Kinetic aspects of the interaction of blood clotting enzymes

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Kinetic Aspects of the Interaction of Blood-Clotting Enzymes

VI. Localization of the Site of Blood-Coagulation Inhibition by the Protein Induced by Vitamin K Absence (PIVKA)

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In previous papers we have shown that the absence of functional vitamin K results in the appearance in the circulation of an anticoagulant protein. This protein we called PIVKA (Protein Induced by Vitamin K Absence or Antagonists) (8). This result had been found with one reagent only, namely the Thrombotest reagent (13). It thus seemed worth while to investigate if the inhibitor was specific for this reagent or if it had to be considered a generally acting inhibitor of the blood coagulation process. If so the site of action of this inhibitor had to be found out.

We concluded from experiments reported in another article (4) that it is possible to apply techniques derived from the disciplines of enzyme kinetics to the results of clotting tests. The next step, obviously, is to use the kinetic approach to locate the site at which PIVKA inhibits the coagulation reaction sequence.

Under the conditions prevailing in the test in which the inhibitor was first recognized (i.e. the Thrombotest method), the concentration of factor X has been shown to be rate-limiting for the velocity of the reaction (9). This suggests that inhibition takes place at the site in the coagulation reaction sequence at which factor X is rate-limiting. Since the inhibition appears to be of the competitive type, this finding further suggests that PIVKA might be a substrate analogue of factor X. The evidence, however, cannot be accepted as being more than suggestive. Furthermore, there may be other sites of inhibition that are not recognized by the Thrombotest method. It is therefore evident that the effect of PIVKA must be localized more precisely.

It should be stressed once again that care must be taken not to confuse the rectilinear plots obtained by the graphical representation of clotting time as a function of the inverse of the concentration of the rate-limiting clotting factor (i.e. $t_0 - 1/C$ plots) with Lineweaver-Burk plots such as can be obtained in the simpler and therefore betterdefined systems used in most enzyme kinetic work.

Uncontrollable circumstances such as the concentration of clotting factors in the plasma used as a reagent, make it impossible to draw conclusions about the actual magnitude of the reaction constants of the system under study, or to draw any conclusion whatsoever from comparison of experiments carried out with different reagent plasmas. Nevertheless, the construction of $t_0 - 1/C$ plots is useful because these plots show, at the intercept of the line obtained from the experiment and the X-axis, the so-called minimal clotting time ($t_{min}$). This minimal clotting time is the clotting time that would be obtained, under the circumstances of the test, in the presence of an infinite amount of the clotting factor, the inverse concentration of...
which is rendered on the X-axis. By application of this procedure, one variable of the clotting reaction, i.e. the concentration of one among the factors II, V, VII, or X, can be eliminated. This enables us to draw conclusions as to the type and site of inhibition, since the recognition of inhibition and the determination of the type of inhibition does not require estimation of reaction constants.

**Materials and Methods**

Except for minor changes in the composition of the reaction mixtures as indicated in the next section, materials and methods are the same as those indicated in preceding articles (4, 8, 9). It may be reminded that the reagent plasmas in the specific tests where congenitally deficient human plasmas except for the factor V reagent, which was Ba-Stearate adsorbed normal human plasma (10) and the second factor X reagent which was plasma from a patient with amyloidosis which showed an acquired specific factor X deficiency. The thromboplastin used was always human brain thromboplastin prepared according to Owren and Aas (14).

