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Conformational Change of Human Prothrombin Induced by Calcium Ions: An X-ray Scattering Study†

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ABSTRACT: The scattered X-ray intensities from dilute solutions of prothrombin and Ca^{2+}–prothrombin at 21 °C in 0.1 M Tris-HCl buffer of pH 7.4 indicate that the prothrombin molecule attains a more extended conformation when Ca^{2+} ions are bound. This is indicated from the distance distribution function: the largest distance within the prothrombin molecule increases from 130 to 150 Å when Ca^{2+} ions are bound; the radius of gyration increases from 35.5 to 39.5 Å. A comparison of the experimental scattering curves with theoretical scattering curves calculated for various triaxial bodies shows that the shape of the prothrombin molecule can be represented by two ellipsoids and that the effect of Ca^{2+} binding can be represented by a change in the angle between their major axes.

The last zymogen activation step in the blood coagulation "cascade" is the conversion of prothrombin into thrombin via the cleavage of two peptide bonds in prothrombin by factor X_a (Davie & Fujikawa, 1975; Mann, 1976; Suttie & Jackson, 1977). Thrombin then catalyzes the limited proteolysis of soluble fibrinogen which yields insoluble fibrin. Prothrombin as well as other vitamin K dependent plasma proteins binds Ca^{2+} ions; in the presence of Ca^{2+} ions each of these proteins forms complexes with phospholipid surfaces (Suttie & Jackson, 1977; Nelsestuen et al., 1978; Nelsestuen, 1978). Prothrombin seems to bind Ca^{2+} ions via its γ-carboxyglutamic acid residues; they are formed by posttranslational vitamin K dependent carboxylation of the ten glutamic acid residues situated in the amino-terminal end of the molecule (Stenflo & Suttie, 1977; Olson & Suttie, 1977). When prothrombin is cleaved by thrombin, an amino-terminal fragment of 22 000 is formed which is denoted fragment 1 (Mann, 1976; Suttie & Jackson, 1977). This fragment, which contains the Ca^{2+} binding residues, has recently been crystallized (Aschaffenburg et al., 1977). There are several pieces of indirect evidence which indicate that both fragment 1 and prothrombin undergo a conformational change when Ca^{2+} ions are bound (Suttie & Jackson, 1977; Bloom & Mann, 1978; Björck & Stenflo, 1973).

The aim of this work has been to study the interaction of Ca^{2+} ions with prothrombin by analyzing the size and shape of both prothrombin and Ca^{2+}–prothrombin by using the low-angle X-ray scattering method. The results indicate that the prothrombin molecule can be described by one large and one small ellipsoid. When Ca^{2+} ions are bound to prothrombin, the change in the X-ray scattering curve can be explained if it is assumed that the smaller ellipsoid moves so that the molecule attains a more extended conformation.

Materials and Methods

Prothrombin was prepared and checked for dimerization as described in a previous communication (Stenflo, 1976). The concentration of prothrombin was calculated from the absorbance at 280 nm by using the extinction coefficient $E_{1cm}^{1\%} = 14.5$ (Stenflo, 1972).

The X-ray small-angle scattering data were recorded with a camera developed by Kratky & Skala (1958). The entrance slit width of the camera was 0.10 mm and the counter slit width 0.25 mm. The scattering angle was set by an on-line Hewlett-Packard computer, 2100S, which also received and recorded the intensity data (B. G. Wingren, B. Sjöberg, and R. Österberg, unpublished experiments). Monochromatization was achieved with a nickel β filter and a pulse height discriminator in conjunction with a proportional counter. As indicated by both gel electrophoresis and low-angle X-ray scattering of some prothrombin samples, prothrombin behaved as a native protein after the low-angle X-ray scattering curve was recorded. Also, recording of a second X-ray scattering curve with the same sample was in good agreement with the first one, indicating that no major changes occurred during X-ray exposure.

All measurements were made at 21 °C. The sample container was a thin-walled Mark capillary with a diameter of 0.85 mm, and the distance between the sample and the plane of registration was 20.5 cm. The absolute scattered intensities

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were obtained by using a standard Lupolen sample (Pilz et al., 1970a); the Lupolen sample had been previously calibrated at the Graz Institut für Physikalische Chemie.

