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Prothrombin Domains: Circular Dichroic Evidence for a Lack of Cooperativity†

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ABSTRACT: The far-ultraviolet circular dichroism spectra of bovine and human prothrombin, prothrombin fragment 1, prethrombin 1, prothrombin fragment 2, and prethrombin 2 (prethrombin $2_{des(1-13)}$) were determined and the method of Chen et al. [Chen, Y. H., Yang, J. T., & Martinez, H. M. (1972) *Biochemistry 11*, 4120–4131; Chen, Y. H., Yang, J. T., & Chau, K. H. (1974) *Biochemistry 13*, 3350–3359] was used to calculate the apparent α-helix, β-sheet, and random-coil contents of each protein. Prothrombin and its activation components were found to contain a large amount of aperiodic secondary structure and there was little species difference between the spectra and, thus, secondary structures. The hypothesis that the prothrombin activation components exist as relatively noncooperative "domains" within the prothrombin molecule was tested by comparing the circular dichroism spectrum of prothrombin with the sum of the spectra of the components. In support of the hypothesis, no gross alterations in the spectra and, hence, secondary structures of the components were found to have occurred upon activation.

Thrombin, a serine protease, catalyzes the conversion of fibrinogen to fibrin in the blood coagulation process. It also participates in the activation of several coagulation factors (V, VIII, and XIII), as well as in the aggregation of platelets.

During hemostasis, the formation of thrombin is believed to occur via the catalytic conversion of prothrombin by factor Xa. In this reaction, substrate (prothrombin), enzyme (factor Xa), and cofactor (factor Va) are complexed by virtue of mutual adherence to phospholipid via calcium bridges (Papahadjopoulos & Hanahan, 1964; Cole et al., 1965). Upon factor Xa activation, prothrombin undergoes proteolytic cleavage to release the "pro" portion of the molecule and the two-chain thrombin molecule. In addition, the thrombin produced upon activation can cleave prothrombin at one or two sites. The result of the two factor Xa cleavages and the thrombin cleavage(s) is that many activation components of prothrombin are produced (Mann, 1976).

The most readily isolated activation components of bovine and human prothrombin are[1] (Mann, 1976) prothrombin

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fragment 1 and prethrombin 1, produced by the thrombin catalyzed cleavage of prothrombin, and prothrombin fragment 2 and prethrombin 2, produced by a factor Xa catalyzed cleavage of prethrombin 1. The human protein differs from the bovine in that human prothrombin contains an additional thrombin-sensitive site (Downing et al., 1975). This additional site results in the liberation of an amino-terminal peptide of 13 residues from the A chain of human thrombin (and/or prethrombin 2).

The functions and primary sequences of prothrombin and its activation components suggest that the components exist as relatively noncooperative "domains" within the prothrombin molecule. For example, studies of the kinetics of activation of prothrombin and the two thrombin precursors, prethrombin 1 and prethrombin 2, suggest that prothrombin fragments 1 and 2 retain their prothrombin functions upon activation and purification. Prothrombin fragment 1 has been shown to be the Ca^{2+}-dependent phospholipid binding segment of the prothrombin molecule (Gitel et al., 1973; Bajaj et al., 1975), while prothrombin fragment 2 is believed to be the factor Va binding site (Esmon et al., 1973; Bajaj et al., 1975). Prothrombin contains 10 or 11 Ca^{2+} binding sites and, upon activation, these sites are distributed between fragment 1 (about 5 sites) and fragment 2 (4 or 5 sites) (Bajaj et al., 1975).

The primary sequence of prothrombin also gives evidence of the presence of "domains". Fragment 1 and fragment 2 contain homologous regions of amino acid sequences and the last three disulfide bridges of prothrombin fragment 1 are homologous to the three bridges in prothrombin fragment 2. These disulfide bridge patterns have led them to be called "kringle" structures (Magnusson et al., 1975).

The present report examines the secondary structure of bovine and human prothrombin and their respective activation components by circular dichroism. We show that no gross alterations in the secondary structures of the components occur upon activation and, thus, the activation components of prothrombin exist essentially as "domains" within the prothrombin molecule.

