2.1(2); Thr 1.0(1); Glu 3.3(3); Pro 2.1(1); Gly 3.0(3); Ala 1.1(1); Val 4.6(5); Leu 2.1(3); Tyr 0.7(1); Arg 2.0(2); thus identifying it as β^X T-3,4 with a substitution of Leu→Pro (values for residues of the normal β T-3 and β T-4 peptides are listed between parentheses).

FIG. 5a/2. Separation of the tryptic peptides of the β^X chain by reversed phase HPLC.

The substitution was confirmed and localized at codon 31 through sequencing of amplified DNA. Two primers (synthesized on a Beckman System 1 DNA Synthesizer) were used to amplify a 710 bp fragment that was sequenced in both directions. Fig. 5a/3 illustrates the results of the direct sequencing; two nucleotides (T and C) are present at the second position of codon 31, resulting in a CTG→CCG (or Leu→Pro) mutation.

Since the parents of the boy did not have this abnormality, paternity studies were undertaken. The polymorphisms in the HLA DQ α locus were evaluated using the Perkin Elmer Cetus HLA DQ α Forensic DNA Amplification and Typing Kit. The results revealed genotype 1.1/1.1 for the mother, 4.4 for the father, and 1.1/4 for the proband, thus confirming the parentage of the proband.

FIG. 5a/3. Direct sequence analysis of the amplified DNA of the proband. Two nucleotides (T + C) are present at the second position of codon 31. The sequences of the amplification primers (numbers given in parentheses are relative to the Cap site) were: Direct: 5'-GCCAAGGACAGGTACGGCTGTCATC-3' (-140 through -116); reverse: 5'-CCC TTCCTATGACATGAACCTTAACCAT-3' (+566 through +540). The primer used for sequencing was: 5'-CAGGTTGGTATCAAGGTTA-3' (+139 through +158).

DISCUSSION

The variant, with a Leu→Pro substitution at position 31 of the β chain, was described before as Hb Yokohama (1). Introduction of a proline residue at β 31 disrupts the heme globin contact, thus making the variant extremely unstable. This instability leads to the transfusion dependent hemolytic anemia. In this paper we present an observation of Hb Yokohama in a 10-year-old Yugoslavian boy. He exhibited moderately severe, transfusion-dependent hemolytic anemia, and was splenectomized at the age of 2.5 years. Since the parents of the boy were not carriers of this variant, the possibility of a *de novo* event was studied by evaluating the polymorphisms in the HLA DQ α locus. According to the genotype frequencies among Caucasians (14), the PI index of 1.97 and W value of 66% were calculated (15). These results led us to consider this mutation as a *de novo* event.

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CHAPTER 5b

HB VOLGA [$\beta 27(B9)ALA \rightarrow ASP$]: DETECTION OF A DE NOVO MUTATION BY AVA II DIGESTION OF PCR-AMPLIFIED DNA

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ABSTRACT

Hb Volga was observed as a de novo mutation in a 5-year-old boy from Tuzla, Bosnia and Hercegovina, who exhibited severe Heinz body hemolytic anemia. The variant was detected and quantitated at 10.6% by a reversed phase HPLC procedure. Structural characterization was done by HPLC analysis. An easier approach for the detection of Hb Volga by Ava II digestion of PCR-amplified DNA is described.

INTRODUCTION

Hb Volga was first described in a Russian boy (1), and shortly after that the same abnormality was observed in a few members of a Dutch family (2). Since then it has been reported several times (3-6). We here describe a detection of Hb Volga as a *de novo* mutation in a young boy from Tuzla, Bosnia and Hercegovina. An easier approach for the detection of this variant by *Ava* II digestion of PCR-amplified DNA is given.

MATERIALS AND METHODS

Blood samples from the patient and his family were collected in vacutainers with EDTA as anticoagulant. Hematological data were obtained with an automated cell counter and routine hematological procedures. Red cell lysates were studied by starch gel electrophoresis at alkaline pH (7), and PAGE in the presence of urea, acid, and Triton X-100 (8). Heat stability of the Hb was tested at 52°C, 58°C, and 62°C (9). The stability was also analyzed by isopropanol and PCMB tests (9). The percentage of Hb A₂ and Hb F was determined by microcolumn chromatography (10) and an alkali denaturation procedure (11), respectively. The abnormal β chain was quantitated by reversed phase HPLC on a large-pore C₄ column (12).

The abnormal Hb was isolated by precipitation with 17% isopropanol in 0.1 M Tris/HCl, pH 7.4, at 37°C for 30 minutes. The abnormal β chain was separated from the α chain by CM-52 column chromatography (13) and digested with trypsin for 6 hours at room temperature and pH 8.5. The tryptic peptides were separated by reversed phase HPLC on a C₁₈ column (14). The amino acid composition of the peptides was determined with a Beckman 6300 amino acid analyzer (Beckman, Palo Alto, CA, USA).

DNA was isolated from the leukocytes (15). A 706 bp fragment from the β -globin gene, including exon 1 and exon 2, was PCR-amplified (16). The primers used were 5'-GCCAAGGACAGGTACGGCTGTCATC-3' (direct) at positions -140 to -116, and 5'-CCCTTCCTATGACATGAACCTAACCAT-3' (reverse) at positions +566 to +540, both relative to the Cap site of the β -globin gene. The 706 bp amplified product was digested with *Ava* II for 4 hours at 37°C, electrophoresed on 2.5% agarose gel, stained with ethidium bromide, and visualized under UV light.

The polymorphisms in the HLA DQ α locus were evaluated with a Perkin Elmer Cetus (Norwalk, CT, USA) HLA DQ α Forensic DNA Amplification and Typing kit using the procedure recommended by the manufacturer.

RESULTS AND DISCUSSION

The propositus (J.E.) is a 5-year-old boy from Tuzla, Bosnia and Hercegovina. Anemia was noted when he was 3 months old. He was hospitalized in the Clinic of Pediatrics in Belgrade in December 1990 because of high temperature, anemia, and subicterus. Physical examination revealed a normally developed boy with pale complexion. Cervical, supraclavicular, axillar, and inguinal lymph nodes were palpable. The liver and spleen

were palpable 3 cm below the costal margin. He received two blood transfusion while in hospital. A few weeks later he was referred to our institution for detailed investigation.

Hematological data for the propositus and his parents are given in Table 5b/1. A peripheral blood smear from the propositus revealed anisocytosis, spherocytosis, and poikilocytosis. Heinz bodies were detectable after incubation of the blood at 37°C for 2 hours. The heat stability test at 52°C, 58°C, and 62°C indicated the presence of 5-10% unstable Hb. The isopropanol test was also positive. Starch gel electrophoresis of the precipitate obtained after PCMB treatment revealed the presence of β^A , β^X , and α^A chains. No abnormal Hb was detected by starch gel electrophoresis and anion exchange chromatography. PAGE showed the presence of α^A and β^A chains only. The abnormal β chain was observed by reversed phase HPLC and estimated to represent 10.6% of the total Hb (Fig. 5b/1). The Hb A₂ and Hb F fractions amounted to 4.1 and 5.5%, respectively. The parents had normal hematological and Hb findings.

TABLE 5b/1. Hematological Data

Subject	Hb g/dl	PCV l/l	RBC 10 ¹² /l	MCV fl	MCH pg	Retics. (%)	A ₂ ^a %	F ^b %	X ^c %
J.E.	9.2	.310	3.2	96.9	28.7	13.1	4.1	5.5	10.6
Mother	13.8	.440	4.4	100.0	31.4	n.d.	2.5	0.9	0
Father	14.2	.430	4.8	89.6	29.6	n.d.	2.5	0.3	0

^a By microcolumn chromatography (10).

^b By alkali denaturation (11).

^c By reversed phase HPLC (12).

n.d. = not determined.

Structural analysis of the β^X chain was done by reversed phase HPLC. The amino acid analysis of the abnormal tryptic peptides, eluting just in front of β T-1, showed the composition of the β T-3 peptide with the substitution of alanine by aspartic acid or asparagine. The only alanine in the normal β T-3 peptide is at position 27. A single base GCC→GAC substitution at that position would lead to an Ala→Asp replacement specific for the Hb Volga variant, while at least two base substitutions, namely GCC→AAC, would be required for an Ala→Asn replacement.

Since the GCC→GAC substitution creates a new Ava II restriction site, the Hb Volga gene can be detected by Ava II digestion. A 706 bp fragment (position -140 to position +566, relative to the Cap site of the β -globin gene) from the patient and a normal control was PCR-amplified and digested with Ava II. This fragment contains one constant Ava II site at position +293 and one polymorphic site at position +507. An Ava II digest of the 706 bp fragment from the chromosome which lacks the polymorphic site would result in two fragments of 433 and 273 bp, while the fragment from the chromosome with the polymorphic site present would give three fragments of 433, 214, and 59 bp, respectively. If a GCC→GAC substitution at position +131 is present, a 433 bp fragment would be further divided into two smaller fragments of 271 and 162 bp. An Ava II restriction map of a 706 bp

FIG. 5b/1. Separation of β^X , β^A , and α^A chains present in the red cell lysate of the propositus, by reversed phase HPLC.

PCR-amplified fragment from the normal chromosome and the chromosome with the Hb Volga mutation is shown in Fig. 5b/2. Fragments of 433, 273, and 214 bp were obtained from the normal control, while four fragments of 433, 271 (273), 214, and 162 bp were obtained from the patient. Only the 162 bp fragment is specific for Hb Volga, since the 271 bp fragment cannot be separated from the normal 273 bp fragment by agarose gel electrophoresis. The polymorphic site at position +507 is present on one chromosome only, both in the patient and in the normal control.

Since we could not detect this variant in the parents of the patient, we undertook paternity studies using the Perkin Elmer Cetus HLA DQ α Forensic DNA Amplification and Typing kit. The results revealed genotype 1.2/2 for the mother, 4/4 for the father, and 1.2/4 for the propositus. According to the genotype frequencies among Caucasians (17) the PI index of 25.6 and W value of 96.6 were calculated (18), which made us consider this variant to have occurred as a de novo event.

In conclusion, we have described a fast, easy and new approach for detection of the Hb Volga mutation. The whole procedure can be performed in less than two days in laboratories that lack facilities for chemical analysis of proteins. We also described the first observation of Hb Volga in the Balkan peninsula.

FIG. 5b/2. An Ava II restriction map of the 706 bp fragment of the β -globin gene from a patient with Hb Volga and a normal control.

