Studies on the vitamin K-dependent carboxylase in bovine liver : properties, purification procedure and a possible reaction mechanism

CHAPTER 9

CONCLUSIONS AND SUMMARY

Vitamin K is involved in the carboxylation of a great number of proteins (amongst which four coagulation factors). By this reaction some of the glutamic acid residues in the polypeptide chain are converted into γ-carboxyglutamic acid residues. In this thesis we describe some characteristics of the vitamin K-dependent carboxylation reaction in cow liver.

When cows are fed with vitamin K-antagonists, the vitamin K-dependent reactions are blocked, which results in the appearance of descarboxyfactors in the blood and in an accumulation (50-100 fold) of clotting factor precursors in the liver (chapter 2 and 4). In our in vitro system these precursors serve as an endogenous substrate for carboxylase and they mainly consist of precursors of factor X (60%) and prothrombin (25%). The hepatic factor X precursor is a single chain protein. Since plasma factor X is a two-chain molecule, this observation strongly suggests, that factor X is synthesized as a single polypeptide chain, which is cleaved during the last stage of its maturation. The almost complete identification of the reaction products of carboxylase could only be obtained in the bovine system. In rat carboxylase for instance, only 25% of the endogenous substrate was identified (1).

In chapter 3 we have shown that descarboxyprothrombin and descarboxy-factor X, purified from blood, hardly affect the in vitro carboxylation reaction at plasma concentrations. It could be established that descarboxy-prothrombin precursors are substrate molecules albeit with a high K_m. A proteolytic degradation product of descarboxyprothrombin (the amino acids 13-29) was a far better substrate than the intact molecule. The K_m for carboxylase was lowered at least 1000 fold after degradation with subtilisin. The minimal requirement for a good substrate has not been established and it is unknown which other descarboxyfactors contain the intrinsic information to be a good substrate for the carboxylase prep-aration used. It is not unlikely, however, that at least the clotting factor X and prothrombin are carboxylated by the same enzyme since carboxylase purified with the aid of immunoadsorption to antifactor X, is

still active in the carboxylation of descarboxyprothrombin and its degradation products. The specificity of carboxylase preparations from various organs is a subject of current studies.

The development of low molecular weight substrates for the vitamin K-dependent carboxylase (2,3) has rendered the possibility to study the carboxylation reaction without being dependent on the presence of endogenous substrates. With one of the substrates (Phe-Leu-Glu-Glu-Leu) it could be demonstrated that warfarin treatment of cows does not affect the level and the properties of hepatic vitamin K-dependent carboxylase (chapter 2).

When measured in the microsomal fraction, the carboxylation reaction can be driven by vitamin K quinone, vitamin K hydroquinone as well as by vitamin K epoxide. The highest carboxylation rate is obtained with vitamin K hydroquinone. Vitamin K hydroquinone is believed to be the form which is driving the carboxylation event (3,4) and this implies that vitamin K is first reduced by vitamin K reductase before entering the carboxylating enzyme system. In the presence of vitamin K hydroquinone carboxylase may function independent of the reductase, when the latter enzyme is blocked by warfarin. Vitamin K epoxide is formed in parallel with the carboxylation reaction and can be reduced by vitamin K epoxide reductase. In our system the reduction of vitamin K epoxide (and vitamin K) could be uncoupled from the carboxylation by using an enzyme preparation without any substrate for the carboxylase. So both carboxylase and reductase may function independently.

Nevertheless it seems probable that carboxylase and reductase are associated within the microsomal membrane (chapter 2). Furthermore we concluded that an intimate link exists between the endogenous substrate and the enzyme, because the complex is retained on a column on which antibodies against the endogenous substrate are present (chapter 4). Also, we showed (chapter 6) that phospholipid (primarily phosphatidylcholine) is an essential constituent of the vitamin K-depedent carboxylase. Therefore we postulate the existance of an enzyme complex of carboxylase, reductase, endogenous substrate (if present) and phospholipid. The phospholipid moiety might function in the transport and/or storage of various forms of vitamin K e.g. vitamin K hydroquinone produced by the reductase.

