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Calorimetric Evaluation of the Existence of Separate Domains in Bovine Prothrombin†

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ABSTRACT: At least two endothermal (denaturing) transitions, at 53.1 ± 1.5 °C and 58.2 ± 1.5 °C, of a total ΔH of 324 ± 12 kcal mol^{-1}, are observed, by differential scanning calorimetry (DSC), for solutions of bovine prothrombin. Studies with the appropriate isolated fragments show that the low-temperature transition arises from the prethrombin 1 portion of the molecule and the higher temperature transition originates from the fragment 1 region. We have compared the transition temperature midpoints, T_m, the total ΔH values of the thermal transitions, and the ratio of the calorimetric to van't Hoff enthalpies of these isolated regions of prothrombin with those of the prothrombin molecule. The data obtained strongly suggest that they exist as independent domains in the intact bovine prothrombin molecule. On the other hand, when prethrombin 1 is digested to fragment 2 and prethrombin 2, DSC experiments performed on the isolated components, in the presence or absence of Ca^{2+}, strongly support the conclusion that these regions contribute in a cooperative fashion to the structure of intact prethrombin 1 (and prothrombin). Addition of saturating (5 mM) levels of Ca^{2+} to intact bovine prothrombin results in essentially no change in the T_m of the prethrombin 1 domain, an increase in the T_m of the fragment 1 domain, and a decrease in the total ΔH of the thermal transitions. Further, the endotherm for the prethrombin 1 region is markedly sharpened in the presence of Ca^{2+}. Addition of Ca^{2+} causes no alteration in the T_m of 52.6 ± 1.5 °C or the ΔH of 200 ± 10 kcal mol^{-1} of isolated prethrombin 1, and the sharpening effect induced by Ca^{2+} in the prethrombin 1 region of bovine prothrombin was not observed with isolated prethrombin 1. The T_m of the fragment 1 region of intact bovine prothrombin is increased to 64.8 ± 1.5 °C upon addition of 5 mM Ca^{2+} to the solution. Similar changes are also noted in isolated fragment 1 as a result of addition of Ca^{2+}. Here, the T_m of fragment 1 of 58.5 ± 1.5 °C and the ΔH of fragment 1 of 134 ± 10 kcal mol^{-1} are altered to 64.5 ± 1.5 °C and 102 ± 10 kcal mol^{-1}, respectively, as a result of addition of 5 mM Ca^{2+}. Other metal ions such as Mg^{2+} and Mn^{2+} induce similar alterations in the thermal transitions of prothrombin and isolated fragment 1.

The conversion of fibrinogen to fibrin is catalyzed by thrombin, a serine protease derived from a plasma precursor, prothrombin. Bovine prothrombin is a 72000 molecular weight glycoprotein (Heldebrant et al., 1973a) whose structure (Magnusson et al., 1975), function, and activation mechanism (Esmon et al., 1974; Esmon & Jackson, 1974a,b; Bajaj et al., 1975; Heldebrant et al., 1973a) have been extensively investigated. The efficient activation of prothrombin to thrombin requires the cooperative effect of two proteins, factor Xa and factor Va, in addition to phospholipid and calcium (Nesheim et al., 1979b).

Several proteolytic cleavages occur in the prothrombin molecule upon activation, giving rise to various intermediate fragments (Esmon et al., 1974; Esmon & Jackson, 1974a,b;

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Bajaj et al., 1975). Enzymatic cleavage of prothrombin by factor Xa produces a 37000 molecular weight polypeptide (Mann, 1976), prethrombin 2, and a 35000 molecular weight fragment originating from the amino-terminal region of the molecule, fragment 1.2 (Mann, 1976). The production of thrombin from prethrombin 2 is accomplished by factor Xa, resulting in the two-chain thrombin molecule. Enzymatic cleavage of prothrombin with thrombin results in the formation of prethrombin 1, a 50000 molecular weight polypeptide, and fragment 1, a 22000 molecular weight polypeptide originating from the amino terminus of the zymogen (Heldebrant et al., 1973b).

Studies on the purified fragments generated during prothrombin activation have demonstrated that fragment 1 is a phospholipid binding region of the molecule (Gitel et al., 1973; Benzon et al., 1973). Phospholipid binding is calcium dependent, and fragment 1 has been shown to contain about five of the ten calcium sites found in the molecule (Bajaj et al., 1975; Prendergast & Mann, 1977). Occupancy of the fragment 1 sites by Ca^{2+} induces conformational changes in this peptide (Prendergast & Mann, 1977).