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CHAPTER 6

γ CHAIN VARIANTS IDENTIFIED BY HPLC

6a	Hb F-Jiangsu, the First γ Chain Variant With a Valine-Methionine Substitution: $\alpha_2^A\gamma_{2134}$ (H12)Val-Met	113
6b	Hb F-Catalonia or $\alpha_2^G\gamma_{215}(A12)Trp-Arg$	119
6c	Hb F-Brooklyn or $\alpha_2^G\gamma_{266}(E10)Lys-Gln$	125

CHAPTER 6a

**HB F-JIANGSU, THE FIRST γ CHAIN VARIANT WITH
A VALINE- $\rightarrow$ METHIONINE SUBSTITUTION: $\alpha_2\gamma_{12134}(\text{H12})\text{VAL}\rightarrow\text{MET}$**

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(Adapted from: Hemoglobin, 14:177-183, 1990)

ABSTRACT

During a survey of cord blood samples of healthy Chinese babies an A_γ variant was detected by its partial separation from normal A_γ in an HPL chromatographic experiment. Structural analysis of the isolated A_γ^X chain identified a Val $\rightarrow$ Met substitution at position $A_\gamma 134$; this substitution created a rather unique Met-Met sequence. The ratio of A_γ^X to A_γ was about 2 to 3; thus, Hb F with this variant A_γ chain appears to have approximately normal stability.

INTRODUCTION

Fifty-four γ chain variants (1) have been detected through September 1989; 28 of these result from mutations in the G_γ -globin gene and 17 from mutations in the A_γ -globin gene; six have two substitutions including the Ile $\rightarrow$ Thr substitution at position $A_\gamma 75$, while for three variants the $A_\gamma 75$ information is not available. Institutions in the People's Republic of China have shown an increased interest in identifying γ chain abnormalities and this led to the detection of Hb F-Urumqi [$G_\gamma 22(B4)Asp\rightarrow Gly$], Hb F-Xinjiang [$A_\gamma 125(B7)Gly\rightarrow Arg$], Hb F-Shanghai [$G_\gamma 66(E10)Lys\rightarrow Arg$], and Hb F-Xin-Su [$A_\gamma 73(E17)Asp\rightarrow His$]; references are listed in Ref. 1.

In this communication we describe the fifth γ chain variant observed among Chinese, named Hb F-Jiangsu. The substitution involves a valine $\rightarrow$ methionine at position 134 of the A_γ chain. This is the first mutation resulting in the introduction of a methionine residue; moreover, the -Met-Met-sequence at positions 133-134 is unique among the Hb polypeptide chains.

METHODOLOGY

The blood sample was shipped by air from Shanghai to Augusta, GA. Collection was made in EDTA. Red cell lysates were studied by IEF (2) with commercially available agar plates (Isolab, Inc., Akron, OH, USA), and by reversed phase HPLC (3,4). Isolation of the normal and abnormal A_γ chains was by preparative reversed phase HPLC with a 30.0 x 2.15 cm C_{18} column (5). Isolated chains were digested with trypsin for 6 hours at pH 8.5 and at room temperature. The resulting peptides were separated by a reversed phase HPLC procedure described before (6). A few peptides were rechromatographed by a similar method but with water-acetonitrile-TFA developers (7). Amino acid analyses were made with the Waters Pico Tag amino acid analysis system and sequence analyses with a microsequencing procedure described by Chang et al (8).

RESULTS AND DISCUSSION

The variant was detected unexpectedly when several cord blood samples from healthy babies of the Shanghai area were analyzed by reversed phase HPLC to determine possible variations in the relative quantities of the G_γ and A_γ chains. Fig. 6a/1 (top) shows the initial chromatogram indicating the possible presence of two different A_γ chains. The ratio of A_γ^X and normal A_γ was about 2 to 3; this ratio remained the same upon storage of the sample. The abnormal γ chain (A_γ^X) was also separated from G_γ and A_γ by preparative reversed phase HPLC with a large C_{18} column (Fig. 6a/1, bottom). Several such chromatograms provided a few mg each of the A_γ^X and the A_γ chains for structural analyses.

Fig. 6a/2 illustrates the separation of the soluble peptides from tryptic digests of the two A_γ chains by reversed phase HPLC methodology (6). The two chromatograms showed the presence of many minor zones which

FIG. 6a/1. Separation of globin chains by reversed phase HPLC (3,4). **Top:** An analytical chromatogram from the large-pore Vydac C₄ column. **Bottom:** A preparative chromatogram from a C₁₈ column (5). The amounts of Hb applied to the columns are estimated at 50 to 60 μ g (top) and 5 to 6 μ g (bottom).

are presumptive contaminants collected during the isolation of the small quantities of the two chains. However, the γ chain peptides (T-1, T-2, T-3, T-4, T-5, T-6, T-7, T-8, T-9, T-10, T-14, T-15, T-16) were readily recognized. All had the expected mobilities in this chromatographic system, except T-15 of the $A_{\gamma X}$ chain which eluted a few minutes later than the comparable peptide of the A_{γ} chain. Peptide T-15 of $A_{\gamma X}$ was rechromatographed by reversed phase HPLC to obtain an acceptable analysis. Table 6a/1 lists the amino acid composition of this peptide which indicates a Val $\rightarrow$ Met replacement. As peptide T-15 contains two valine residues, an additional quantity of this fragment was isolated and purified for sequence analysis. The result of that study gave:

133 134 135 136 137 138 139 140 141 142 143 144

Met-Met-Thr-Gly-Val(Ala,Ser,Ala,Leu,Ser,Ser,Arg)

which located the Val→Met replacement at position $\gamma 134$. Amino acid analysis of peptide T-9 indicated the presence of one isoleucine residue and one threonine residue, thus excluding an Ile→Thr substitution at position $\gamma 75$. These results identified the variant as $\alpha_2\gamma_2 134(\text{H12})\text{Val} \rightarrow \text{Met}$; this substitution is likely due to a GUG→AUG mutation in CD 134. The variant was named Hb F-Jiangsu for the infant's residence.

FIG. 6a/2. Separation of peptides present in the tryptic digests of the normal A γ and abnormal A γ^X chains by a reversed phase HPLC method (6).

Because of the small volume of cord blood available, the low quantity of Hb F-Jiangsu in this sample (<20%), and the inability to separate Hb F-Jiangsu from normal Hb F, functional and physicochemical analyses were not conducted.

This Val→Met replacement is the first observed among the γ chain variants. (See Ref. 1 for a comparison of the structures of the 54 abnormal γ chains.) It is of some interest to compare its chromatographic behavior with that of the rather mysterious M γ chain which is characterized by a Leu→Met replacement in the same γ T-15 peptide but at position $\gamma 141$ (9,10). The M γ chain (10), although it has alanine at residue 136 as does the F-Jiangsu A γ^X chain, elutes before the G γ chain in the Vydac C₄ chromatographic system, and considerably earlier than the normal A γ and F-Jiangsu A γ^X chains. The M γ T-15 peptide also has a much shorter retention time than the normal A γ T-15, but the F-Jiangsu A γ T-15^x elutes after the

TABLE 6a/1. Amino Acid Compositions of Peptides T-9 and T-15 of the $A_{\gamma}X$ Chains^a

Amino Acid	Peptide T-9 ^b	Peptide T-15 ^c
Aspartic acid	1.59 (1)	
Serine	1.12 (1)	2.71 (3)
Glycine	1.25 (1)	<0.05 (0)
Arginine		0.98 (1)
Threonine	0.91 (1)	0.99 (1)
Alanine	1.24 (1)	3.10 (3)
Valine	1.00 (1)	1.04 (2)
Methionine		1.69 (1)
Isoleucine	1.17 (1)	
Leucine	2.07 (2)	1.06 (1)
Lysine	0.89 (1)	

^a In moles/peptide. The expected numbers of residues are listed between parentheses.

^b This peptide was not purified by rechromatography which may explain the somewhat high levels of Asp, Ser, Gly, and Ala.

^c This peptide was rechromatographed by reversed phase HPLC with water-acetonitrile-TFA developers (7).

normal $A_{\gamma}T$ -15. Clearly these discrepancies in behavior cannot be attributed solely to differences in the polarities and sizes of the valine, leucine, and methionine residues, since these differences are relatively minor (5). Thus, it appears that the location of the second methionine residue significantly influences the chromatographic properties of these three types of A_{γ} chains, as well as of their T-15 peptides.

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CHAPTER 6b

Hb F-CATALONIA OR $\alpha_2^G\gamma_{15}(A12)TRP \rightarrow ARG$

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ABSTRACT

Hb F-Catalonia, a G_γ chain variant with a Trp→Arg substitution at position $\gamma_{15}(A12)$, was observed in two Spanish newborn babies from North-eastern Spain. Analyses were performed with different reversed phase HPLC procedures that allowed the identification of the amino acid replacement in only a minute quantity of the isolated G_γ^X chain.

INTRODUCTION

The testing of newborn babies for the presence of Hb abnormalities has become popular worldwide; it has also resulted in the detection of numerous γ chain variants (see Ref. 1 for a list and references). A new variant was recently detected during a survey of newborn babies from the northeastern part of Spain. The abnormality was present in two infants living in Tortosa, Tarragona Province, which is located in the delta of the Ebro River; its characterization is described here.

MATERIALS AND METHODS

Blood samples were collected with EDTA as anticoagulant and studied at the laboratory of the local institution in Barcelona, while part was shipped (as frozen washed red cells) by air to Augusta, GA. Informed consent from the parents was obtained.

Red cell lysates were studied by IEF (2), cation exchange HPLC (3,4), and by reversed phase HPLC (5,6). A larger quantity of the variant was isolated by DEAE-cellulose chromatography (7), while the α and γ^X chains of the isolated Hb Fx were separated by reversed phase HPLC using a preparative C18 column (8). The relative quantity of the γ^X chain (as % of total γ) was determined by reversed phase HPLC using a large-pore Vydac C4 column (5,6).

The γ^X chain was digested with trypsin for 6 hours at room temperature and at pH 8.2. The tryptic peptides were separated by reversed phase HPLC (9) and their amino acid compositions determined with a Waters Pico Tag automated amino acid analyzer.

RESULTS AND DISCUSSION

The two babies had identical Hb patterns in different electrophoretic and chromatographic experiments. Structural analyses of the γ^X chains from both infants showed that the substitution was the same; data for the variant present in the larger sample are presented here.

Detection of the abnormal Hb was by cellulose acetate electrophoresis and IEF; it had a mobility between those of Hbs S and C in both systems. The parents of the infants did not have the abnormality. It was readily isolated on a column of DEAE-cellulose. Globin chain analysis on a reversed phase HPLC system showed that the γ^X chain eluted slightly faster than the normal G_γ chain (Fig. 6b/1A). The γ^X and G_γ chains together accounted for 72.2%; this normal value was considered to indicate that γ^X was a mutant of the G_γ chain. The pattern for the Hb in the red cell lysate of the second baby was the same except for an additional $A_{\gamma T}$ heterozygosity; the four γ chains, $A_{\gamma T}$, $G_{\gamma X}$, G_γ , and A_γ , were eluted in that order and were present for 11.5%, 45.5%, 31.3%, and 11.7%, respectively (the higher $G_{\gamma X}$ value is likely due to an incomplete separation of $A_{\gamma T}$, $G_{\gamma X}$, and G_γ). Isolated Hb Fx was nearly pure and contained α and $G_{\gamma X}$.

chains only (Fig. 6b/1B). As only a small quantity of Hb F_X was available, the α and G _{γ} ^X chains were separated by preparative reversed phase HPLC rather than by the common CM-cellulose chromatographic procedure; the separation of α and G _{γ} ^X was partially complete (Fig. 6b/1C).