**Experimental**

First of all it was established that PIVKA does inhibit in a system different from Thrombotest. The results are shown in Fig. 1. It is seen that in a reagent consisting

![Fig. 1. The effect of PIVKA in a “prothrombin-time” estimation using reagents of human origin. The reaction mixture consisted of 0.1 ml Al(OH)₃ adsorbed human plasma, 0.1 ml human brain thromboplastin; 0.1 ml sample (if diluted, diluted with Michaelis buffer); 0.1 ml CaCl₂ 20 mM. The figures give the means of 50 determinations. White squares: normal plasma; the regression line through these points intercepts the Y-axis at t₁min = 12.3 sec ± 0.2 sec (sd). Black squares: mixed plasma from 6 patients on long term anticoagulant treatment; the regression line through these points intercepts the Y-axis at t₂min = 23.4 sec ± 0.4 sec (sd). The significant difference between t₁min of the normal plasma and t₂min of the abnormal plasma indicates the action of PIVKA (see ref. 8).](image-url)
of human brain thromboplastin (prep. according to Owren & Aas) and adsorbed
human plasma the inhibitive action of PIVKA can be demonstrated, but to a lesser
extent than with the Thrombotest method, and even to an extend which can be
barely observable when the experiments are not carried out in multiple. In Fig. 1
however the prolongation of $t_{min}$ obtained with Dicumarol plasma and vitamin K
deficient plasma is significant and so the fact that PIVKA can inhibit in a system
consisting entirely of material of human origin seems proven.

A first indication of the site of action of PIVKA is found in Table 1. In this ex-
periment, two plasmas with extremely low concentrations of factor II, VII, IX, and
X, and with a considerable amount of PIVKA present, were tested. The slope of the
t - D line obtained with Thrombotest and the amount of inhibitor could not be
assessed directly in these plasmas because of the extremely long coagulation times.
Consequently, the amount of inhibitor and the slope were estimated in a mixture of
equal parts of normal plasma and the unknown plasma. Because the slope and the
amount of inhibitor have been shown to be additive quantities in mixtures (8), the
values of the original plasmas could be calculated from the values obtained with the
mixtures.

Table 1. Coagulation Times in Specifically Deficient Systems in the Presence of Plasma from
Severely Vit. K Deficient Patients.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Buffer</th>
<th>Pat. R</th>
<th>1/1</th>
<th>1/10</th>
<th>Pat. B</th>
<th>1/1</th>
<th>1/10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor II cong.</td>
<td>46</td>
<td>42</td>
<td>45</td>
<td>37</td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Factor II art.</td>
<td>89</td>
<td>46</td>
<td>65</td>
<td>38</td>
<td>56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Factor V art.</td>
<td>195</td>
<td>15</td>
<td>19</td>
<td>16</td>
<td>19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Factor VII/X art.</td>
<td>149</td>
<td>290</td>
<td>110</td>
<td>158</td>
<td>123</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Factor VII cong.</td>
<td>104</td>
<td>96</td>
<td>104</td>
<td>73</td>
<td>87</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Factor X acquired def.</td>
<td>46</td>
<td>95</td>
<td>47</td>
<td>54</td>
<td>51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Factor X cong.</td>
<td>50</td>
<td>76</td>
<td>55</td>
<td>71</td>
<td>55</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Patient R: Attempted suicide by prolonged intake of Sintron
Slope t-D curve: 1%, Inhibitor: 1.8 U.
Patient B: Bile obstruction by carcinoma of the stomach
Slope t-D curve: 2%, Inhibitor: 2.0 U.

The figures give the means of 10 clotting time estimations expressed in sec.

It can be seen from Table 1 that as soon as factor X plays a rate limiting role in
the determination, a singular phenomenon occurs: the clotting time obtained with
the sample present is longer than the clotting time obtained with buffer substituted
for the sample. This finding suggests that PIVKA inhibits at the level of factor X.
These results again indicate that the inhibition is not limited to the Thrombotest
system, all reagents used in this experiment being of human origin.

It has been shown previously that a rectilinear plot is obtained when the clotting
time in a specific one-stage test is plotted against the inverse of the concentration of
the rate-limiting clotting factor (4). This mode of plotting provides a means of locat-
ing the site of inhibition more precisely.

The clotting time in the presence of a competitive inhibitor can be represented by
the following formula (6)

$$t_0 \cdot h = \frac{1}{E} + \frac{D}{X} \left(1 + \frac{Km}{E}\right) + \frac{I}{E \cdot X} \cdot \frac{Km + E}{Ki + E}$$
in which $t_c$ = clotting time; $h$ = a constant; $E$ = enzyme concentration; $D$ = dilution factor; $X$ = substrate factor concentration in the undiluted plasma; $I$ = concentration of inhibitor in the undiluted plasma; $K_m$ and $K_i$ = reaction constants.