Recently, evidence has been presented suggesting that fragment 1, fragment 2, and prethrombin 2 exist as noninteracting domain regions (Bloom & Mann, 1979) within prothrombin. This conclusion was based on circular dichroism data, showing that very little alteration in the secondary structure of these three polypeptides occurred following isolation of the individual fragments. Use of a thermodynamic method would represent a much more suitable and informative manner in which to investigate the possibility of independent domains in a protein molecule. In the present study, differential heat capacity calorimetry has been used to obtain information concerning the structure of the prothrombin molecule and the alterations in conformation that occur upon metal ion binding.

Materials and Methods

Materials. Bovine blood was obtained from the Gentner Packing Co. (South Bend, IN) or Martin's Custom Butchering Co. Wakarusa, IN). Thrombin standard (21.7 units/mg) Lot B-3 was obtained from the Division of Biological Standards, National Institutes of Health, Bethesda, MD. Crude Russell's viper venom was purchased from Calbiochem (La Jolla, CA). Factor X deficient plasma and rabbit brain cephalin were purchased from Sigma Chemical Co. (St. Louis, MO). DEAE[1]-cellulose, QAE–Sephadex, and SP-Sephadex were also purchased from Sigma Chemical Co. Sephacryl S-200 was obtained from Pharmacia Inc. (Piscataway, NJ). *N*-2-Hydroxyethylpiperazene-*N'*-2-ethanesulfonic acid (Hepes) was purchased from Sigma and Chelex-100 from Bio-Rad Laboratories (Richmond, CA).

Proteins. Bovine prothrombin and factor X were isolated from fresh citrated plasma. The method utilized for their simultaneous isolation was essentially that of Bajaj & Mann (1973).

The factor X activating protein of Russell's viper venom (RVV-X) was purified as described by Furie et al. (1974) and insolubilized to Sepharose 4B, as previously described (Byrne & Castellino, 1978). Factor Xa was prepared by activation with insolubilized RVV-X (Strickland & Castellino, 1980). The specific activity, as determined by a one-stage clotting assay (Bachmann et al., 1958) was ~300 units of Xa mg^{-1}.

Thrombin was obtained from the activation of bovine prothrombin by a bovine factor Xa–Va–Ca–phospholipid system. A total of 35 mL of solution, containing 60 mg of bovine prothrombin, 1 mg of factor Xa, 300 units of factor Va, 0.24 μmol of (phospholipid) rabbit brain cephalin, and 200 μmol of Ca^{2+}, was incubated at room temperature. After 15 min, the activation was inhibited by the addition of 0.7 mL of 500 mM benzamidine hydrochloride (10 mM final). Thrombin was purified as described by Lundblad et al. (1976), and factor V was isolated as described by Nesheim et al. (1979a).

Preparation and Isolation of Prethrombin 1 and Fragment 1. Prethrombin 1 and prothrombin fragment 1 were obtained from the proteolytic digest of thrombin on bovine prothrombin. The methods for the proteolysis and subsequent isolation of these fragments were essentially as described by Heldebrant et al. (1973a). Further purification of fragment 1 was achieved by gel filtration on a Sephacryl S-200 column (2.5 × 80 cm) in a buffer system consisting of 50 mM Tris-HCl–0.2 M NaCl–1 mM benzamidine, pH 7.4, at a flow rate of 12 mL/h. $DodSO_4$ gel electrophoresis, in the presence and absence of β-mercaptoethanol, was utilized as a criterion of fragment purity. Extinction coefficients ($\epsilon_{1cm}^{1\%}$ at 280 nm) utilized were 16.4 (prethrombin 1) and 10.5 (fragment 1) (Mann, 1976).

Preparation and Isolation of Prethrombin 2 and Fragment 2. Prethrombin 2 and prothrombin fragment 2 were obtained from proteolytic digests of bovine factor Xa and prethrombin 1. The methods for the proteolysis and subsequent isolation of these fragments were essentially those described by Heldebrandt et al. (1973a). Further purification of prethrombin 2 was achieved by ion-exchange chromatography on QAE-Sephadex, as described by Owen et al. (1974). $DodSO_4$ gel electrophoresis was utilized as a criterion of purity of these fragments. Extinction coefficients, $\epsilon_{1cm}^{1\%}$ at 280 nm, utilized were 19.5 (prethrombin 2) and 12.5 (fragment 2) (Mann, 1976).