FIG. 6b/1. Separation of the globin chains by reversed phase HPLC (5,6). A: Whole red cell lysate; B and C: Hb F_X, isolated by DEAE-cellulose chromatography. Chromatograms A and B were developed with an analytical large-pore Vydac C₄ column, and chromatogram C with a preparative C₁₈ column.

Fig. 6b/2 illustrates the separation of the peptides present in a tryptic digest of the G _{γ} ^X chain; the partial separation of α and G _{γ} ^X on the reversed phase C₁₈ column (Fig. 6b/1C) was responsible for the presence of notable quantities of α chain peptides. All soluble γ chain peptides were recovered and identified. Their amino acid compositions were normal except for those of peptides T-2^X and T-1,2^X which showed an extra arginine residue that replaced the normally occurring tryptophan (Table 6b/1). This substitution occurred at position γ 15 and Hb F_X, therefore, has as structure $\alpha_2\gamma_2$ 15(A12)Trp-Arg. The data obtained for peptides T-8,9 and T-15 (Table 6b/1) confirmed the suspicion that the γ ^X chain was a variant of the G _{γ} chain (peptide T-15 has one glycine and two alanine residues; peptide T-8,9 had one isoleucine and one threonine residue).

FIG. 6b/2. Separation of tryptic peptides of the γ^X chain with reversed phase HPLC (9). Small quantities of tryptic peptides of the contaminating α chain were also observed; these have not been indicated in the chromatogram.

TABLE 6b/1. Amino Acid Compositions of Some Tryptic Peptides of the γ^X Chain^a

Amino Acid	T-2 ^X	T-1,2 ^X	T-8,9	T-15
Aspartic acid		1.24(1)	0.99(1)	
Glutamic acid		1.94(2)		
Serine	1.20(1)	1.01(1)	0.89(1)	2.98(3)
Glycine		0.97(1)	1.07(1)	0.96(1)
Histidine		0.88(1)		
Arginine	1.00(0)	0.84(0)		0.95(1)
Threonine	1.93(2)	2.45(3)	0.93(1)	0.85(1)
Alanine	1.38(1)	0.94(1)	1.02(1)	1.73(2)
Valine			1.00(1)	1.73(2)
Isoleucine	1.18(1)	1.03(1)	1.01(1)	
Methionine				0.80(1)
Leucine	1.48(1)	1.15(1)	2.17(2)	1.01(1)
Phenylalanine		0.96(1)		
Lysine		1.02(1)	2.00(2)	
Residue number	9-15	1-15	65-76	133-144

^a In moles/mole; numbers between parentheses are those expected for the tryptic peptides of the normal γ chain.

The Hb F_X has been named F-Catalonia for the area where it was discovered. It is unknown if the γ 15 Trp→Arg substitution has any effect on the functional or physicochemical properties of the fetal Hb; the studies required to determine this were not possible due to a complete lack of material.

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CHAPTER 6c

HB F-BROOKLYN OR $\alpha_2\text{G}_{\gamma 266}(\text{E10})\text{LYS}\rightarrow\text{GLN}$

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Routine screening of blood samples from newborn babies or infants for Hb abnormalities often results in the discovery of (minor) abnormal Hb components which are difficult to characterize but are most frequently γ chain variants. This was true for a 2-month-old Caucasian female; her red cell lysate exhibited a minor, fast-moving, variant when examined by alkaline electrophoresis. About 5 ml blood containing 500 mg Hb was available for further analysis.

The variant was detectable by IEF (1) and by cation exchange HPLC (2); quantitation by the latter procedure estimated Hb F_x to be about 23% of the total Hb. The best separation was seen in reversed phase HPLC (3,4). The abnormal γ chain ($\text{G}_{\gamma}\text{X}$) eluted between the normal G_{γ} and A_{γ} chains (Fig. 6c/1); its quantity was 25.1% of the total γ chains which corresponds to 17.4% of the total Hb. Preliminary identification of the abnormal chain as a variant G_{γ} chain is based on the ratio of ($\text{G}_{\gamma} + \text{G}_{\gamma}\text{X}$) to A_{γ} of 64.4 to 35.6%, which is normal for most newborn babies.

The variant Hb was isolated by DEAE-cellulose chromatography (5) and the $\text{G}_{\gamma}\text{X}$ and α chains were separated by preparative reversed phase HPLC (6). Tryptic digests of the $\text{G}_{\gamma}\text{X}$ chain was at room temperature and at pH 8.5 for a period of 6 hours. When the soluble tryptic fragments were separated by a reversed phase HPLC procedure (7), all except the T-9 peptide had their expected positions in the chromatogram. Peptide T-9 did not appear in its normal position between T-14 and T-15, but a shoulder was observed on the early side of T-15. Rechromatography with a slightly different HPLC procedure (8) separated the two peptides; their amino acid compositions are presented in Table 6c/1. One was a normal $\text{G}_{\gamma}\text{T-15}$ and the other was T-9 plus glutamic acid or glutamine. A manual sequencing

procedure (9) showed the latter peptide to contain at its amino terminus -Gln-Val-Leu. Therefore, lysine at position $\gamma 66$ is replaced by glutamine. This conclusion is further supported by the absence of free lysine ($\gamma T-8$) from the front zone of the chromatogram. These results identify a Lys $\rightarrow$ Gln substitution at position $\gamma 66$. The variant was named Hb F-Brooklyn after the patient's residence. The amino acid composition of $\gamma T-15$ identified the presence of one glycine residue and two alanine residues, thus confirming that Hb F-Brooklyn is a G_Y variant.

FIG. 6c/1. Separation of globin chains in a red cell lysate of the baby with Hb F-Brooklyn or $\alpha_2 G_Y \gamma 66(E10) \text{Lys} \rightarrow \text{Gln}$ by a reversed phase HPLC procedure.

TABLE 6c/1. Amino Acid Composition of Two Peptides (G_Y -Brooklyn)^a

Amino Acid	T-8,9	T-15
Aspartic Acid	1.04 (1)	
Threonine	0.97 (1)	0.91 (1)
Serine	0.97 (1)	2.53 (3)
Glutamic Acid	1.12 (0)	
Glycine	1.08 (1)	1.00 (1)
Alanine	1.11 (1)	1.98 (2)
Valine	0.99 (1)	1.87 (2)
Methionine		0.80 (1)
Leucine	1.99 (2)	0.96 (1)
Isoleucine	0.92 (1)	
Lysine	0.93 (2)	
Arginine		1.00 (1)

^a In moles/peptide; the numbers between parentheses represent the expected number of residues for the normal peptide.

Hb F-Brooklyn is the second G_{γ} chain variant in which lysine at position $\gamma 66$ is replaced; the first is Hb F-Shanghai or $\alpha_2 G_{\gamma 266(E10)}\text{Lys} \rightarrow \text{Arg}$ (10). Two β chain variants have been found in which lysine at the same position is replaced by glutamic acid in Hb I-Toulouse (11) and by threonine in Hb Chico (12). The instability of Hb I-Toulouse is likely caused by the loss of the nonpolar "plug" which normally seals the protein surface; the substitution to glutamic acid allows water to enter the heme crevice (13,14). Hb Chico is less unstable and does not cause an overt hemolytic disorder. Similar data for both Hb F-Shanghai [$G_{\gamma 66(E10)}\text{Lys} \rightarrow \text{Arg}$] and Hb F-Brooklyn [$G_{\gamma 66(E10)}\text{Lys} \rightarrow \text{Gln}$] are not available as material for detailed stability analyses was not available.

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CHAPTER 7

**HB MONTREAL, A NEW VARIANT WITH AN EXTENDED β CHAIN DUE TO
A DELETION OF ASP, GLY, LEU AT POSITIONS 73, 74, AND 75,
AND AN INSERTION OF ALA, ARG, CYS, GLN AT THE SAME LOCATION**

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ABSTRACT

The unstable Hb Montreal with a deletion of three amino acid residues (Asp, Gly, Leu) at positions 73, 74, and 75 of the β chain and an insertion of four residues (Ala, Arg, Cys, Gln) at the same location was observed in a 7-year-old Canadian boy suffering from a moderate hemolytic anemia. The introduction of an extra amino acid residue and of other changes in the crevice where the heme group is located is the likely cause of the instability of this Hb variant. The above listed changes were detected through analyses of tryptic peptides of the β -Montreal chain, sequencing of amplified DNA, and hybridization of amplified DNA with appropriate, ³²P-labeled, oligonucleotide probes. It is suggested that a mispairing involving the AGTG sequences at CDs 66 and 67 and at CDs 72 and 73 of the normal β gene caused a repetition of a 16 bp segment, while a deletion of 10 nts due to recombination or slippage, followed by a second short deletion during DNA repair, resulted in the modified sequence of the β -Montreal gene.

INTRODUCTION

In our continued survey of patients with hemolytic disease caused by the presence of an unstable Hb variant, we studied a young Canadian patient with a most unusual Hb abnormality which had a deletion of three amino acids in the β chain and an insertion of four different amino acids at the same location. Here we describe details of this investigation.

MATERIALS AND METHODS

Blood samples were collected in vacutainers with EDTA as anticoagulant and shipped by overnight mail from Montreal, Canada to Augusta, GA. Informed consent was obtained.

Hematological data were obtained with automated cell counters and with routine hematological procedures. Red cell lysates were studied by IEF (1) with commercially available agar plates (Isolab, Inc., Akron, OH, USA). The abnormal Hb was tested for heat stability (2). The variant was quantitated by cation exchange HPLC (3,4) and by reversed phase HPLC (5,6).

The variant was isolated by preparative DEAE-cellulose chromatography (2); it eluted behind Hb A₂ in this system. The α and β^X chains were separated by a preparative reversed phase HPLC procedure with a large (21.5 x 300 mm) C₁₈ column (7). The isolated β^X chain was aminoethylated (8) and digested with trypsin for 8 hours at pH 8.5 and at room temperature. The separation of the tryptic peptides was with a reversed phase HPLC procedure (9), while the amino acid composition of each isolated peptide was determined with a Waters Pico Tag amino acid analyzer. Sequence analysis of two peptides was with the method of Chang et al (10).

DNA was isolated by the method of Poncz et al (11). An appropriate segment of genomic DNA was amplified as described before (12,13) and sequenced by the Sanger dideoxy procedure (14). The changes in DNA sequence were verified by dot-blot analysis with ³²P-labeled probes with sequences listed in the legend of Fig. 7/3. Methodology has been described in earlier studies (15,16).