According to this formula, the presence of an inhibitor results in a relatively less important prolongation of the clotting time the greater the dilution of the sample containing the inhibitor. This holds because an increase of the dilution factor ($D$) causes an increase of the second term of the formula, but leaves the third term unchanged since $D$ does not figure in that term whereas it is the only term in which the concentration of the inhibitor figures. We can use this property to locate inhibition in the following way:

From a normal standard plasma various dilutions are made, and a $t_c - 1/C$ plot is constructed as previously indicated. This plot gives the relation between clotting time and clotting-factor concentration, and can therefore be used to read the clotting-factor concentration belonging to a clotting time found with an unknown sample. When the same sample is tested in various dilutions, multiplication of the concentration read on the standard curve by the dilution factor gives the concentration of the original sample. When no inhibitor is present, the concentration thus calculated to be present in the original sample will be the same, independent of the dilution from which the calculation has been made.

We have shown above that when an inhibitor is present, the inhibition becomes less important the more the plasma sample containing the inhibitor is diluted. Therefore, with a sample containing a competitive inhibitor more realistic, i.e. higher values, will be found when the estimation is done by testing the sample in a high dilution than when the sample is tested in relatively concentrated form.

The best approximation will be found with the sample in its highest dilution. Of course, the practical aspects of the estimation of clotting times set a limit to the dilution that can be used.

The procedure is illustrated in Tables 2 and 3, from which it is evident that Dicumarol plasma shows essentially the same amount of factor II no matter from what dilution of the sample this amount has been calculated. Factor X, however, shows a level that appears to be low when calculated from a low dilution and when calculated from a high dilution appears to be high. This indicates that an inhibitor of the rate-limiting reaction in a factor X estimation is present in the unknown sample.

**Table 2. Estimation of the Factor II Content in Marcoumar Plasma.**

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Clotting time (sec)</th>
<th>Concentration in sample (%)</th>
<th>Concentration in plasma (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:4</td>
<td>32.9</td>
<td>6.85</td>
<td>27.4</td>
</tr>
<tr>
<td>1:6</td>
<td>39.3</td>
<td>4.95</td>
<td>29.1</td>
</tr>
<tr>
<td>1:8</td>
<td>41.8</td>
<td>4.00</td>
<td>32.0</td>
</tr>
<tr>
<td>1:10</td>
<td>47.7</td>
<td>2.98</td>
<td>29.8</td>
</tr>
<tr>
<td>1:12</td>
<td>53.5</td>
<td>2.42</td>
<td>29.0</td>
</tr>
<tr>
<td>1:14</td>
<td>57.5</td>
<td>2.12</td>
<td>29.7</td>
</tr>
<tr>
<td>1:16</td>
<td>61.5</td>
<td>1.80</td>
<td>28.8</td>
</tr>
</tbody>
</table>

The clotting times have been obtained in a specific one stage estimation of factor II using congenitally factor II deficient plasma as a substrate. The third column gives the concentration present in the (diluted) sample according to a reference curve obtained with normal plasma. The fourth column gives the concentration in the undiluted Marcoumar plasma as calculated from columns 1 and 3.
Table 3. Estimation of the Factor X Content in Marcumar Plasma.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Clotting time (sec)</th>
<th>Concentration in sample (%)</th>
<th>Concentration in plasma (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:4</td>
<td>80.7</td>
<td>1.87</td>
<td>7.5</td>
</tr>
<tr>
<td>1:6</td>
<td>69.7</td>
<td>2.50</td>
<td>15.0</td>
</tr>
<tr>
<td>1:8</td>
<td>72.1</td>
<td>2.33</td>
<td>18.6</td>
</tr>
<tr>
<td>1:10</td>
<td>73.6</td>
<td>2.22</td>
<td>22.2</td>
</tr>
<tr>
<td>1:12</td>
<td>71.3</td>
<td>2.38</td>
<td>28.5</td>
</tr>
<tr>
<td>1:14</td>
<td>74.4</td>
<td>2.15</td>
<td>30.1</td>
</tr>
<tr>
<td>1:16</td>
<td>79.6</td>
<td>1.92</td>
<td>30.6</td>
</tr>
</tbody>
</table>