Small-Angle X-ray Scattering Measurements. Prothrombin solutions for X-ray scattering were prepared by dialyzing a prothrombin solution of 0.1 M Tris-HCl buffer (pH 7.4) against 0.1 M Tris-HCl of pH 7.4 containing 0.1 mM EDTA. Ca^{2+}–prothrombin solutions were prepared by dialyzing a similar sample of prothrombin against 0.1 M Tris-HCl of pH 7.42 containing 2 mM $CaCl_2$. After dialysis a series of diluted samples were prepared, and these samples were then stored frozen (−80 °C) until studied. Before each sample was subjected to the X-ray beam, it was allowed to equilibrate at 21 °C for 30 min. X-ray scattering data were recorded for prothrombin solutions containing 4.7, 8.0, 13.6, 23.4, and 57.6 mg/mL and for the Ca^{2+}–prothrombin solutions containing 4.1, 8.2, 12.3, 20.6, and 41.7 mg/mL. X-ray scattering from a Tris buffer of the same kind as that used for the samples and recorded under identical conditions was subtracted from the scattering curves in order to correct for the background scattering.

Results

When the normalized intensity ($\tilde{I}/c$) was plotted against the scattering angle, interparticle scattering was indicated. It was found that the interparticle-scattering effect was considerably more pronounced for prothrombin than for Ca^{2+}–prothrombin, but in both cases the effect is rather small and in accordance with what could be expected from theory (Fournet, 1951). It was corrected for by extrapolating the $\tilde{I}/c$ data to zero concentration. This extrapolation most probably also eliminated any possible contribution of dimers to the intensity (Prendergart & Mann, 1977), since the molecular weights determined agreed with that calculated from the primary structure (see below). The extrapolated data were used for calculating the distance distribution function $p(r)$

$$p(r) = \frac{r}{2\pi^2}\int_{h=0}^{\infty} I(h)h \sin(hr)\, dh \qquad (1)$$

Here, $h = (4\pi/\lambda)\sin\theta$, where θ is half the scattering angle and λ is the wavelength of the monochromatic radiation (Guinier & Fournet, 1955). The $p(r)$ function was calculated as an indirect Fourier transform by using the computer program developed by Glatter (1977). As a step in this data analysis, the primary data are desmeared; the desmeared scattering curves are shown in Figures 1 and 2. The $p(r)$ functions are shown in Figure 3.

It follows from the distance distribution function, $p(r)$, shown in Figure 3, that the maximal distance within the prothrombin molecule increases about 20 Å when Ca^{2+} ions are bound. In the absence of Ca^{2+} the maximum distance observed for prothrombin, 130 Å, is within the same order of magnitude as that reported by Lamy & Waugh (1953), 119 Å. As further shown by Figure 3, there is one maximum and a tendency to at least one shoulder, most clearly indicated in the $p(r)$ curve of the Ca^{2+}–prothrombin system. One way to explain the presence of one or more shoulders in the $p(r)$ curve is that the molecule contains more than one structural domain.

The radius of gyration was determined to be 35.5 ± 1.5 Å for prothrombin and 39.5 ± 1.5 Å for Ca^{2+}–prothrombin; slit correction was done according to the computer program developed by Glatter (1974). These results essentially agree with the radii of gyration obtained by integration of the $p(r)$ function, which were 35.0 and 39.0 Å, respectively.

The molecular weight was determined by the formula described by Kratky (1963). By use of a digital densitometer

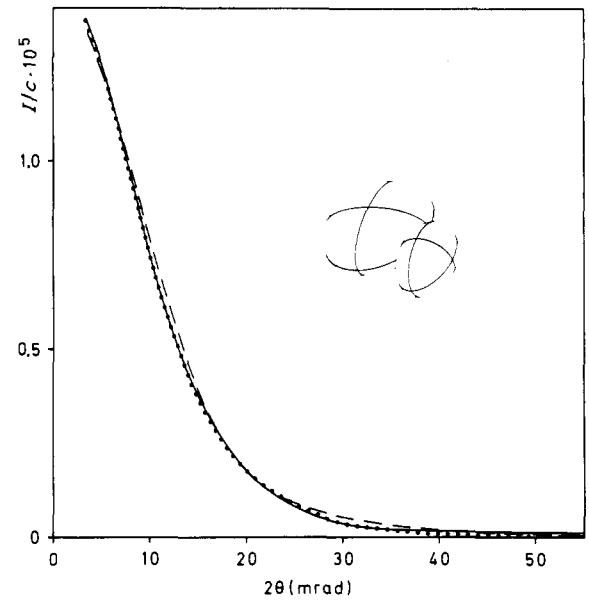


FIGURE 1: Experimental and calculated X-ray scattering curves of prothrombin in the absence of Ca^{2+} ions. (Full-drawn curve) Experimental curve extrapolated to zero concentration and corrected for the collimation effect; (dashed curve) scattering curve calculated for the best-fitting ellipsoid using a least-squares computer program; (dotted curve) scattering curve calculated for a model involving two ellipsoids arranged as indicated with the semiaxes $a_1 = b_1 = 40$, $c_1 = 20$ Å and $a_2 = 30$, $b_2 = c_2 = 20$ Å.