Materials and Methods

Proteins. Prothrombin was purified from citrated bovine plasma and from human Cohn fraction 3 (a gift of Dr. Charles Heldebrant, Abbott Laboratories) or from Red Cross factor IX concentrate (gift of Dr. Yu-Lee Hao, the American National Red Cross) as previously described (Downing et al., 1975; Bajaj & Mann, 1973). Prothrombin fragment 1 and prethrombin 1 were prepared by proteolytic cleavage of prothrombin by thrombin and were separated as described elsewhere (Downing et al., 1975; Heldebrant et al., 1973). Prothrombin fragment 2, bovine prethrombin 2, and human prethrombin $2_{des(1-13)}$ were prepared by proteolytic cleavage of prethrombin 1 by factor Xa and were separated as previously described (Downing et al., 1975; Heldebrant et al., 1973). The protein preparations were greater than 95% homogeneous as evaluated electrophoretically by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Weber & Osborn, 1969).

Protein concentrations were determined and spectrophotometrically corrected for Rayleigh scattering using the equation

$$A_{280} = A_{280abs} - 1.706A_{320abs}$$

The extinction coefficients ($E_{280}^{1\%}$) used were: 14.7 for human prothrombin, 11.9 for human prothrombin fragment 1, and 17.8 for human prethrombin 1 (Butkowski et al., 1977). The coefficients used for the other proteins were those of the bovine system as reported by Mann (1976). The mean residue weights and molecular weights of the proteins were calculated from the primary structures (Butkowski et al., 1977; Elion et al., 1976; Walz et al., 1977; Magnusson et al., 1975). Molecular weights were computed based upon 8.2% carbohydrate in prothrombin and a symmetrical three-chain distribution (Hudson et al., 1975). The human (bovine) molecular weights and mean residue weights are listed respectively: prothrombin, 71 600 (72 100) and 113.1 (113.7); prothrombin fragment 1, 21 700 (22 000) and 114.6 (115.4); prothrombin fragment 2, 12 866 (12 791) and 109.0 (108.4); prethrombin 1, 49 900 (50 200) and 112.5 (113.2); prethrombin 2, 37 000 (37 400) and 113.9 (115.0).

In preparation for analysis, the proteins were dialyzed in plastic containers, first against 5 mM EDTA in 0.01 M Tris, 0.10 M NaCl, pH 7.4, to ensure minimal Ca^{2+} contamination and then exhaustively against 0.01 M Tris, 0.10 M NaCl, pH 7.4, to ensure minimal residual EDTA.

Circular Dichroism Measurements. Circular dichroism (CD) spectra were obtained using a Jasco Model J-20A spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan). The far-ultraviolet spectra were recorded at room temperature (25 °C) with protein concentrations of 0.2–0.6 mg/mL in a 0.05-cm path length cuvette. Base line deviations were subtracted from all spectra. All spectra were measured in at least duplicate and the averaged values have been reported. The results have been expressed in terms of either the molar ellipticity, $[\theta]$, or mean residue ellipticity, $[\theta']$:

$$[\theta]_\lambda \text{ (or } [\theta']_\lambda) = \frac{\theta_{obsd}\text{MW (or MRW)}}{10dc}$$

where λ = wavelength; θ_{obsd} = observed ellipticity in degrees; MW = molecular weight; MRW = mean residue weight; c = concentration in grams per milliliter; d = path length in centimeters. Ellipticity has the units of deg cm^2 dmol^{-1}.

The far-UV CD spectra of prothrombin and its activation components were analyzed by a technique which assumes that only three types of fundamental protein conformations contribute to a protein's structure in solution: "α helix", "β sheet", and "random coil". Chen et al. (1972) have found that the CD spectrum of a protein can be expressed as

$$X(\lambda) = f_H X_H(\lambda) + f_\beta X_\beta(\lambda) + f_R X_R(\lambda) \quad (1)$$