The clotting times have been obtained in a specific one stage estimation of factor X using congenitally factor X deficient plasma as a substrate. The third column gives the concentration present in the (diluted) sample according to a reference curve obtained with normal plasma. The fourth column gives the concentration in the undiluted Marcumar plasma as calculated from columns 1 and 3.

As already indicated, the level found with the sample in its highest dilution is the best approximation of the real amount present. By using this approximation, we can design experiments showing inhibition in clotting tests in a set-up analogous to the classical Lineweaver-Burk plot. In these experiments a fixed amount of Dicumarol plasma is mixed with an equal amount of various dilutions of normal plasma. Consequently, in these mixtures a fixed amount of PIVKA is present along with variable amounts of the clotting factors. When these mixtures are subjected to a specific test, the amount of the rate-limiting clotting factor present in that test is known because we know the (variable) amount present in the diluted normal plasma as well as the amount present in the reagent (4), and we have a reasonable approximation of the

---

Fig. 2. The effect of PIVKA in a factor II estimation. The clotting time obtained in an one stage factor II estimation is plotted against the inverse of the factor II concentration present; in absence (open dots) or in presence (black dots) of a fixed amount of PIVKA. No difference is observed.

Fig. 3. The effect of PIVKA in a factor X estimation. The clotting time obtained in an one stage factor X estimation is plotted against the inverse of the factor X concentration. Congenitally factor X deficient plasma was used as a reagent in the factor X estimation. A pronounced inhibition is evident in presence of PIVKA (upper curve). At infinite factor X concentration, i.e. at the intercept with the Y-axis PIVKA has no effect. This indicates a competitive type of inhibition.
amount present in the Dicumarol plasma. Comparing the results obtained in the presence and in the absence of PIVKA, we see again that the inhibitory action is obvious in factor X estimations but is not manifested in a factor II estimation (Figs. 2, 3 and 4).

Fig. 4. The effect of PIVKA in a factor X estimation. As in Fig. 3 except that plasma with an acquired factor X deficiency due to amyloidosis has been used as a reagent.

Fig. 5. The effect of PIVKA in a factor V estimation. The clotting time obtained in a one stage factor V estimation is plotted against the inverse of the factor V concentration in absence (lower curve) and in presence (upper curve) of a fixed amount of PIVKA. A slight inhibition is observed.

Fig. 6. The effect of PIVKA in a factor VII estimation. The clotting time obtained in a one stage factor VII estimation is plotted against the inverse of the factor VII concentration in absence (lower curve) and in presence (upper curve) of a fixed amount of PIVKA. A slight inhibition is observed.

Fig. 7. The effect of PIVKA in the intrinsic coagulation system. Reaction mixture: 0.2 ml plasma diluted with BaSO₄ adsorbed plasma; 0.1 ml suspension of Kaolin (5 mg/ml) and phospholipid; 0.1 ml CaCl₂; 25 mM. The plasma used was either normal plasma (lower curve) or Marcoumar plasma (upper curve). In this type of graph, where the inhibitor is diluted along with the substrate, the fact that the Marcoumar plasma gives a straight line that does not extrapolate to the same point at the Y-axis as the line obtained with normal plasma does again indicate the presence of a competitive inhibitor (ref. 6, 8).
The results obtained with factor V and factor VII estimations are shown in Figs. 5 and 6 respectively. Both show a certain amount of inhibition, although much less than the inhibition in a factor X estimation. Fig. 7 shows that the inhibitor is also active in a system in which the intrinsic rather than the extrinsic clotting system is operative.