RESULTS

Case Report. The proband is a 7-year-old male born to a healthy French-Canadian couple. His mother noted scleral icterus at 8 months of age and his anemia was noted at about 15 months. He was first seen at the Hôpital Sainte-Justine in Montreal at 2½ years. Hematological data for him and his parents at the latest collections are listed in Table 7/1. Heinz bodies were readily detectable after 1-2 hours of incubation of blood at 37°C, suggesting the presence of an unstable Hb variant. This was confirmed by a heat stability test which showed that some 15-20% of the Hb in a red cell lysate readily precipitated at 61°C. The Hb variant was also detected by IEF; its mobility was similar to that of a common Hb D.

Quantitation was by two HPLC procedures; the β^X and β^A chains were partially separated on a reversed phase C_4 column (Fig. 7/1, top), while the Hb X eluted from a cation exchange HPLC column well separated from the other Hb types (Fig. 7/1, bottom). Its quantity before splenectomy was some 16-19%, and after splenectomy this value had decreased to 12%. The Hb A₂ value of 4 to 4.5% was definitely higher than normal (2-3%); Hb F was also slightly increased (2-3% versus <1% as normal value). The parents of the boy were healthy and had no Hb abnormality. There are two other normal children in this family, neither with any anemia.

TABLE 7/1. Hematological Data

	Patient F.P.		Father P.	Mother P.
Sex-Age	M- 7		M-31	F-28
Hb (g/dl)	8.3	8.9 ^a	15.5	14.5
PCV (l/l)	0.285	0.305	0.44	0.405
RBC ($10^{12}/l$)	2.59	2.63	5.28	4.52
MCV (fl)	110.0	116.0	83.0	90.0
MCH (pg)	32.0	33.8	29.4	32.1
MCHC (g/dl)	29.1	29.2	35.2	35.8
Hb F (%) ^b	3.0	2.1	<1.0	<1.0
Hb A ₂ (%) ^b	4.4	4.1	2.6	2.8
Hb X (%) ^b	16.7	12.0	0	0

^a Data a few weeks after splenectomy.

^b By cation exchange HPLC (3,4).

Structural Analyses. Fig. 7/2 illustrates the separation of the tryptic peptides of the AE- β^X chain. Nearly all peptides were recovered, had their expected position in the chromatogram, and had normal amino acid compositions. The β T-9 was missing and two small fragments were found; one eluted behind β T-1 and the second between β T-11 and β T-13. Their amino acid compositions are listed in Table 7/2; both fragments were rather pure and probably replaced the first eight and the last eight residues of peptide β T-9. Peptide β T-9^A of Hb X shared six amino acid residues with β T-9^A of Hb A and an Asp and Gly were replaced by Arg and Ala; sequence analyses identified the five amino terminal residues as being the same as those found for β T-9 (Hb A). Peptide β T-9^B (Hb X) shared seven residues with the C-terminal segment of β T-9 (Hb A), contained one Cys residue and one Glu (or Gln) residue not present in β T-9^B (Hb A), and had one less Leu residue. Its sequence determination was complicated by a probable -Cys-Gln- amino terminus and by a low recovery of the peptide. From these results we derived the tentative amino acid sequence for the β T-9 A and B fragments as listed in Fig. 7/2. The suggested sequence of β T-9 (Hb X) compared with that of Hb A was as follows:

	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82		
Hb A:	Val	Leu	Gly	Ala	Phe	Ser	---	Asp	Gly	Leu	---	Ala	His	Leu	Asp	Asn	Leu	Lys
Hb X:	Val	Leu	Gly	Ala	Phe	Ser	ALA	ARG	CYS	GLN	Ala	His	Leu	Asp	Asn	Leu	Lys	

These preliminary data suggested a deletion of the three amino acid residues (Asp, Gly, Leu) and an insertion of four amino acid residues (Ala, Arg, Cys, Gln).

FIG. 7/1. Identification of Hb Montreal by HPLC. Top: Partial separation of the β^X (= β -Montreal) and β^A chains in reversed phase HPLC (5,6). Bottom: Separation of Hb X (= Hb Montreal) and the other Hb components by cation exchange HPLC (3,4).

TABLE 7/2. Amino Acid Composition of the Two Fragments of Peptide T-9^a

Amino Acid	T-9A	T-9B
Aspartic Acid	0 (1)	1.97 (2)
Glutamic Acid		1.13 (0)
Serine	0.84 (1)	
Glycine	0.93 (3)	
Arginine	1.14 (0)	
Histidine		0.78 (1)
Alanine	2.17 (1)	0.88 (1)
Valine	0.95 (1)	
Leucine	0.97 (1)	1.88 (3)
Phenylalanine	0.97 (1)	
Lysine		1.00 (1)
AE-Cysteine		0.68 (0)
Positions in normal β chain	67-74	75-82

^a In residues/mole. The numbers between parentheses refer to the numbers of residues observed for the positions in the normal β^A chain.

FIG. 7/2. Separation of peptides in a tryptic digest of the AE- β^X (= β -Montreal) chain by reversed phase HPLC (9). The abnormal peptides indicated by peaks with dashed lines; the insert shows the sequence of these peptides.

DNA Analyses. The DNA from the patient was amplified asymmetrically and sequenced in both directions. The data identified a frameshift starting at CD 73 with the direct sequencing primer, and at CD 75 with the reverse sequencing primer (Fig. 7/3). This made it possible to characterize the sequences of the normal chromosome and the abnormal chromosome as indicated in Fig. 7/3. These data confirmed the tentative results obtained by the analyses of the abnormal β^X chain. Final confirmation was obtained by dot-blot analyses of amplified DNA from the patient and his parents with ^{32}P -labeled oligonucleotide probes; the deletion/insertion was identified only in the DNA of the patient (Fig. 7/3). The Hb variant was named Hb Montreal after the city of residence of the proband.

DISCUSSION

Hb Montreal is clearly a most unusual Hb variant because its abnormal β chain has a deletion of three amino acid residues (Asp, Gly, Leu at β 73, β 74, and β 75) and an insertion of four different residues (Ala, Arg, Cys, Gln) at the same location. The evidence is based on data obtained through chemical analysis of two isolated tryptic fragments of the β chain (T-9^A and T-9^B), on sequence data of amplified DNA, and finally, on data from hybridization of the amplified DNA with ^{32}P -labeled probes specific for this change in DNA sequence. The location of this deletion/insertion is of some interest because two other β chain variants have been discovered which are characterized by a deletion of Leu at β 75 [Hb Vicksburg (17)] and of Gly-Leu at β 74 and β 75 [Hb St. Antoine (18)].

FIG. 7/3. Left: Reverse sequence analysis of amplified DNA of the propositus F.P. The start of the double-banding is located in CD 76 (CAG for Gln) in β^X and CD 75 (CTG for Leu) in β^A . The direct sequence identified two CDs at position 73 (GCT for Ala in β^X and GAT for Asp in β^A ; data not shown). The sequences of the amplification primers were: Direct: 5'-GCCAAGGACAGGTACGGCTGTCATC-3' (positions -140 through -116 relative to the Cap site); reverse: 5'-CCCTTCCTATG ACATGAACCTTAACCAT-3' (positions +566 through +540 relative to the Cap site). Right: The results of the hybridization of amplified DNA with 32 P-labeled probes; the sequences of the probes were: Mutant: 5'-AGTGCTCGGTGCCAGGCTC-3'; normal: 5'-AGTGATGGCCTGGCTCACC-3'.

The deletion of Asp-Gly-Leu and the insertion of Ala-Arg-Cys-Gln occurred at the C-terminal end of the E helix (positions E17, E18, E19) which is part of the crevice where the heme group is located. Although none of the three deleted residues is involved in any major contact, i.e. with heme or between chains, it is evident that the introduction of an extra amino acid in this helix, and the presence of the large arginine residue should affect the stability of the protein and perhaps its function. A considerable instability of the variant was indeed noticed by hematological observations such as Heinz body formation and an increase in precipitation of Hb during the heat stability test. MethHb (= ferriHb) was not observed and functional studies were not conducted.

The β -Montreal variant is presumed to be the result of a *de novo* genetic event because the parents of this young patient were normal and did not have the Hb variant; paternity testing was not performed. The mechanism(s) responsible for the noted changes is (are) not clear. However,

it is well-known that small deletions and also small insertions can result from slippage and mispairing of two homologous DNA sequences during DNA replication. This may result either in the removal or repetition of the DNA between these small homologous sequences. Fig. 7/4 illustrates a possible mechanism for the formation of the β -Montreal gene. The repetitive sequence AGTG is present in CDs 66 and 67 and CDs 72 and 73 of the normal β gene. As a result of a mispairing, the AGTG sequence and the CTCGGTGCCTTT sequence between the two repetitive sequences (a total of 16 bp) are repeated. A similar phenomenon has been extensively described in a bacterial system (19). A deletion of 10 nts (TGATGGCCTG) may have arisen from recombination or slippage between short sequence repeats (perhaps the TG dinucleotide) followed by a second deletion of the TTT sequence during the subsequent DNA repair. The final result of these structural alterations is a modified sequence which affects only CDs 73 through 76 of the β -Montreal gene and does not otherwise alter the reading frame.

FIG. 7/4. A scheme providing a possible mechanism for the formation of the β -Montreal gene.

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CHAPTER 8

DISCUSSION

Since the discovery of Hb S in 1949 (1), more than 100 Hb variants have been described (2). The extensive progress made in the studies of Hb variants was made possible primarily by technological advances, namely the development of more sensitive and rapid methods for their detection and characterization. Most of the common Hb variants were detected some 10 to 20 years ago because of their altered electrophoretic mobilities. Variants that have been described in recent years are usually rare hemoglobins, that have mutations that are difficult to detect by electrophoretic procedures or a higher structure requiring advanced methodology for their characterization. Fig. 8/1 shows the number of different abnormalities detected between 1975 and 1992. The increase in the number of Hb variants with amino acid deletions or extended chains, of γ and δ chain variants, and of those with the mutations that were detected in the last few years is likely due to the application of improved technology.

DISCUSSION

Fig. 8/1. The number of different Hb variants detected between 1975 and 1992 (from Ref. 3).

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DISCUSSION

Since the discovery of Hb S in 1949 (1), more than 600 Hb variants have been described (2). The extensive progress made in the studies of Hb variants was made possible primarily by technological advances, namely the development of more sensitive and rapid methods for their detection and characterization. Most of the common Hb variants were detected some 20 to 30 years ago because of their altered electrophoretic mobilities. Hb variants that have been described in recent years are usually rare abnormalities, that have mutations that are difficult to detect by electrophoretic procedures or a rather complex structure requiring advanced methodology for their characterization. Fig. 8/1 shows the number of different Hb abnormalities detected between 1978 and 1992. The increase in the number of Hb variants with amino acid deletions or extended chains, of γ and δ chain variants, and of those with two mutations that were detected in the last few years is likely due to the application of improved methodology.

FIG. 8/1. The number of different Hb variants detected between 1978 and 1992 (from Ref. 3).