where $f_H + f_\beta + f_R = 1$ and all f's ≥ 0. $X(\lambda)$ is the ellipticity at any wavelength, and the f's are the fractions of α helix (H), β sheet (β), and random coil (R). Chen et al. (1972) used f values empirically determined for five proteins whose structures were already known from X-ray studies to compute $X_H(\lambda)$, $X_\beta(\lambda)$, and $X_R(\lambda)$ at numerous wavelengths through a least-squares method. With the tabular data of Chen et al. (1974) and the assumption that the five proteins used in his calculations of $X_H(\lambda)$, $X_\beta(\lambda)$, and $X_R(\lambda)$ are a representative sample, the contributions of α helix, β sheet, and random coil to the secondary structure of any protein can be computed from its CD spectrum. The solution of eq 1 is a special case of an overdetermined system of linear equations where the third variable can be expressed in terms of the other two. The least-squares approximation computer program used to solve eq 1 for each spectrum was written by Mr. Aloysius Chu and Dr. Barry K. Gilbert and solved on a CDC 3500 computer.

[1] The nomenclature used in this paper is based on the recommendations of the Task Force on the Nomenclature of Blood Clotting Zymogens and Zymogen Intermediates.

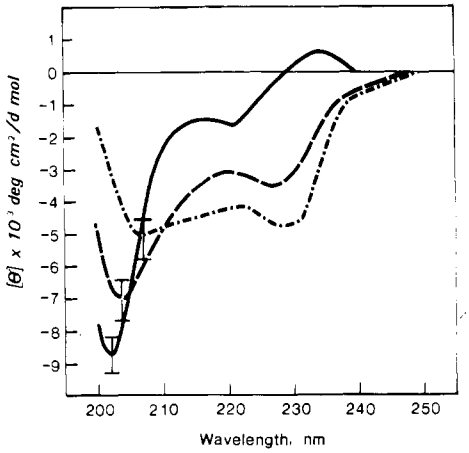

FIGURE 1: Far-ultraviolet CD spectra of bovine prethrombin 1 (---), prothrombin fragment 2 (—), and prethrombin 2 (•••). The proteins are in 0.01 M Tris, 0.10 M NaCl, pH 7.4, at concentrations of prethrombin 1, 0.375 mg/mL; fragment 2, 0.317 mg/mL; prethrombin 2, 0.217 mg/mL. [θ′] is the mean residue ellipticity.

Table I: Calculated Apparent α-Helix, β-Sheet, and Random-Coil Contents (%) of Prothrombin and Its Activation Components

	α helix	β sheet	random coil
human prothrombin	8	15	77
bovine prothrombin	10	15	75
human prothrombin fragment 1	6	31	63
bovine prothrombin fragment 1	9	33	58
human prothrombin fragment 2	0	20	80
bovine prothrombin fragment 2	3	18	79
human prethrombin 1	11	3	86
bovine prethrombin 1	13	5	82
human prethrombin $2_{des(1-13)}$	17	1	82
bovine prethrombin 2	18	0	82

An obvious source of error in the Chen et al. (1972, 1974) method of analysis is in the choice of reference spectra. However, there are other sources of error in CD spectral interpretations other than that due to the choice of reference spectra. For these reasons, the percentages of α helix, β sheet, and random coil calculated in this paper must not be literally interpreted as being accurate estimates of structure.

Results

CD Spectra of Prothrombin and Its Activation Components. The CD spectra, in the range of 200–250 nm, of bovine and human prothrombin, prothrombin fragment 1 and 2, prethrombin 1 and prethrombin 2 (prethrombin $2_{des(1-13)}$) were determined. The CD spectra of human prothrombin and prothrombin fragment 1 have been reported previously (Bloom & Mann, 1978) and the prothrombin fragment 1 spectrum is in agreement with that reported by Gabriel et al. (1975). The far-UV CD spectrum for bovine prothrombin is similar to that obtained by Bjork & Stenflo (1973). The bovine prothrombin fragment 2, prethrombin 1, and prethrombin 2 CD spectra are shown in Figure 1. The calculated apparent α-helix, β-sheet, and random-coil contents of human and bovine prothrombin and their activation components are given in Table I.

The conclusions to be drawn from these experiments are twofold. (1) Prothrombin and its activation components contain between 60% and 90% unordered (or aperiodic) structure. The Chen et al. (1972, 1974) method of CD spectral analysis, however, applies to proteins containing a moderate to high amount of α helix (19–77%) and, thus, may not be applicable in the present case. (2) Little species difference was observed between human and bovine prothrombin and their activation fragments.