The carboxylase is a constituent of the rough endoplasmic reticulum

(5,6), which can be extracted therefrom with a number of detergents. After solubilization a considerable purification could be achieved with immunoadsorption onto antibodies against the endogenous substrate (chapter 4). No other satisfactory purification methods have been published until now. In a number of cases the difficulties with the purification might be explained by a loss of the enzyme activity caused by the separation of phospholipid and proteins in the presence of detergents. At least in the bovine system phospholipids are essential for carboxylase activity (chapter 6).

As has been pointed out in chapter 5 the semi-purified carboxylase, after elution from the solid phase, is a high molecular weight moiety consisting of phospholipid and various proteins. For further purification carboxylase has to be re-solubilized and until now this has not been achieved without loosing the main part of the enzyme activity. We have no indications that cofactors other than vitamin K (e.g. heme) are involved in the carboxylation reaction (chapter 7).

Sp-carboxylase is a useful enzyme preparation for studying the vitamin K-dependent carboxylation reaction. Most properties are similar to those of other known carboxylase preparations and the purification procedure is simple. The attachement to the Sepahrose beads is advantageous when a quick washing procedure is needed e.g. after the phospholipase treatment (chapter 6). Sp-carboxylase differs from other preparations in several ways: it is more pure, it does contain a low amount of vitamin K-reductase, the carboxylation rate of Phe-Leu-Glu-Glu-Leu is linear for a longer period, the carboxylation reaction has a higher optimal temperature and the reaction is inhibited by low detergent concentrations.

It is generally believed that the vitamin K-dependent carboxylation reaction is coupled to the epoxidation of vitamin K hydroquinone (see chapter 1). Radicals and a hydroperoxide intermediate might be involved in this epoxide formation. The idea that hydroperoxide vitamin K is involved was substantiated by the peroxidase inhibition and the carboxylation with t-butylhydroperoxide present instead of vitamin K hydroquinone (7). The t-butylhydroperoxide driven carboxylation was small however and might be effected by an impurity in the carboxylase preparation (8). With the semi purified Sp-carboxylase we could clearly demonstrate that the properties of the t-butylhydroperoxide driven carboxylation were similar to the properties of the vitamin K-dependent carboxylation (chapter 5).

The epoxidation is not absolutely coupled to the carboxylation (4). In most systems more vitamin K epoxide is formed than CO_2 incorporated (on a molar basis). With CN^- the carboxylation could even be completely blocked without blocking the epoxidation (chapter 7, ref. 9).

In chapter 8 we showed that carboxylation can occur without the concurrent formation of vitamin K epoxide. In the presence of sulphite a hydroxyvitamin K was formed instead of the epoxide. We concluded that the hydroxyvitamin K (and vitamin K epoxide when no sulphite is present) was formed from a hydroperoxide intermediate after a heterolytic fission of the peroxide bond. A the same time a carbanion is formed which can be carboxylated easily. Obviously the proposed mechanism has to be verified in a purified system.

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SAMENVATTING

Vitamine K is nodig bij de carboxylering van een groot aantal eiwitten, waaronder verschillende stollingsfaktoren. Bij deze carboxyleringsreactie worden enkele glutaminezuur (glu) resten omgezet in γ-carboxyglutaminezuur (gla) resten. Na het toedienen van vitamine K antagonisten (hydroxycoumarinederivaten) zakt het plasma niveau van de vitamine K-afhankelijke stollingsfactoren en neemt de kans op ongewenste stolselvorming af. De orale antistollingstherapie met coumarinederivaten neemt dan ook een belangrijke plaats in bij de bestrijding en preventie van thrombose. In dit proefschrift wordt het onderzoek naar de vitamine K-afhankelijke carboxylering in runderlever beschreven.

Na het toedienen van vitamine K-antagonisten worden de vitamine K-afhankelijke reacties geremd. Descarboxyfactoren (stollingsfactoren met glu resten in plaats van gla resten) verschijnen dan in het bloed en precursors van de stollingsfactoren hopen zich op in de lever (hoofdstuk 2 en 4). In het door ons ontwikkelde in vitro systeem kunnen deze precursors vitamine K-afhankelijk gecarboxyleerd worden. In hoofdstuk 4 is aangetoond dat het grootste geddelte van de carboxyleerbare eiwitten uit precursors van factor X (ca. 60%) en prothrombine (ca. 25%) bestaat. De factor X precursor uit de lever bleek uit één eiwitketen te bestaan, terwijl het factor X uit plasma uit twee eiwitketens bestaat.