Technological Advances in the Studies of Hb Variants. Electrophoretic techniques have unquestionably played an important role in the studies of Hb variants. The first electrophoretic technique was the moving boundary type that was conducted in a liquid media and was used in the original

study of Pauling and his colleagues when they discovered the abnormal electrophoretic behavior of Hb S (1). This discovery stimulated a search for other Hb abnormalities, and within a short time, two new Hb variants with altered electrophoretic mobilities, namely Hb C (4) and Hb D (5) were described, and many others thereafter. Although the moving boundary electrophoresis offered a high degree of accuracy and precision, it was time-consuming and difficult to perform. This, and an increased interest in the abnormalities, stimulated a search for more simple electrophoretic techniques that could be used for screening purposes. The development of the electrophoretic technique that uses filter paper as a support medium (6,7) permitted a larger number of Hb variants to be detected. Improvements in electrophoretic techniques have included the use of starch gel (8), agar gel (9), and other support media, as well as the development of IEF methods (10). Different modifications of these techniques have been introduced that have permitted higher resolution of Hbs and have contributed significantly to the identification of many abnormal Hbs.

Currently, a large number of electrophoretic techniques are available; most popular is IEF, that has a high resolving power and the ability to detect minor Hb components. In IEF, the different Hbs are fractionated according to their isoelectric point (pI) along a pH gradient; an excellent resolution is possible even for variants with pIs that differ by only 0.02 pH unit (11). The analyses of IEF are fast, many samples can be simultaneously analyzed, and common Hb variants such as Hbs S, C or G-Philadelphia are readily detected; this makes this procedure the method of choice for large-scale screening programs. Various methods, such as acid citrate-agar electrophoresis, solubility testing of deoxyHb or immunological procedures, can confirm the presence of these common Hb variants. Although electrophoretic techniques are still the most common methods used for the initial detection of abnormal Hbs, they fail to discriminate many Hb variants from Hb A and Hb F.

Other methodological advances that have played an important role in the studies of Hb variants are the chromatographic procedures. Different macrochromatographic methods using different ion exchangers and developers have been developed (12); these allowed an easy separation and quantitation of the normal and many abnormal Hbs with a high degree of resolution. Furthermore, the same methods permitted the isolation of various abnormal Hbs in pure form, and have replaced electrophoretic techniques previously used for that purpose. The first absorbent to be used was the cation exchanger Amberlite IRC-50; a simple qualitative technique was developed by Huisman and Prins in 1957 (13) that was later replaced by more accurate procedures (14,15). Later refinements in chromatographic methods have included the use of different cation exchangers such as CM-cellulose and CM-Sephadex, as well as the anion exchangers DEAE-Sephadex and DEAE-cellulose. CM-cellulose and CM-Sephadex were introduced in extensive experiments by Huisman and his collaborators (16-19). DEAE-Sephadex was used for the first time by Dozy et al in 1968 (20) and improved by Huisman and Dozy in 1969 (21); almost immediately this procedure became the most popular chromatographic method for quantitation and isolation of abnormal Hbs. The later developed DEAE-cellulose chromatographic method with glycine-KCN-NaCl developers (22) offered additional advantages; it was faster, simpler, and provided

improved separation of Hbs. This method has replaced all other procedures for the quantitation and isolation of Hb variants, and in many laboratories, is still widely used for the isolation of larger quantities of certain abnormal Hbs. Although all these chromatographic methods are reproducible and offer quantitative data, they have the disadvantage of being time-consuming and labor-intensive, thus making them unsuitable for screening purposes.

The search for a rapid and improved methodology for the quantitation of Hb A₂, and the detection and quantitation of some common abnormal Hbs such as Hbs S and C, led to the development of several microchromatographic procedures. Chromatography on small columns was first described by Huisman and Dozy in 1962 (23), but the micro procedures became really popular in 1973 when Schroeder, Huisman, and their collaborators introduced miniature columns and developing systems useful for a rapid detection of Hbs S and C at birth (24), and for the quantitative determination of Hb A₂ (25,26). Since then, other microchromatographic methods or modifications of existing ones have been devised for the quantitation of different Hb types such as Hbs A₂, F, Bart's, and A_{1c} (27). Although these microchromatographic techniques are less accurate for the detection and quantitation of the major abnormal Hbs, they are most useful for a rapid and accurate quantitation of Hb A₂. As a result, these methods are still widely used in many laboratories.

Chromatographic procedures were also valuable for structural analyses of Hb variants. The separation of Hb chains is the first important step in the procedures for structural characterization of abnormal globin chains; many chromatographic techniques for separation and isolation of Hb chains have been described (28). However, the CM-cellulose chromatographic method using a sodium-ion gradient in 8 M urea-mercaptoethanol phosphate buffers, developed by Clegg et al (29) in 1966, was the best procedure that replaced all other techniques developed for that purpose. This procedure is still used for isolation of abnormal chains when a sufficient amount of sample material is available.

The structural characterization of Hb variants is usually done by examination of the globin tryptic peptides. The first technique used for this purpose was the fingerprinting procedure (paper electrophoresis followed by partition chromatography) that was introduced by Ingram in 1956 (30). This procedure has been used in modified forms for many years and has contributed to the identification of many Hb variants. Macrocolum chromatographic procedures for analysis and separation of globin peptides were introduced by Jones (31) and included the automatic peptide chromatography and Dowex macrocolumn chromatography described by Schroeder (32,33). These techniques permitted the isolation of large quantities of globin peptides in pure form, and represented an important advance in methodology for the structural analyses of Hb variants.

The development of HPLC methods has brought great improvement in the studies of Hb abnormalities because highly sensitive and rapid procedures became available for the detection, quantitation, and isolation of abnormal Hbs, globin chains, and peptides. These techniques are accurate and repro-

ducible, rapid, sensitive, and have high resolving power. Therefore, they have rapidly replaced all other available technology for the detection, quantitation, and structural characterization of Hb variants.

An anion exchange HPLC procedure for the detection and quantitation of normal and abnormal Hbs was first described by Gooding et al in 1979 (34). Several modifications appeared in the literature during the next few years (35,36). This procedure provided a rapid method for the quantitation of Hbs A, S, C, and F in newborn and adult samples, as well as for the separation and quantitation of many other abnormal Hbs. The disadvantage of this technique is its inability to separate Hb F from the minor Hb A₁ components, thus preventing the quantitation of Hb F in adult samples with a low Hb F percentage.

Cation exchange HPLC appeared to be a more promising method for the separation and particularly for the quantitation of normal and abnormal Hbs (37-40). The general rule defined by macro cation exchange chromatography applies, i.e. the higher the positive charge, the longer the elution time. However, the analyses of numerous Hb variants with the same type or with comparable amino acid substitutions have shown some differences in their RET values, suggesting that the location of the substitution can affect the elution of Hb variants in this type of chromatography. Variants due to substitutions within the helical region elute faster than those in a prehelical region or in an interhelical segment. Additional factors, such as an extra positive charge on some of the globin chains, and the formation of salt bonds that eliminate the extra positive charge, may also influence the elution of some Hb variants in this system (41). The differences in RET values are reproducible, allowing the chromatographic identification of certain common Hb abnormalities; their presence can be confirmed by some of the methods mentioned above.

Preparative anion and cation exchange HPLC columns are also available; the value of these columns is that they allow separation and isolation of Hb components that are present in very small amounts. For instance, the preparative polyCAT HPLC column allows the complete separation of Hb F and Hb A_{1c}, and the isolation of Hb F in pure form when Hb levels in the samples are as low as 1 or 2% (40). Thus, this procedure is important for the isolation of some abnormal Hbs that are present in small amounts; several chromatograms can provide sufficient material for the structural characterization by sensitive HPLC procedures.

Different reversed phase HPLC procedures for the separation of Hb chains have been developed (42-44) and the one introduced by Shelton et al (45), using a large-pore C₄ column and acetonitrile-water-TFA developers has replaced all other methods. Separation by this procedure is based on the differences in the polarities, sizes, and pK values between the substituted and introduced amino acids. As a general rule, the more polar (less hydrophobic) chains elute earlier in the chromatogram. There is an excellent correlation between predicted (according to the polarities of the amino acids) and obtained RET values, although there are some exceptions, suggesting that some additional factors such as the location of the amino acid substitution in the globin chain may influence the elution

of the variant globin chain (41). The usefulness of this method in detecting Hb variants with neutral substitutions has been confirmed many times in recent years, and has resulted in the detection of an increased number of Hb variants with neutral substitutions.

Preparative reversed phase HPLC columns for chain separation are also available; they have facilitated the isolation of some abnormal globin chains that have neutral substitutions and/or are present in small amounts. This procedure is particularly valuable for the isolation of the γ chains in pure form.

A reversed phase HPLC method for the separation of globin peptides was introduced by Wilson et al in 1979 (46). The resolution of peptides in this method is, in part, based on differences in molecular weight, on the hydrophobic nature of the compounds, and to a much lesser extent, to differences in charge. This procedure has shown distinct advantages over the other procedures for peptide separation; it allows separation of micro quantities of tryptic peptides within a short time. Most peptides eluted are pure and uncontaminated, and can be analyzed without further purification with some of the sensitive amino acid analyzers presently available. Small changes in the elution profile can be observed and are frequently indicative of a specific peptide being abnormal in structure. This procedure, and modifications introduced later (47), have greatly facilitated the identification of numerous Hb variants.

Application of DNA Methodology to the Identification of Hb Variants.

Since the genetics of the normal human Hbs and their variants were well defined long before the development of the recombinant DNA technology, Hb has served as a model for the implementation of recombinant DNA technology to human genetics. Globin genes were one of the first human genes that were cloned and isolated from DNA libraries, resulting in an early characterization of the chromosomal arrangement of the globin genes and their fine structure. In the same time, the application of recombinant DNA technology has dramatically increased the possibilities for the diagnosis and prenatal detection of the hemoglobinopathies.

Restriction enzyme mapping, or gene mapping, was developed by Southern in 1975 (48), and was one of the first techniques used in the studies of globin genes. The well-defined restriction enzyme map of the β -globin gene cluster allowed the application of this technique to the identification of hybrid Hbs. Gene mapping analyses readily detected the deletions of 7 and 22.5 kb DNA fragments in the Lepore Hbs and Hb Kenya, respectively (49,50). These analyses have brought additional evidence that the hybrid chains of the Lepore and anti-Lepore Hbs are the products of non-equal homologous crossovers between misaligned chromosomes, and have also allowed the more precise localization of the recombination event (51).

Gene mapping analyses can also be used for the identification of abnormal Hbs due to single nt changes that result in the loss or creation of a restriction enzyme site. The first abnormal Hb that was detected by gene mapping analyses was Hb S. The A $\rightarrow$ T substitution in the sixth CD of the β^S gene eliminates the recognition site for the restriction enzymes

Dde I and Mst II. This approach has been employed for the prenatal diagnosis of Hb S (52), and for the identification of several other β , and even some γ , chain abnormal Hbs (53,54). Although the technique represented a great improvement over existing methods, particularly for prenatal diagnosis, it was still rather time-consuming.