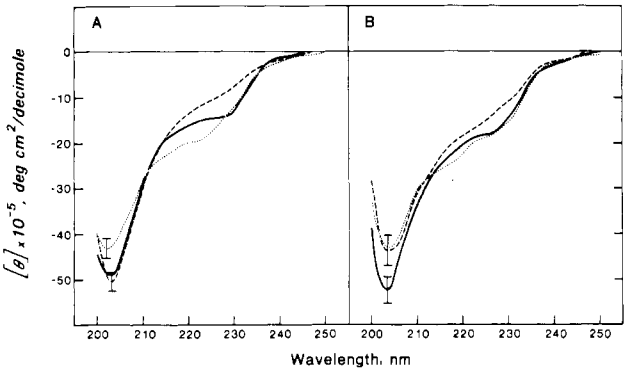

FIGURE 2: (A) CD spectrum of human prothrombin (---). (—) Represents the sum of the individual CD spectra of human prethrombin 1 and prothrombin fragment 1. (•••) Represents the sum of the individual CD spectra of human prothrombin fragment 1, prothrombin fragment 2, and prethrombin $2_{des(1-13)}$. The proteins are in 0.01 M Tris, 0.10 M NaCl, pH 7.4, at concentrations of prothrombin, 0.585 mg/mL; prethrombin 1, 0.318 mg/mL; fragment 1, 0.318 mg/mL; fragment 2, 0.323 mg/mL; prethrombin $2_{des(1-13)}$, 0.306 mg/mL. (B) CD spectrum of bovine prothrombin (---). (—) Represents the sum of the individual CD spectra of bovine prethrombin 1 and prothrombin fragment 1. (•••) Represents the sum of the individual CD spectra of bovine prothrombin fragment 1, prothrombin fragment 2, and prethrombin 2. The proteins are in 0.01 M Tris, 0.10 M NaCl, pH 7.4, at concentrations of prothrombin, 0.319 mg/mL; prethrombin 1, 0.375 mg/mL; fragment 1, 0.327 mg/mL; fragment 2, 0.317 mg/mL; prethrombin 2, 0.217 mg/mL. [θ] is the molar ellipticity.

Combined Spectra of the Prothrombin Activation Components. The hypothesis that the prothrombin activation components exist as "domains" within the prothrombin molecule was tested by examining the CD spectrum of prothrombin and the sum of the spectra of the components for evidence of gross perturbations of the secondary structures of the components upon activation. The CD spectra of the activation components were summed in terms of molar ellipticity and compared with the molar ellipticity spectrum of prothrombin.

The comparisons of the spectrum of prothrombin with that of prethrombin 1 plus prothrombin fragment 1 and that of prothrombin fragment 1 plus prothrombin fragment 2 plus prethrombin 2 (prethrombin $2_{des(1-13)}$) are given in Figure 2. Within experimental error, the CD spectrum of human prothrombin (Figure 2A) and the sum of the spectra of prethrombin 1 plus fragment 1 have minima of identical magnitudes at about 203 nm. In the region 215–235 nm, however, the sum of the spectra of prethrombin 1 plus fragment 1 has a more negative ellipticity than the prothrombin spectrum. The summed spectra of fragment 1, fragment 2, and prethrombin 2 have a minimum at 203 nm that is smaller in magnitude than the prothrombin spectrum but have a greater negative ellipticity than the sum of the spectra of prethrombin 1 plus fragment 1 in the region 213–227 nm.