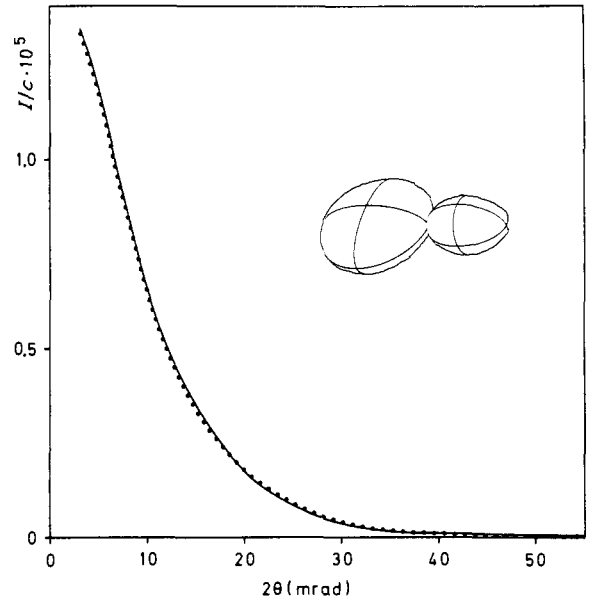


FIGURE 2: Experimental and calculated X-ray scattering curve of prothrombin in the presence of 2 mM Ca^{2+} ions. (Full-drawn curve) Experimental curve extrapolated to zero concentration and corrected for the collimation effect; (dotted curve) scattering curve calculated for a model involving two ellipsoids (see legend to Figure 1) arranged as indicated.

(Kratky et al., 1969) the partial specific volume, $\bar{v}$, was determined to be 0.713 cm^3/g. This is larger than the value of 0.70 cm^3/g reported by Lamy & Waugh (1953). For both prothrombin and Ca^{2+}–prothrombin the molecular weight was then calculated, and the result was 78 000 ± 4000. This is in agreement with the molecular weight calculated from the primary structure assuming 12% carbohydrate, 74 000 (Magnusson et al., 1975).

Shape of Prothrombin and Ca^{2+}–Prothrombin. (1) Prothrombin. The experimental intensity data were first compared with smeared theoretical curves calculated for one triaxial

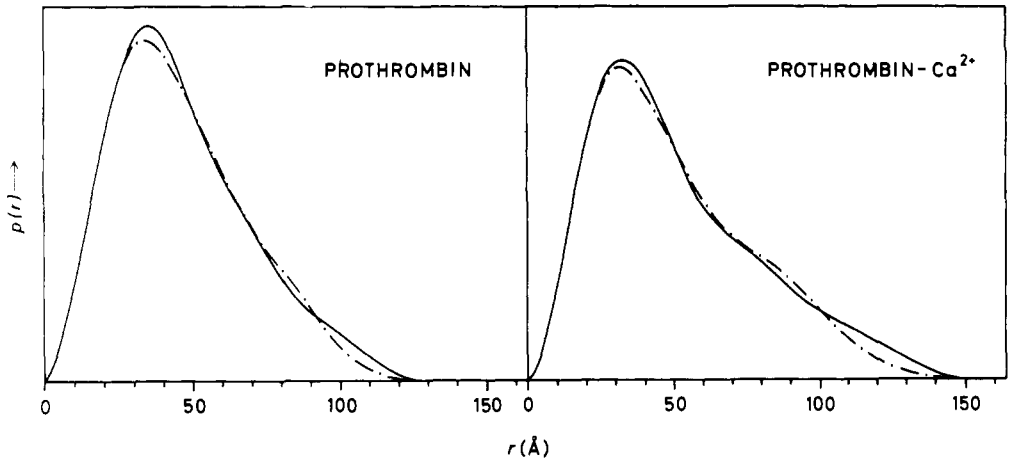


FIGURE 3: Distance distributions, $p(r)$, of prothrombin in the absence of Ca^{2+} ions and in the presence of 2 mM Ca^{2+} ions. The full-drawn curves are obtained from the experimental data, and the dashed-dotted curves are calculated for the models shown in Figures 1 and 2, respectively.