The discovery of the amplification PCR procedure (55,56) has had a major impact on the analyses and diagnoses of the hemoglobinopathies. The introduction of this technique has led to a variety of new approaches for rapid and accurate identification of the globin gene mutations. The technique allows the amplification of a target DNA segment from small amounts of DNA over a period of a few hours. The amplified DNA fragment can be directly analyzed in an ethidium bromide stained gel, with allele-specific oligonucleotide probes, by restriction enzyme digestion, or by direct sequencing. These techniques, all widely used for detecting α - and β -thal alleles, have also been applied to the studies of Hb variants.

PCR has provided a new approach for the detection of the Lepore Hbs. Using a combination of δ - and β -specific amplification primers, it is possible to directly detect the Lepore Hbs by electrophoretic analysis of the PCR products, provided amplification primers are located at either side of the deletion.

Hb variants that alter restriction enzyme sites can be easily and rapidly detected by digestion of the amplified DNA with these enzymes. The PCR method is so powerful, and it produces so much DNA, that the fragments obtained after digestion with restriction enzymes can be analyzed directly on the ethidium bromide stained gel.

Another approach that facilitates the detection of known mutations is a combination of PCR and hybridization with ASO probes (57). Hybridization with ASO probes was first applied to genomic DNA digested with a restriction endonuclease. The combination of ASO hybridization with PCR made this technique faster, simpler, and much more sensitive. Even a minute quantity of DNA, isolated from a dried blood spot collected on filter paper, can be analyzed by this approach. Both ASO hybridization and restriction enzyme digestion of amplified DNA are easy, rapid, and accurate methods for the localization and confirmation of mutations that are determined through protein analyses or are predicted on the basis of electrophoretic and chromatographic analyses.

The development of PCR has led to a new approach for sequencing human globin genes, bypassing the need to clone the gene of interest. The PCR produces enough DNA that can be used directly for dideoxy sequencing by the method of Sanger et al (58) and direct sequence analysis of amplified DNA has been applied to identify structurally different Hb variants.

Application of Micromethodology to the Identification of 10 Hb Variants. The studies reported in this dissertation clearly demonstrate the advantages of the HPLC and DNA methodologies in the characterization of Hb variants. The identification of 10 Hb variants (two α , four β , three γ , and one extended β chain variants) has been presented; seven of these are

new, while three were previously described in only a few patients. Six were clinically silent, while four were unstable Hb variants (Sun Prairie, Yokohama, Volga, and Montreal) resulting in a hemolytic anemia in the carriers. Many were detected and characterized by different HPLC procedures. However, for some Hb variants, a simultaneous identification at the protein and DNA levels is given to show the advantage and necessity of the DNA methodology for their complete characterization.

All the Hb variants described were initially analyzed by an electrophoretic procedure (IEF or starch gel electrophoresis). Five Hb variants, namely Hbs Sun Prairie, Zengcheng, Yokohama, Volga, and F-Jiangsu, did not show an altered electrophoretic mobility and could not be separated from Hbs A or F on IEF or starch gel electrophoresis. Comparable results were obtained with the cation exchange HPLC procedure; Hb variants with an altered electrophoretic mobility were also detectable by this method. The only exception was Hb Iowa, that was detected by IEF as a band very close to Hb A but could not be separated on cation exchange HPLC.

All abnormal globin chains were detected and quantitated on reversed phase HPLC, even when other procedures failed to indicate the presence of an abnormal Hb. However, although all of the variants presented here were detectable by this method, other known Hb variants cannot be detected because of the equal polarities of the substituted and introduced amino acid residues. An example is the abnormal β chain of the unstable Hb variant Hb Köln. Fortunately, a combination of reversed phase HPLC for chain separation and IEF or cation exchange HPLC for the Hb separation, allows the detection of most variants. Currently, these three procedures are the methods of choice for the detection and quantitation of Hb variants.

The precise molecular characterization of a particular Hb variant is greatly facilitated by HPLC methodology and DNA analyses, the advantages of which are best shown in the studies of the three Hb variants presented in Chapters 3b, 5a, and 7. The detection and characterization of the unstable α chain variant Hb Sun Prairie would have been nearly impossible without HPLC methodology. The presence of an unstable Hb variant would probably not have been considered because the stability tests were essentially negative. The detection of a small zone in the Vydac C₄ HPL chromatogram was the first definitive indication for the presence of an abnormal chain. Preparative reversed phase HPLC has allowed the isolation of the abnormal chain in pure form and in a sufficient amount for peptide analyses, and identification of the Ala- $\rightarrow$ Pro substitution at position 130 of the α chain by amino acid analyses. The confirmation of the G- $\rightarrow$ C mutation in CD 130, and its localization on the $\alpha 2$ -globin gene, was obtained by dot-blot analyses of amplified $\alpha 2$ - and $\alpha 1$ -globin genes with the CD 130 Ala and CD 130 Pro probes.

The mutation leading to Hb Yokohama has been characterized at both the protein and DNA level. Apart from the positive stability test, the only evidence for the presence of an abnormal Hb was the extra zone on the reversed phase HPLC for chain separation. The identification of the Leu- $\rightarrow$ Pro substitution was obtained after isolation of the abnormal Hb with high concentrations of salts, separation and isolation of the abnormal β

chain by CM-52 column chromatography, followed by separation of the tryptic peptides by reversed phase HPLC and amino acid analyses. This was not only a time-consuming but also a difficult approach, since the isolated β^X chain was contaminated with normal β chain. In order to confirm the observed substitution, and to present an easier approach for its characterization, DNA analyses were made. The direct sequence analysis of amplified DNA readily localized the T→C substitution at CD 31 of the β gene. This was indeed a much easier and faster approach for the identification of this abnormal variant.

Finally, in the case of Hb Montreal, the direct DNA sequence analysis was not only easier, it was also the only approach to be taken for determining the nt changes and predicting the likely mechanism(s) responsible for the occurrence of this most unusual Hb variant. The protein structural analyses of Hb Montreal predicted the deletion of three amino acid residues (Asp, Gly, and Leu) at positions 73, 74, and 75, and an insertion of four different residues (Ala, Arg, Cys, and Gln) at the same location in the β chain. These were indeed very unusual changes, and the nt alterations could not be predicted from the amino acid variations. The data from the DNA sequence analyses showed the sequence changes in the β -globin gene of the abnormal chromosome and allowed the prediction of the molecular mechanism leading to the observed changes. The repetitive AGTG sequences flanking the observed changes suggested that a mispairing involving the AGTG sequences at CDs 66 and 67 and at CDs 72 and 73 of the normal β -globin gene caused a duplication of a 16 bp segment. Two short deletions (3 and 10 nts) occurring in the 3' end of the duplication and further downstream must have resulted in the sequences observed in the β -Montreal gene.

The above data demonstrate that HPLC is indeed a sensitive and powerful technique, and is the method of choice for structural analyses of abnormal Hbs in protein chemistry laboratories. However, the introduction of direct sequence analysis of amplified DNA has provided a new and promising approach to the identification of abnormal Hbs. This approach has many advantages over protein analyses; it is faster and more sensitive, it does not require costly equipment, and allows the direct detection of the gene defect. It appears that the identification of Hb variants, especially unstable variants, that are often present in minute amounts, will increasingly depend on this approach.

Unstable Hb variants represent a major group of clinically significant Hb variants. The term "unstable Hb variant" refers to the group of variants that have an instability sufficient to produce intracellular Hb precipitation, resulting in a hemolytic anemia in the carriers. The disease varies according to the Hb variant present and it may produce a mild, moderate, or severe hemolytic anemia. However, acute episodes of severe hemolytic anemia may be seen in all cases, secondary to bacterial or viral infections, and exposure to chemical oxidants. The most important factor in the treatment of the hemolytic anemias, secondary to the presence of unstable Hbs, is an early diagnosis. Once the diagnosis is reached, supportive and preventive measures can be instituted, such as administration of folic acid, prevention and prompt treatment of infections, and avoidance of oxidant drugs. Splenectomy can be beneficial, and partial correction

of the anemia has been achieved in some instances. An early diagnosis of the unstable Hb variant can prevent unnecessary diagnostic procedures and inappropriate therapeutic interventions.

CONCLUSIONS

Methodology developed for detection and identification of Hb variants has been greatly modified over the years. IEF for initial detection of abnormal Hbs, cation exchange HPLC for separation and quantitation of abnormal Hbs, and reversed phase HPLC for separation and quantitation of abnormal globin chains, are currently the methods of choice. Although reversed phase HPLC for peptide analyses is the most useful technique for structural characterization of abnormal Hbs at the protein level, the identification of Hbs with complex structures and with distinct physicochemical properties, will increasingly depend on the application of DNA methodology, i.e. direct sequence analysis of amplified DNA.

Recent developments in the studies of Hb and its variants have led to revolutionary insights into modern molecular genetics; the contribution of the Hb genes and hemoglobinopathies to our understanding of molecular genetics continues. However, this has not been a one-way benefit, because the increasing technology of molecular genetics has improved our understanding of the clinical disorders comprising the hemoglobinopathies, and has greatly facilitated their diagnosis. There is no better example than this blending of science and clinical medicine to the mutual benefit of both fields.

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CHAPTER 2

SUMMARY

A major aim of the studies reported in this dissertation is to demonstrate the usefulness of the HPLC techniques and HPLC methodology for the identification of rare Hb variants, and to contribute to our understanding of molecular mechanisms that are responsible for the occurrence of these unusual Hb abnormalities.

A review of the literature is presented in Chapter 1 and includes a description of structure and structure-function relationships of the Hb molecule, organization and fine structure of the globin genes, and study of abnormal expression. General features of the normal human Hb, including those predominantly present in the embryo, fetus, and newborn baby, as well as that in the normal adult, are reviewed. This chapter also includes a summary of the molecular events responsible for the occurrence of these Hb variants. Most abnormal Hbs have occurred as a result of mutations in the coding regions of the globin genes that change the sequence. Thus, most of the abnormal Hbs are characterized by single amino acid substitutions, but some have two amino acid substitutions within the same chain, or have longer or shorter chains. Others are fusion or split. The last have arisen through processes of chromosomal rearrangement. Clinically significant variants are characterized as follows: they are classified in four groups: The Hb S syndrome, the unstable Hbs resulting in hemolytic anemia in the carriers, the Hbs with high O₂ affinity leading to cyanotic syndromes, and the Hbs with low O₂ affinity and Hb E variant, both leading to congenital cyanosis. An updated alphabetical list of known Hb variants is provided as an appendix to this chapter.

SUMMARY

The methodologies used for the identification of the Hb variants described in this dissertation are presented in Chapter 2. Chapter 3 concerns the identification of two Hb variants by HPLC. Chapter 4 describes the characterization of a new Hb variant with an Asn at the distal site at position 68. The variant Hb Denmark, was detected in 40 members of a Danish family, with an autosomal mode of the type 1:1 inheritance, followed by the separation of the mixture of hemoglobins by reversed phase HPLC, and amino acid sequence analysis of a few of these hemoglobins, greatly facilitated the identification of this substitution. The Asn is not involved in any water contact with the heme group or between chains subunits, and its replacement by another amino acid is not likely to affect Hb stability. Carriers of Hb Denmark are therefore not expected to be clinically affected.