As an additional test for activation component interactions, human prethrombin 1 and fragment 1 were mixed in equimolar amounts in a cuvette and the far-UV spectrum was recorded (not shown). The minimum at 203 nm was identical within experimental error with the sum of the individual spectra of fragment 1, fragment 2, and prethrombin 2, while in the region 215–250 nm the spectrum was identical within experimental error with that of prothrombin. Thus, any interaction between prethrombin 1 and fragment 1 that may have occurred in

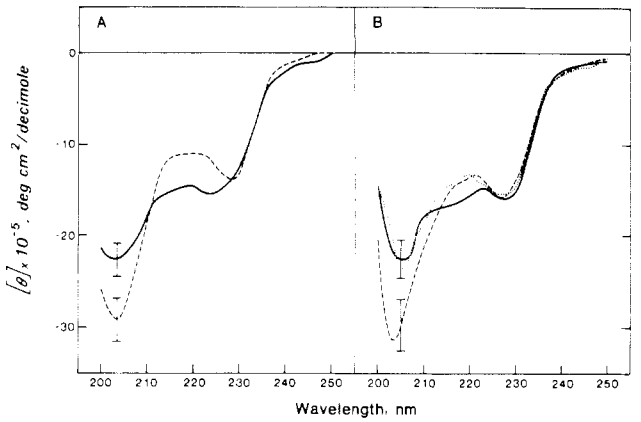

FIGURE 3: (A) CD spectrum of human prethrombin 1 (---). (—) Represents the sum of the individual CD spectra of human prothrombin fragment 2 and prethrombin $2_{des(1-13)}$. The proteins are in 0.01 M Tris, 0.10 M NaCl, pH 7.4, at concentrations of prethrombin 1, 0.318 mg/mL; fragment 2, 0.323 mg/mL; prethrombin $2_{des(1-13)}$, 0.306 mg/mL. (B) CD spectrum of bovine prethrombin 1 (---). (—) Represents the sum of the individual CD spectra of bovine prothrombin fragment 2 and prethrombin 2. (···) represents the spectrum of an equimolar mixture of bovine prothrombin fragment 2 and prethrombin 2. The proteins are in 0.01 M Tris, 0.10 M NaCl, pH 7.4, at concentrations of prethrombin 1, 0.375 mg/mL; fragment 2, 0.317 mg/mL; prethrombin 2, 0.217 mg/mL. $[\theta]$ is the molar ellipticity.

solution had little effect upon the secondary structure. The lack of gross spectral differences between the CD spectrum of the equimolar mixture of human prethrombin 1 and fragment 1 and the sum of the individual spectra also confirms the precision of the spectral summation technique.

The CD spectrum of bovine prothrombin (Figure 2B) and the sum of the spectra of prothrombin fragment 1, fragment 2, and prethrombin 2 have identical minima at 203–204 nm. However, in the region 215–235 nm, the summed spectra of fragment 1, fragment 2, and prethrombin 2 have a more negative ellipticity than the prothrombin spectrum. The minimum at 203–204 nm of the summed spectra of prethrombin 1 plus fragment 1 is more negative than the prothrombin spectrum, but the spectrum is essentially identical with the sum of the spectra of fragment 1, fragment 2, and prethrombin 2 in the region 215–250 nm. Thus, no gross structural alteration has occurred in the activation of prothrombin to prethrombin 1 plus fragment 1 or to fragment 1 plus fragment 2 plus prethrombin 2.

Bovine prothrombin fragment 1, fragment 2, and prethrombin 2 were mixed in equimolar amounts in a cuvette, and the far-UV spectrum was recorded (not shown) as an additional test for activation component interaction. The minimum of the spectrum of the mixture at 203–204 nm was identical within experimental error with the spectral minimum of the sum of the individual spectra of fragment 1 plus fragment 2 plus prethrombin 2 and to the prothrombin spectral minimum. In the region 215–250 nm, the spectrum of the mixture was identical with the spectral sum of the individual fragments and with the spectrum of prethrombin 1 plus fragment 1. Thus, any interaction in solution between fragment 1, fragment 2, and prethrombin 2 appears to have a minimal effect upon the CD spectra of the molecules.