In hoofdstuk 3 hebben we aangetoond dat descarboxyprothrombine, geisoleerd uit runderbloed, substraat is voor de vitamine K-afhankelijke carboxylase uit runder- en rattelever. De affiniteit van descarboxyprothrombine voor het carboxylase is echter bijzonder laag (K_m 0.3 - 0.4 mM). Door proteolytische afbraak van descarboxyprothrombine met subtilisine Carlsberg werd een fragment verkregen met een veel hogere affiniteit voor het carboxylase (K_m = 0.001 - 0.003 mM). Het fragment bestond uit de aminozuren 13-29 van descarboxyprothrombine.

In hoofdstuk 2 wordt de vitamine K-afhankelijke carboxylase uit de lever van normale en geantistolde koeien vergeleken. Behalve in de hoeveelheid precursors, die substraat zijn voor carboxylase, werd geen verschil gevonden tussen de beide preparaten. Tevens wordt in dit hoofdstuk aangetoond dat de vitamine K-afhankelijke carboxylering en de reductie van vitamine K onafhankelijk van elkaar kunnen verlopen; de enzymen die deze

reakties katalyseren (carboxylase en reductase) zijn echter wel nauw met elkaar verbonden.

In hoofdstuk 4 wordt de zuivering van carboxylase uit gesolubilizeerde levermicrosomen beschreven. Wij maakten gebruik van het feit dat de factor X precursors, die substraat zijn voor het carboxylase, gecomplexeerd zijn met het carboxylase. Met behulp van immunospecifieke adsorptie aan antilichamen tegen de factor X precursors werd (uitgaande van de microsomale fractie) een 100-voudige zuivering verkregen. Dit gedeeltelijk gezuiverde preparaat is gebonden aan Sepharose deeltjes en wordt daarom Sp-carboxylase (Solid phase carboxylase) genoemd.

Enkele eigenschappen van Sp-carboxylase worden beschreven in hoofdstuk 5 en 6. De belangrijkste eigenschappen zijn vergelijkbaar met die van een ongezuiverd preparaat. Het Sp-carboxylase bevat nog verschillende eiwitcomponenten, waarvan twee vitamine K binden. Voorts bestaat het preparaat voor ca. 30% uit phospholipiden (voornamelijk phosphatidylcholine). Het preparaat kon met behulp van phospholipases of detergentia phospholipiden-vrij gemaakt worden. Reconstitutie tot ruim 80% van de oorspronkelijke activiteit was mogelijk door het toevoegen van micellen, die phosphatidylcholine en cholaat bevatten.

In hoofdstuk 7 wordt de remming van de vitamine K-afhankelijke carboxylering door cyanide beschreven. Het bleek een niet-lineaire remming te zijn, competitief met CO_2. De epoxidering van vitamine K hydroquinone werd niet geremd door cyanide. De remming door cyanide bleek geen argument voor de deelname van een haem groep aan de vitamine K-afhankelijke carboxyleringsreaktie. Haem kon bovendien niet aangetoond worden in Sp-carboxylase.

In hoofdstuk 8 tenslotte wordt beschreven dat de vitamine K afhankelijke carboxyleringsreaktie gekoppeld is aan de vorming van vitamine K-epoxide. In aanwezigheid van sulfiet kan de carboxyleringsreaktie echter gekoppeld worden aan de vorming van hydroxy vitamine K. De carboxyleringsreaktie werd tevens gestimuleerd in aanwezigheid van sulfiet, zowel de vitamine K-afhankelijke als ook de t-butylhydroperoxide afhankelijke carboxylering. In aanwezigheid van peroxidases werd de carboxyleringsreaktie en de vorming van vitamine K epoxide geremd, terwijl hydroxyvitamine K wel gevormd werd (ook in afwezigheid van sulfiet). Deze bevindingen leidden tot de hypothese dat de carboxylering van glutaminezuur resten gekoppeld is aan een heterolytische splitsing van een peroxide binding met de gelijktijdige vorming van vitamine K epoxide.