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The second observation of the Hb San Francisco (Hb S-F) variant is described in Chapter 5. The variant was detected in a healthy 40-year-old female with clinical and hematological features of sickle cell anemia. The identification of this variant would have been virtually impossible without HPLC; the only definitive indication for the presence of an abnormal Hb was the small extra peak on the reversed phase HPLC chromatogram. The preparative reversed phase HPLC permitted the separation of the

CHAPTER 9

SUMMARY

A major aim of the studies reported in this dissertation is to demonstrate the usefulness of the HPLC techniques and DNA methodology for the characterization of rare Hb variants, and to contribute to our understanding of molecular mechanisms that are responsible for the occurrence of some unusual Hb abnormalities.

A review of the literature is presented in Chapter 1 and includes a description of structure and structure-function relationship of the Hb molecule, organization and fine structure of the globin genes, and their developmental expression. General aspects of the normal human Hb, including those predominantly present in the embryo, the fetus, and newborn baby, as well as that in the normal adult, are presented. This chapter also includes a summary of the molecular defects responsible for the occurrence of known Hb variants. Most abnormal Hbs have occurred as a result of mutations in the coding regions of the globin genes that change the sequence. Thus, most of the abnormal Hbs are characterized by single amino acid substitutions, but some have two amino acid substitutions within the same chain, or have longer or shorter chains. Others are fusion or hybrid Hbs that have arisen through crossovers between misaligned chromosomes. Clinically significant variants are reviewed separately; they are classified in four groups: The Hb S syndromes, the unstable Hbs resulting in hemolytic anemia in the carriers, the Hbs with high O₂ affinity leading to erythrocytosis, and the Hbs with low O₂ affinity and Hb M variants, both leading to congenital cyanosis. An updated alphabetical list of known Hb variants is provided as an addendum to this chapter.

The methodologies used for the identification of the Hb variants described in this dissertation are presented in Chapter 2.

Chapter 3 concerns the identification of two α chain variants by HPLC. Chapter 3a describes the characterization of a new Hb variant with an Asp $\rightarrow$ His substitution at position $\alpha 78$. The variant, Hb Davenport, was detected in two members of a Caucasian family. Mild acidic hydrolysis of the large $\alpha T-9$ peptide, followed by the separation of the resulting fragments by reversed phase HPLC, and amino acid sequence analysis of a few of these fragments, greatly facilitated the identification of this substitution. Asn $\alpha 78$ is not involved in any major contact with the heme group or between globin subunits, and its replacement by another amino acid is not likely to affect Hb stability; carriers of Hb Davenport are therefore not expected to be clinically affected.

The second observation of the Hb Sun Prairie [$\alpha_2 130(H13)Ala \rightarrow Pro\beta_2$] anomaly is described in Chapter 3b. The variant was detected in a 20-year-old Indian male with clinical and hematological features of chronic hemolytic anemia. The identification of this variant would have been virtually impossible without HPLC; the only definitive indication for the presence of an abnormal Hb was the small extra peak on the reversed phase HPL chromatogram. The preparative reversed phase HPLC permitted the isolation of the

abnormal chain in pure form. Dot-blot hybridization analysis of the amplified $\alpha 2$ - and $\alpha 1$ -globin genes, using CD 130 Ala and CD 130 Pro oligonucleotide probes, localized the G→C substitution in the $\alpha 2$ -globin gene. The study provided the interesting observation that the α -Sun Prairie chain has a greater affinity for the δ chain than for the β chain.

The characterization of two new β chain variants by HPLC is presented in **Chapter 4**. **Chapter 4a** deals with the identification of Hb Iowa, which was observed in a Black infant and her mother. The baby was also heterozygous for Hb S. Structural characterization through amino acid analysis of tryptic peptides of the AE- β^X chain showed a Gly→Ala substitution at position $\beta 119$ (GH2). The replacement of the $\beta 119$ Gly does not affect the function of the Hb molecule, despite its involvement in molecular contacts between the $\alpha 1$ and $\beta 1$ chains.

The use of IEF, cation exchange HPLC, and reversed phase HPLC for screening Hb abnormalities has led to the detection of a new β chain variant, namely Hb Zengcheng. The identification of this variant is described in **Chapter 4b**. Hb Zengcheng [$\alpha 2\beta 2114$ (G16)Leu→Met] was observed in a Chinese newborn. The leucine residue at position $\beta 114$ is internally located and is not involved in any major contact with the heme or globin chains; the Leu→Met replacement is not expected to alter the physicochemical or functional properties of the variant.

Chapter 5 concerns the detection of two unstable β chain variants. Their identification was made by both protein and DNA analyses. Hb Yokohama or $\alpha 2\beta 231$ ($\beta 13$)Leu→Pro, was observed in a young boy from the former Yugoslavia, who presented with a severe transfusion-dependent hemolytic anemia. The detection of this Hb variant by reversed phase HPLC is presented in **Chapter 5a**. Its characterization was by HPLC analyses on material obtained by high salt precipitation and by direct sequencing of amplified DNA. The detection of Hb Volga or $\alpha 2\beta 227$ ($\beta 9$)Ala→Asp in a 5-year-old boy from Bosnia and Hercegovina, who suffered from severe hemolytic anemia, is presented in **Chapter 5b**. The variant was observed by reversed phase HPLC and the structural characterization was by HPLC analysis. An easier approach for the detection of the Hb Volga mutation by Ava II digestion of amplified DNA is also described. The patients with Hb Yokohama and Hb Volga were born to healthy parents, without a Hb abnormality. Paternity studies included an evaluation of the polymorphisms of the HLA DQA locus and provided data that confirmed a de novo mutation in both patients.

Chapter 6 describes the detection of three new γ chain variants, and their characterization by HPLC. Preparative reversed phase HPLC allowed the isolation of the abnormal γ chains in pure form, thus greatly facilitating their characterization. During a survey of cord blood samples from healthy Chinese newborns, a new A_γ variant was detected. The structural characterization of this abnormal variant, named Hb F-Jiangsu, is presented in **Chapter 6a**. Structural analyses of the isolated A_γ^X chain demonstrated a Val→Met substitution at position $A_\gamma 134$, thus creating a rather unique Met-Met sequence. The location of the second methionine residue in the γ T-15 peptide significantly influences the chromatographic properties of the abnormal A_γ chain. The identification of Hb F-Catalonia or $\alpha 2\gamma 215$ (Al2)Trp→Arg is presented in **Chapter 6b**. The variant was observed in

two Spanish babies. Different HPLC procedures allowed the identification of the Trp→Arg substitution in only a minute quantity of the $G_{\gamma}X$ chain. **Chapter 6c** describes the structural characterization of Hb F-Brooklyn. The variant was detected in a 2-month-old Caucasian female during a routine screening of blood samples from newborn babies. The variant was detectable on IEF, cation exchange HPLC, and reversed phase HPLC. Structural analyses identified a Lys→Glu substitution at position $G_{\gamma}66$. It is not yet known whether the substitutions observed in these three new γ chain anomalies have any effect on the functional or physicochemical properties of the variants; such studies were not possible due to lack of material.

Chapter 7 presents the identification of the unstable Hb Montreal with a deletion of three amino acid residues (Asp, Gly, Leu) at positions 73, 74, and 75 of the β chain, and an insertion of four different residues (Ala, Arg, Cys, Glu) at the same location. The variant was observed in a 7-year-old boy suffering from a moderate hemolytic anemia. The parents of the boy and two of his siblings were normal. The introduction of an extra amino acid residue, and of the changes in the crevice where the heme pocket is located, is the likely cause of the instability of this abnormal Hb. The above listed changes were detected by analyzing the tryptic peptides of the β -Montreal chain by direct sequencing of amplified DNA, and through hybridization of amplified DNA to specific oligonucleotide probes. DNA sequence analysis allowed the prediction of the likely mechanism(s) responsible for the occurrence of this unusual variant. It is suggested that a mispairing involving the repetitive AGTG sequence at CDs 66 and 67 and at CDs 72 and 73 of the normal β gene caused a duplication of a 16 bp segment, while a deletion of 10 nts due to recombination or slippage, followed by a second short (3 bp) deletion during DNA repair could have resulted in the Hb Montreal anomaly.

The extensive progress made in the studies of Hb variants was made possible primarily because of the development of more sensitive and rapid methods for their detection and characterization. Some of the more important methodological achievements in the studies of Hb variants are briefly reviewed in **Chapter 8**. Different electrophoretic and chromatographic techniques have been developed in the past; they have played an important role in the detection and characterization of numerous Hb variants. Because of their altered electrophoretic properties most of the common Hb variants were detected some 20 to 30 years ago. The Hb variants that have been described in recent years are rare abnormalities, and often have mutations that are difficult to detect by electrophoretic procedures or have a rather complex structure. Advanced methodologies such as HPLC procedures and DNA analyses are required for their characterization.

Currently, IEF, cation exchange HPLC, and reversed phase HPLC are the methods of choice for the initial detection and quantitation of Hb variants. Reversed phase HPLC for peptide analyses is a most useful technique for structural characterization of abnormal Hbs at the protein level, but will be replaced by sequencing of amplified DNA involving the α -, β -, δ -, or γ -globin genes. The identification of Hbs with more complex structures and with distinct physicochemical properties will depend completely on the application of DNA methodology.

CHAPTER 10

SAMENVATTING

Een hoofddoel van de studies, die in dit proefschrift worden beschreven, is de geschiktheid aan te tonen van HPLC-technieken en DNA-methodologie bij het karakteriseren van zeldzame Hb-varianten, en bij te dragen tot een beter begrip van de moleculaire mechanismen, die verantwoordelijk zijn voor het optreden van enkele ongewone Hb-abnormaliteiten.

In Hoofdstuk 1 wordt een overzicht van de literatuur gegeven en ook wordt de structuur en het structuur-functie verband van het Hb-molecule beschreven. Bovendien wordt de organisatie en de fijn-structuur van de globine-genen beschreven, en hoe de genetische informatie tot uitdrukking wordt gebracht. Algemene aspecten van normaal humaan Hb worden gepresenteerd, inclusief de belangrijkste aspecten van Hb afkomstig van de embryo, de foetus, de pasgeboren baby en de volwassene. In dit hoofdstuk wordt bovendien een samenvatting gegeven van de moleculaire defecten welke verantwoordelijk zijn voor het voorkomen van bekende Hb-varianten. De meeste abnormale Hb's zijn het gevolg van een mutatie in de coderende gebieden van de globine genen, waardoor de volgorde is veranderd. Daarom worden de meeste abnormale Hb's gekarakteriseerd door een enkele aminozuur substitutie, maar enkele hebben twee aminozuur substituties in dezelfde keten, of hebben een langere of kortere keten. Andere zijn fusie of hybride Hb's die zijn ontstaan door cross-overs van chromosomen. Klinische belangrijke varianten worden apart besproken. Een indeling in vier klassen is gemaakt: de Hb S syndromen, de onstabiele Hb's, waardoor de dragers hemolytische anemie oplopen, de Hb's met hoge affiniteit voor O₂ hetgeen leidt tot erythrocytosis, en tenslotte de Hb's met lage affiniteit voor O₂ en de Hb M varianten, die beide congenitale cyanosis veroorzaken. In een bijlage van dit hoofdstuk wordt een bijgewerkte alfabetische lijst gegeven van bekende Hb-varianten.