Discussion

In the first place it must be remarked that the inhibition by PIVKA is not a unique feature of the Thrombotest reagent. It is also observed when using reagents of human origin both in the extrinsic and the intrinsic system (Figs. 1 and 7).

The discussion of the experiments designed to locate the site of the action of PIVKA is necessarily based on our present concept of the uninhibited reactions involved in thrombin formation. On the basis of recent studies (1, 3, 4, 5, 7), a fairly detailed reaction scheme can be written:

\[
\begin{align*}
Xll & \rightarrow XIIa, \\
XIIa + XI & \rightarrow A. C. P. \\
IX & \rightarrow IXa \\
IXa + VIII + Ph. lip. + Ca^{++} & \rightarrow X-ase \\
\text{A. C. P. or T. F.} & \rightarrow VIIa \\
X & \rightarrow Xa \\
Xa + V + Ph. lip. + Ca^{++} & \rightarrow \text{prothrombinase} \\
\text{prothrombinase} & \rightarrow \text{Thrombin}
\end{align*}
\]

(T. F. = tissue factor; Ph. lip. = phospholipid; A. C. P. = activated contact product)

Factor VIII probably must be acted upon by thrombin before it can react in reaction D (15). Factor V possibly must be activated by thrombin or in some other way before it can take part in reaction F (12). The reasons why this scheme has been preferred to the original cascade scheme (2, 11) have been discussed elsewhere (4, 5, 7). The location of the site at which PIVKA is inhibitory will be discussed in terms of this reaction scheme.

Fig. 2 shows that when factor II is rate-limiting, PIVKA does not influence the reaction rate. This excludes reaction II as a possible site of action of PIVKA.

A strong competitive inhibition is observed when factor X is present in rate-limiting amounts (Fig. 3). This indicates that PIVKA inhibits a reaction in which factor X is involved (i.e., either reaction F or reaction G). The fact that the inhibition is of a purely competitive type suggests that PIVKA is a substrate analogue of factor X. To exclude the possibility that the inhibition was due to a specific property of the congenitally deficient plasma used as a reagent, another reagent was also tested, which gave essentially the same results (Fig. 4).

From Figs. 5 and 6 it can be seen that a certain amount of inhibition can be observed in both the factor V and factor VII test. A possible explanation of this phenomenon might be found in the assumption of the existence of factor V and factor VII analogues in Dicumarol plasma.
In view of the fact that factor V is known to be unaffected by the presence or absence of vitamin K, this possibility must be regarded as unlikely in the case of factor V, especially since an alternative explanation is available that does not require the postulation of more than one abnormal protein.

The inhibition of factor VII conversion is apparently not very extensive and is not of a clearly competitive type. If a factor VII analogue were present as well as a factor X analogue, one would expect an easily recognizable competitive inhibition. Although the possibility that a slightly inhibitory factor VII analogue is present cannot be ruled out, the experiments do not seem to justify postulation of its existence, since the observed inhibition can also be explained on the basis of the existence of a factor X analogue only.

If we assume that PIVKA present in the plasma in vitamin K deficiency is an analogue of factor X and that it can be converted into an analogue of activated factor X (called PIVKA* henceforward) by the enzyme for which factor X is the normal substrate, i.e., by either activated factor VII or tenase, we have explained the fact that PIVKA is not observed in the serum obtained from Dicumarol plasma (8). At the same time, we also have an explanation for the inhibition observed in a factor VII test. Since factor VII is tested in the presence of an excess of its normal substrate (factor X), we would not expect the amount of PIVKA introduced with the sample to give rise to a marked inhibition. If the product of the action of factor VII on PIVKA is adsorbed onto phospholipid in the same way as its analogue factor Xa, this would furnish an explanation for the inhibition observed in factor V tests. The “activated” PIVKA would react with phospholipid and factor V to form an inactive prothrombinase according to the reaction formula:

\[
\text{PIVKA}^* + \text{Ca}^{++} + \text{V} + \text{Ph. lip} \rightarrow \text{inactive prothrombinase} \quad (I)
\]

In this complex a certain amount of the rate-limiting factor V would be taken up, and therefore a certain amount of inhibition would be apparent. Again, this inhibition cannot be expected to be very extensive, since an excess of normal factor X is present under these circumstances.