body. Using the least-squares program developed by Sjöberg (1978), we obtained a minimum in the error square sum for an oblate ellipsoid having the semiaxes $a = b = 51$, $c = 13$ Å. As follows from Figure 1 the scattering from this two-parameter model describes the data in the most proximal range but deviates considerably for angles $2\theta > 24$ mrad. Therefore, in agreement with the $p(r)$ function, it seems as if more than one triaxial body is required in order to describe the data.

When theoretical curves were calculated for models of two ellipsoids, we used the procedure described by Pilz et al. (1970b). The best fit to the experimental data was obtained by the scattering from the model shown in Figure 1; this model involves one large and one small ellipsoid with the following semiaxes: $a_1 = b_1 = 40$, $c_1 = 20$ Å and $a_2 = 30$, $b_2 = c_2 = 20$ Å. It is indicated from Figure 1 that this model essentially describes the data in the present angular range. Also, for this model a $p(r)$ curve was calculated from the theoretical intensity by using eq 1; and the resulting $p(r)$ curve agrees with the experimental one within the experimental errors (Figure 3). The radius of gyration of the model is 35.9 Å; its volume is 184 300 Å^3 which corresponds to a water content of 47%. It should be noted that the shape of prothrombin suggested by Lamy & Waugh (1953), corresponding to a single ellipsoid with the semiaxes $a = 59.5$, $b = c = 17$ Å, cannot be correct. By use of the molecular weight of 74 000 and the partial specific volume of 0.713 cm^3/g determined in this work, it can be calculated that the volume of such an ellipsoid is only 72 028 Å^3 which is less than the dry volume of prothrombin (=87 700 Å^3). The same appears to be true for the model suggested by Lim et al. (1977) which yields a water content of only 3%.

(2) *Ca^{2+}–Prothrombin.* For this system the $p(r)$ data more clearly indicate that more than one triaxial body is required in order to describe the data (Figure 3). Also, the X-ray scattering data yield a larger radius of gyration and larger maximal distance as compared to those of prothrombin. Therefore, we attempted to orientate the two ellipsoids representing the model for prothrombin (Figure 1) so that the scattering from them should explain the data obtained for Ca^{2+}–prothrombin; the result is shown in Figure 2. It follows from this figure that the angle between the largest axes (a_1 and a_2) for the two bodies described in Figure 1 has been increased from 90 to 180° when going from prothrombin to Ca^{2+}–prothrombin; the two largest axes (a_1, b_1, and a_2, b_2) of each ellipsoid are supposed to remain in the same plane. For this model a $p(r)$ curve was calculated by using the theoretical intensity data and eq 1; the result indicates that, within the experimental errors, the theoretical $p(r)$ curve describes the experimental one (Figure 3).

Discussion

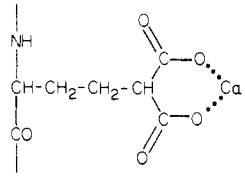
The results presented in the previous section indicate that Ca^{2+} ions induce a conformational change in the prothrombin molecule. The data (Figures 1 and 2) can be explained if it is assumed that, as a result of Ca^{2+} binding, the masses of the two globules of the prothrombin molecule become further apart from each other (cf. Figure 3). This is illustrated in Figures 1 and 2 by the models consisting of two ellipsoids; as a result of Ca^{2+} binding, the angle between the main axes of the ellipsoids, in the plane of the two main axes of both ellipsoids, increases from 90 to 180°. In agreement with these results, the antigenic structure as well as the circular dichroism spectra changes when Ca^{2+} ions are bound to prothrombin (cf. Bloom & Mann, 1978; Stenflo & Ganrot, 1972, 1973; Henriksen & Jackson, 1975; Nelsestuen, 1976). It should be noted that our model of two ellipsoids involves the minimum number of parameters required in order to describe the data. Other models involving more parameters may equally well explain the data.