Differential Scanning Calorimetry. The MC-1 Scanning Calorimeter, purchased from Microcal (Amherst, MA), consists of twin platinum cells (sample and reference cells) of ~1.0-mL capacity and is similar to that described by Krishnan & Brandts (1978). The exact volume of the sample placed into the cells was determined by water weight analysis of an equivalent volume delivered by a calibrated microsyringe. Both the reference and sample were deaerated under reduced pressure for 10 min prior to their delivery into the cells. Equivalent volumes of sample and reference were placed in the cells and allowed to temperature equilibrate under vacuum. The calorimetric scan was obtained under a constant pressure of ~3.0 cmHg at 20 °C/h.

Protein concentrations ranging from 2 to 10 mg/mL were utilized for these studies. Metal-free protein solutions were passed over a Chelex-100 column and subsequently dialyzed against Chelex-treated buffer. The buffer system for these studies consisted of 50 mM Hepes–150 mM NaCl, pH 7.4. This buffer, in the absence or presence of metal ions, was the reference system.

The treatment of the calorimetric data was as described by Jackson & Brandts (1970). The calorimeter directly monitors the differential heat capacity (ΔC_p), in calories per degree, of the sample cell with respect to the reference cell, as a function of temperature. Absolute values of the heat flow were obtained by using a calibration pulse, which is a design feature of the instrument. The temperature at which the rate of heat flow reaches a maximum is the transition temperature, T_m, between the low-temperature and high-temperature conformational states of the protein.

[1] Abbreviations used: DEAE, diethylaminoethyl; Tris, 2-amino-2-(hydroxyethyl)-1,3-propanediol; CD, circular dichroism.

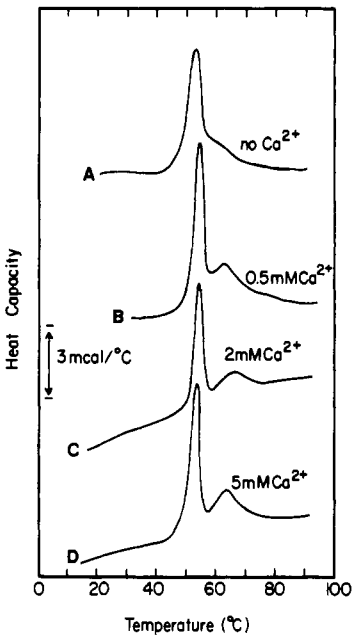

FIGURE 1: Thermal denaturation endotherms of bovine prothrombin in the presence and absence of Ca^{2+}. (A) Prothrombin (10.4 mg/mL) in the absence of Ca^{2+}. (B) Prothrombin (11.8 mg/mL) in the presence of 0.5 mM Ca^{2+}. (C) Prothrombin (7.92 mg/mL) in the presence of 2 mM Ca^{2+}. (D) Prothrombin (12.6 mg/mL) in the presence of 5 mM Ca^{2+}. In all cases, the heating rate was 20 °C/h.

The difference in enthalpy, ΔH, between the native and denatured states of the protein was obtained by integration of the peak as described by Jackson & Brandts (1970). Integration was performed by comparing the weight of the peak area and the precalibrated trace. The value of ΔH, so obtained, is expressed per mole of protein or peptide. In all cases, instrument base lines, obtained by using buffer solutions in each cell, when significant, were subtracted from the protein scans.

Calculation of the ratio of the calorimetric (ΔH_{cal}) to van't Hoff (ΔH_{vH}) enthalpies was accomplished by integration of the experimental heat capacity as described by Sturtevant (1974).

Atomic Absorption Analysis. The Ca^{2+} content of solutions of interest was analyzed with use of a Varian Techtron atomic absorption spectrometer, as previously described Byrne et al. (1980).