The comparison of the spectrum of prethrombin 1 with that of the sum of the spectra of prethrombin 2 plus fragment 2 is given in Figure 3. The CD spectrum of human prethrombin 1 (Figure 3A) has a larger minimum at 203–204 nm but a less negative ellipticity in the region 212–227 nm than the sum of the spectra of fragment 2 plus prethrombin $2_{des(1-13)}$. The spectrum of bovine prethrombin 1 (Figure 3B) has a larger

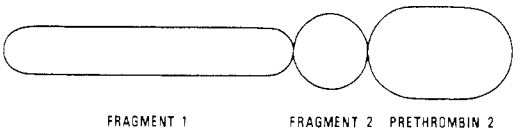

FIGURE 4: A possible configuration for the prothrombin molecule.

minimum at 203–204 nm but a smaller ellipticity in the region 215–223 nm than the sum of the spectra of fragment 2 plus prethrombin 2. Thus, no gross structural differences are apparent between prethrombin 1 and the sum of its activation components: fragment 2 plus prethrombin 2 (prethrombin $2_{des(1-13)}$).

Prothrombin fragment 2 binds noncovalently to prethrombin 2 with a high degree of affinity (Myrmel et al., 1976). To determine whether the secondary structures of the proteins are altered by this interaction, we mixed bovine fragment 2 and prethrombin 2 in equimolar amounts in a cuvette and the far-UV CD spectrum was recorded (Figure 3B). Except in the region 215–220 nm the spectrum of the mixture of fragment 2 plus prethrombin 2 appears to be identical within experimental error with the sum of the individual spectra; only in the approximate area of 200–210 nm does the spectrum differ from that of prethrombin 1. Thus, no gross changes in the CD spectra and, hence, secondary structures of fragment 2 and prethrombin 2 occur either as a result of activation from prethrombin 1 or as a result of the strong noncovalent interaction between the molecules.

Discussion

The far-ultraviolet circular dichroism spectra of bovine and human prothrombin, prothrombin fragment 1, prethrombin 1, prothrombin fragment 2, and prethrombin 2 (prethrombin $2_{des(1-13)}$) were determined, and the method of Chen et al. (1972, 1974) was used to calculate the apparent α-helix, β-sheet, and random-coil contents of each protein. Prothrombin and its activation components were found to contain a large amount of aperiodic secondary structure and there was little species difference between the spectra and, thus, secondary structures.

The CD spectrum of prothrombin and the sum of the spectra of the activation components were compared to determine whether any gross perturbations of the secondary structures of the fragments occurred upon activation. The spectra of equimolar mixtures of the activation fragments were also utilized in the comparisons since prothrombin fragment 2 and prethrombin 2 associate noncovalently with a high degree of affinity (Myrmel et al., 1976). These comparisons showed no gross spectral differences and, hence, no gross alterations in the structures of the fragments upon activation. These results are consistent with the hypothesis that the activation components of prothrombin exist essentially as noninteracting "domains" within the prothrombin molecule.

A possible configuration for the prothrombin molecule is shown in Figure 4. Prothrombin fragment 1 and prethrombin 2 are represented as prolate ellipsoids with axial ratios of 6/1 and 1.6/1, respectively. These axial ratios were calculated from the known physical properties of fragment 1 and prethrombin 2 (Mann & Elion, 1979) with an $s_{20,w}{}^0$ for prethrombin 2 of 3.7 (Seegers et al., 1968) and by assuming 0.2 g of bound water/g of protein (Oncley, 1941). The overall dimensions shown in Figure 4 were calculated from the axial ratios and volumes per mole of each protein. As the $s_{20,w}{}^0$ for prothrombin fragment 2 has not yet been determined, it is represented as a sphere with dimensions calculated from the volume per mole (Mann & Elion, 1979). The axial ratio of

prothrombin in the proposed configuration, where the *b* axis is an average of the three fragments, is 7/1. This value is very close to the axial ratio of prothrombin of 6/1 calculated from the known physical properties of prothrombin (Mann & Elion, 1979).

The presence of activation component "domains" within the prothrombin molecule is not without precedent in other protein systems. An analogous result was obtained in circular dichroism studies of rabbit IgG immunoglobulins. Cathou et al. (1968) found that the far-ultraviolet CD spectrum of rabbit antidinitrophenyl antibody is the sum of separate contributions from the papain fragments, Fab and Fc. On the other hand, Dorrington et al. (1967) found that the separation of the heavy and light chains of rabbit and human myeloma IgG resulted in significant conformational changes in the chains as measured by optical rotatory dispersion. The original conformation was restored upon recombination of the chains, however.

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