When prothrombin is cleaved by thrombin, fragment 1 is formed. This fragment, which has a molecular weight of 22 000, crystallizes into a tetragonal space group (Aschaffenburg et al., 1977). The data indicate that eight molecules might be present in the unit cell (Aschaffenburg et al., 1977), which yields a water content of 61%. After slightly proteolytic degradation, fragment 1 also crystallizes into the space group $P2_12_12_1$ with the unit cell 39 × 54 × 129 Å; four molecules in the unit cell yield a water content of 55% (O. Lindqvist and G. Ohlsson, unpublished experiments). Thus, the water contents of both kinds of crystals compare well with that of our model, 47%. It is tempting to speculate that the minor ellipsoid of our model corresponds to fragment 1, since the minor ellipsoid model easily packs into any of these two crystals using the special crystallographic positions.

Fragment 1 of prothrombin seems to be mainly responsible for its Ca^{2+} binding. As a result of a K vitamin dependent process, ten γ-carboxyglutamic acid residues are formed on fragment 1, and they are supposed to be the ligands of Ca^{2+} (Suttie & Jackson, 1977; Nelsestuen, 1978; Stenflo & Suttie, 1977; Olson & Suttie, 1977). Under physiological conditions, where the free calcium ion concentration, [Ca^{2+}], is approximately 1 mM (Branegård & Österberg, 1974), three or four Ca^{2+} ions are bound strongly to the prothrombin molecule (Suttie & Jackson, 1977; Nelsestuen, 1978; Stenflo & Suttie, 1977; Olson & Suttie, 1977). This agrees with the present X-ray data that are consistent for the concentration range from

0.051 to 0.506 mM of prothrombin at the physiological calcium concentration of 2 mM (at the highest prothrombin concentration maximally four Ca^{2+} ions can be bound per prothrombin molecule).

Thus, the physiological Ca^{2+} binding of prothrombin may involve ten γ-carboxyglutamic acid residues and three or four strongly bound Ca^{2+} ions. The Ca^{2+} complex involves most likely six-membered chelate rings of the type shown by formula I. A similar chelate is formed by malonate, and from the



I

stability of the malonate complex we may estimate how many carboxylate groups may maximally be involved per strongly bound Ca^{2+} ion in prothrombin. We assume that the calcium sites are similar and independent and that, at a free Ca^{2+} concentration of 1 mM [cf. Branegård & Österberg (1974)], 99% of the strong Ca^{2+} sites on prothrombin are occupied. Then for these conditions to be fulfilled, it can be estimated from the formula

$$[CaL]/[L] = [Ca^{2+}]\beta^* \qquad (2)$$

that the stability constant, β^*, for a prothrombin site, L, must have a value of $\log \beta^* \geq 5$, since for $[CaL]/[L] \geq 100$, we have $\beta^* \geq 10^5$. If we compare this value with the stability, $\log K = 1.9$, for the corresponding malonate complex (Sillén & Martell, 1964, 1971), it follows that one malonate residue does not bind Ca^{2+} strongly enough. Two malonate residues per Ca^{2+} ion may yield $\log \beta \leq 3.6$, and three malonate residues may yield $\log \beta \leq 5.7$. The latter value would be enough for the required limit, $\beta^* > 10^5$. So, provided that the environment about the γ-carboxyglutamic acid residues is similar to that of the malonate in water solution, then no less than three of these residues are required for the conditions assumed. This number of three γ-carboxyglutamic acid residues per strongly bound Ca^{2+} ions should be considered a maximum estimate, since the area where these Ca^{2+} ions are bound to prothrombin may be more hydrophobic; a relatively hydrophobic area will increase the stability and thus decrease the number of γ-carboxyglutamic acid residues required. And, therefore, although three Ca^{2+} ions per nine γ-carboxyglutamic acid residues are in good agreement with a number of ten such residues present in prothrombin, we cannot exclude the possibility that more than three Ca^{2+} ions are involved in the conformational change (such as four Ca^{2+} ions, for instance).

These binding data, which tell us that three, or possibly four, Ca^{2+} ions might be involved in the conformational change of the prothrombin molecule, yield very little structural information. The X-ray scattering data, on the other hand, indicate the main conformational change but give very little detailed information regarding the exact molecular nature of the process. At the present stage it seems difficult to speculate what the particular reason is for the conformational change. However, the crude models obtained from this study indicate that when Ca^{2+} ions are bound, the prothrombin molecule attains a more extended conformation (Figure 2). Therefore, it might be tempting to speculate that the conformational change makes it sterically possible for the proteolytic enzyme to approach the susceptible bonds in space so that the clotting process can be activated via the splitting of two specific peptide bonds.

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