Fig. 7 shows that inhibition can be demonstrated to occur in the intrinsic coagulation system, i.e., in a system in which factor VII does not play a rate-determining role but factor X does.

Consequently, all the observed phenomena can be explained by one hypothesis: PIVKA, a substrate analogue of factor X, can be converted into an analogue of factor Xa by either factor VII or tenase. In its converted form it can take the place of normal factor Xa in prothrombinase, which makes this prothrombinase inactive.

The fact that inhibition is observed in the intrinsic system leaves open the possibility that an inhibitor of factor IX conversion is also present in the blood of vitamin K-deficient or Marcoumar treated patients. Because the Thrombotest reaction is essentially insensitive to any variation of the concentrations of factor IX, such an inhibitor could not be the same as the one recognized as PIVKA. Whether or not an inhibiting substrate analogue of factor IX exists will have to be determined by further investigations.

The fact that the inhibitory action of PIVKA is more pronounced with one phospholipid (that in Thrombotest) than with another human brain Thromboplastin in might be explained by one of the following hypotheses:

a) In the reaction mixture prepared from human material factor X is not as strictly rate-limiting as it is in the Thrombotest system; consequently introduction of a specific factor-X-inhibitor (i.e., PIVKA) would have less influence on the reaction rate.
b) The competition for factor $X_a$ binding sites between factor $X_a$ and PIVKA given by the following reactions:

\[
\begin{align*}
K & \quad V + X_a + Ca^{++} + \text{Ph. lip} \rightleftharpoons \text{prothrombinase} \\
R & \quad V + \text{PIVKA} + Ca^{++} + \text{Ph. lip} \rightleftharpoons \text{inactive prothrombinase}
\end{align*}
\]

at given concentrations of the reactants is determined by the ratio of the values $K$ and $R$. This ratio thus determines the efficaciousness of the inhibition. As phospholipid is a reactant in both reactions it can be considered likely that this ratio is dependent upon the kind of phospholipid used.

In conclusion, it may be said that the experiments reported here provide an explanation of the inhibition first found in the Thrombotest reaction, in terms of the postulation of the existence of an analogue of factor X in vitamin K deficiency. The postulation of analogues of the other vitamin K-dependent clotting factors proved to be unnecessary, although the existence of such analogues could not be ruled out. The degree of inhibition is influenced by the kind of phospholipid present in the test. The inhibition is not however specific for one special kind of thromboplastin.

**Summary**

PIVKA, the circulating anticoagulant protein found in vitamin K deficiency can, on kinetical grounds, be recognized as an analogue of factor X. The existence of analogues of other vitamin K-dependent clotting factors cannot be ruled out, but need not be assumed to explain the experimental results.

**Résumé**

PIVKA, la protéine anticoagulante circulante dans les cas de déficience en vitamine K peut être reconnue sur des bases cinétiques comme un analogue du facteur X. L’existence d’analogues des autres facteurs de la coagulation dépendant de la vitamine K ne peut être exclue, mais n’est pas utile pour expliquer les résultats expérimentaux.

**Zusammenfassung**

Das beim Vitamin-K-Mangel gefundene zirkulierende antikoagulierende Eiweiß, PIVKA, wurde auf Grund kinetischer Untersuchungen als ein Protein identifiziert, das dem Faktor X analog ist. Die Existenz von analogen Eiweißen anderer Vitamin-K-abhängiger Faktoren kann nicht ausgeschlossen werden. Sie ist jedoch nicht notwendig, um die experimentellen Resultate zu erklären.

**Acknowledgements**

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**References**

Experiments in which plasma congenitally deficient factor V was used, confirmed the results obtained with the artificially deficient factor V reagent described in this article.

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