De methodologie, die in dit proefschrift wordt gebruikt bij de identificatie van de Hb-varianten, wordt in Hoofdstuk 2 beschreven.

Hoofdstuk 3 behandelt de identificatie van twee α -keten varianten door HPLC. In Hoofdstuk 3a wordt de karakterisering van een nieuwe Hb-variant met een Asp→His substitutie op positie $\alpha 78$ beschreven. De variant, Hb Davenport, werd aangetoond in twee leden van een blanke familie. De identificatie van deze substitutie werd zeer vergemakkelijkt door gebruik van zwak-zure hydrolyse van de grote $\alpha T-9$ peptide, gevolgd door scheiding van de gevormde fragmenten door reversed-phase-HPLC en analyse van de aminozuur volgorde van enkele van deze fragmenten. Het asparagine residu op de $\alpha 78$ positie is niet betrokken bij enig belangrijk contact met de haem-groep of tussen globine sub-eenheden, en zijn vervanging door een ander aminozuur beïnvloedt waarschijnlijk niet de Hb-stabiliteit; dragers van Hb-Davenport hebben daarom naar verwachting geen klinische symptomen.

De tweede waarneming van de Hb-Sun Prairie [$\alpha_2 130(H13)Ala \rightarrow Pro\beta_2$] anomalie wordt in Hoofdstuk 3b beschreven. De variant werd aangetoond in een 20-jarige manlijke Indiaan met klinische en hematologische karakteristieken van chronische hemolytische anemie. De identificatie van deze variant zou praktisch onmogelijk geweest zijn zonder HPLC; de enige definitieve aanwijzing dat het Hb abnormaal was, was de kleine extra piek op het reversed-phase-HPL-chromatogram. Met preparatieve reversed-phase-HPLC was het mogelijk de

abnormale keten in zuivere vorm te isoleren. Met dot-blot hybridisatie analyse van de vermeerderde α_2 - en α_1 -genen en gebruik van CD 130 Ala en CD 130 Pro oligonucleotide probes was het mogelijk de G→C substitutie in het α_2 -globine gen aan te tonen. De studie leidde tot de interessante waarneming dat de α -Sun Prairie keten een grotere affiniteit heeft voor de S keten dan voor de β -keten.

De karakterisering van twee nieuwe β keten varianten door HPLC wordt in Hoofdstuk 4 gepresenteerd. Hoofdstuk 4a behandelt de identificatie van Hb-Iowa, dat werd aangetroffen in een neger kind en haar moeder. De baby was ook heterozygoot voor Hb S. Structurele karakterisering door middel van aminozuur analyse van tryptische peptiden van de AE- β^X -keten toonde aan, dat er een Gly→Ala substitutie was opgetreden op positie $\beta 119$ (GH2). De vervanging van $\beta 119$ Gly heeft geen invloed op de functie van het molecule, ondanks zijn betrokkenheid in moleculaire contacten tussen de $\alpha 1$ - en $\beta 1$ -ketens.

Het gebruik van IEF, cation exchange HPLC en reversed-phase-HPLC bij het zoeken naar Hb abnormaliteiten leidde tot de ontdekking van een nieuwe β -keten variant, namelijk Hb-Zengcheng. De identificatie van deze variant wordt beschreven in Hoofdstuk 4b. Hb-Zengcheng [$\alpha_2\beta_2 114$ (G16)Leu→Met] werd waargenomen in een chinese pasgeborene. Het leucine residu op positie $\beta 114$ ligt binnenin het molecule en is niet betrokken bij enig belangrijk contact met het haem of de globine ketens; daarom wordt niet verwacht dat door de Leu→Met uitwisseling de fysicochemische of functionele eigenschappen van de variant zijn veranderd.

In Hoofdstuk 5 wordt de ontdekking van twee onstabiele β -keten varianten beschreven. Zij werden ontdekt door zowel eiwit- als DNA-analyse. Hb-Yokohama of $\alpha_2\beta_2 31$ (B13)Leu→Pro werd waargenomen in een jongetje uit het voormalige Joegoslavië, dat leed aan ernstige transfusie-afhankelijke hemolytische anemie. Hoe deze variant werd aangetoond door reversed-phase-HPLC wordt in Hoofdstuk 5a gepresenteerd. Zijn karakterisering werd uitgevoerd door HPLC analyse van materiaal dat verkregen werd door hoog zout precipitatie en door directe bepaling van de volgorde van vermeerderd DNA. De detectie van Hb-Volga of $\alpha_2\beta_2 27$ (B9)Ala→Asp in een 5-jaar oude jongen uit Bosnië-Herzegovina, die aan ernstige hemolytische anemie leed, wordt in Hoofdstuk 5b gepresenteerd. Deze variant werd waargenomen door middel van reversed-phase-HPLC en structureel gekarakteriseerd door HPLC analyse. Een eerdere poging om de Hb-Volga mutatie vast te stellen, door Ava II digestie van vermeerderd DNA, is ook beschreven. De patiënten met Hb-Yokohama en Hb-Volga werden geboren uit gezonde ouders zonder Hb abnormaliteiten. In studies bij de voorouders is een evaluatie van de polymorfismen van de HLA DQa locus betrokken, die bevestigde dat een de novo mutatie in beide ouders was opgetreden.

In Hoofdstuk 6 wordt beschreven hoe drie nieuwe γ -keten varianten worden aangetoond en hoe zij gekarakteriseerd zijn met HPLC. Door middel van preparatieve reversed-phase-HPLC was het mogelijk de abnormale γ -keten in zuivere vorm te isoleren, waardoor zijn karakterisering zeer werd vergemakkelijkt. Tijdens een algemene studie van navelstreng bloed monsters van gezonde Chinese pasgeborenen werd een nieuwe A γ -variant aangetoond. De structurele karakterisering van deze abnormale variant, genaamd Hb-F-Jiangsu, wordt in Hoofdstuk 6a gepresenteerd. Met een structurele analyse van de geïsoleerde A γ^X -keten werd een Val→Met substitutie op positie A $\gamma 134$ aangetoond, waardoor een tamelijk unieke Met-Met volgorde werd gecreëerd. De lokatie van het tweede methionine residu in het γ T-15 peptide beïnvloedt de chromatografische eigenschappen van de A γ -keten aanzienlijk. De identificatie van Hb-F-Catalonië of $\alpha_2\gamma_2 15$ (A12)Trp→Arg wordt in Hoofdstuk 6b gepresenteerd. De variant werd waargenomen in

twee Spaanse baby's. Met verschillende HPLC procedures was het mogelijk de Trp→Arg substitutie te identificeren in slechts een geringe hoeveelheid van de G γ X-keten. Hoofdstuk 6c beschrijft de structurele karakterisering van Hb-F-Brooklyn. De variant werd gevonden in een 2-maanden-oud blank meisje tijdens een routine onderzoek van bloed monsters van pasgeboren baby's. De variant kon worden aangetoond met IEF, cation exchange HPLC en reversed-phase-HPLC. Door structurele analyse kon een Lys→Glu substitutie op positie G γ 66 worden aangetoond. Het is nog niet bekend of de substitutie, die wordt waargenomen in drie nieuwe γ -keten anomalieën, enig effect heeft op de functionele of fysicochemische eigenschappen van deze varianten; zulke studies waren niet mogelijk door gebrek aan materiaal.

Hoofdstuk 7 behandelt de identificatie van het onstabiele Hb-Montréal met een deletie van drie aminozuren (Asp, Gly, Leu) op posities 73, 74 en 75 van de β -keten en een insertie van vier verschillende residuen (Ala, Arg, Cys, Glu) op dezelfde lokatie. De variant werd waargenomen in een 7-jaar-oude jongen die leed aan een milde hemolytische anemie. De ouders van de jongen, zijn zuster en zijn broer waren normaal. De introductie van een extra aminozuur residu en de veranderingen in de holte waar de haemzak ligt, zijn de waarschijnlijke oorzaak van de instabiliteit van dit abnormale Hb. De bovengenoemde veranderingen werden gedetecteerd door de tryptische peptiden van de β -Montréal keten te analyseren door de volgorde van vermeerderd DNA direct te meten of door hybridisatie van dit DNA met specifieke oligonucleotide probes. Analyse van de DNA volgorde maakt het mogelijk te voorspellen wat het (de) waarschijnlijke mechanisme(n) is (zijn) voor het optreden van deze ongewone variant. Het wordt gesuggereerd dat foutieve base paring, waarbij de repeterende AGTG volgorde bij CD's 66 en 67 en bij CD's 72 en 73 van het normale β -gen een rol speelt, een duplicaat van het 16 bp segment veroorzaakt, terwijl een deletie van 10 nts dankzij recombinitie of glijden, gevolgd door een korte (3 bp) deletie tijdens DNA reparatie, de abnormaliteit van het Hb-Montréal zou kunnen hebben veroorzaakt.

De aanzienlijke vooruitgang die werd gemaakt bij de studie van Hb-varianten was primair mogelijk door de ontwikkeling van gevoeliger en snellere methoden bij hun detectie en karakterisering. Enkele van de belangrijkste methodologische prestaties in de studie van Hb-varianten worden kort besproken in Hoofdstuk 8. In het verleden zijn verschillende elektroforetische en chromatografische technieken ontwikkeld; deze hebben een belangrijke rol gespeeld bij de detectie en karakterisering van talrijke Hb-varianten. Wegens hun veranderde elektroforetische eigenschappen zijn de meeste gewone Hb-varianten al reeds 20 tot 30 jaar geleden ontdekt. De Hb-varianten, die de laatste jaren zijn ontdekt, zijn zeldzame abnormaliteiten, die vaak een mutatie hebben die moeilijk op te sporen is met elektroforetische procedures, of een tamelijk ingewikkelde structuur hebben. Vergevoerde methodologie zoals HPLC-procedures en DNA-analyses zijn vereist voor hun karakterisering.

Momenteel zijn IEF, cation exchange HPLC en reversed-phase-HPLC de methoden, die als eerste gekozen worden, voor de detectie en de bepaling van het voorkomen van Hb-varianten. Reversed-phase-HPLC voor peptide analyse is een zeer bruikbare techniek voor structurele karakterisering van abnormale Hb's op het eiwit niveau, maar zal worden vervangen door het bepalen van de volgorde van vermeerderd DNA van de α -, β -, δ - of γ -genen. De identificatie van Hb's met complexere structuren en met specifieke fysicochemische eigenschappen zal volledig afhankelijk zijn van de toepassing van DNA-methodologie.

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