Results

When bovine prothrombin is heated in the differential scanning calorimeter, in the absence of added metal ions, at least two thermal denaturing transitions are observed. As can be seen in Figure 1, a major peak is obtained at 53.1 ± 1.5 °C and a distinct shoulder is noted at 58.2 ± 1.5 °C. The total ΔH of the prothrombin thermal transitions, obtained from integration of the total peak areas, is 324 ± 12 kcal mol^{-1}. The ΔH values, as well as the temperature midpoints, T_m, obtained for each peak, were independent of the scan rate, up to at least 40 °C/h, for bovine prothrombin and all other proteins and peptides studied herein, demonstrating that equilibrium conditions prevailed in all experiments. Also, all preparations of bovine prothrombin contained <0.1 mol of Ca^{2+}/mol of protein when examined by atomic absorption analysis. As can also be noted from Figure 1, progressive addition of Ca^{2+} to bovine prothrombin causes a concomitant shift in the shoulder region of the thermogram as well as a marked sharpening of the main endotherm. This effect is saturated at 5 mM Ca^{2+}, at which level two distinct peaks are obtained. In 5 mM Ca^{2+}, the main endotherm possesses a T_m of 53.5 ± 1.5 °C, a value unchanged from metal-free prothrombin. The higher temperature transition of prothrombin, in 5 mM Ca^{2+}, has a T_m of 64.8 ± 1.5 °C. The total ΔH of the bovine prothrombin thermal transitions, in 5 mM Ca^{2+}, is 280 ± 12 kcal mol^{-1}, a value significantly lower than that found for metal-free prothrombin. This suggests that the well-documented Ca^{2+}-mediated conformational alteration in bovine prothrombin is reflected in a lower ΔH between the native and thermally denatured states of the molecule.

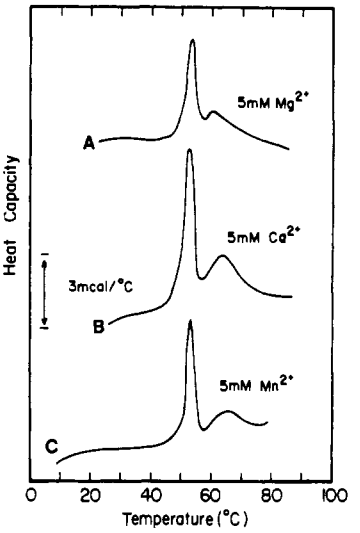

FIGURE 2: Thermal denaturation endotherms of bovine prothrombin in the presence of various divalent cations. (A) Prothrombin (6.43 mg/mL) in the presence of 5 mM Mg^{2+}. (B) Prothrombin (12.6 mg/mL) in the presence of 5 mM Ca^{2+}. (C) Prothrombin (6.15 mg/mL) in the presence of 5 mM Mn^{2+}. In all cases, the heating rate was 20 °C/h.

Table I: T_m and ΔH Values for Bovine Prothrombin in the Presence and Absence of Added Metal Ions

material studied	T_m (°C)	ΔH total[a] (kcal mol^{-1})
bovine prothrombin	53.1 ± 1.5	324 ± 12[b]
	58.2 ± 1.5	
bovine prothrombin + 5 mM Ca^{2+}	53.5 ± 1.5	280 ± 12
	64.8 ± 1.5	
bovine prothrombin + 5 mM Mg^{2+}	53.7 ± 1.5	271 ± 12
	63.2 ± 1.5	
bovine prothrombin + 5 mM Mn^{2+}	53.5 ± 1.5	280 ± 12
	65.0 ± 1.5	

[a] This value represents the total ΔH of all thermal transitions. [b] Error estimate is based on triplicate measurements in all cases.

Figure 2 illustrates DSC thermograms of bovine prothrombin in the presence of saturating levels of two other divalent metal ions, Mg^{2+} and Mn^{2+}. In each case, the T_m values obtained for both the low- and high-temperature endotherms, as well as the total ΔH of the transitions for bovine prothrombin, are nearly identical with those obtained for prothrombin with saturating levels of Ca^{2+}. Thus, all of the above metal ions appear to interact with a similar region of intact prothrombin. The above results are summarized in Table I.

The identification of the structural domains of bovine prothrombin, revealed by DSC, has been attempted by examination of isolated regions of the molecule. In this regard, DSC experiments have been performed with isolated fragment 1, prethrombin 1, fragment 2, and prethrombin 2, in the presence and absence of Ca^{2+}. DSC thermograms of prethrombin 1 and fragment 1 are shown in Figures 3 and 4.

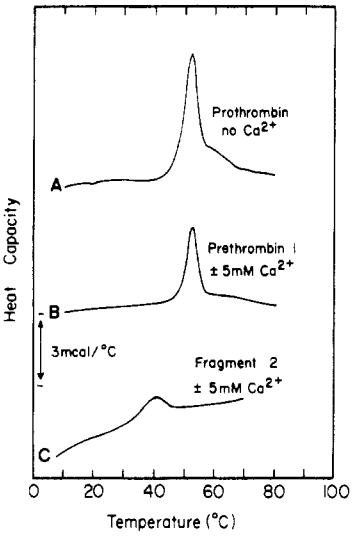

FIGURE 3: Thermal denaturation endotherms of bovine prothrombin activation intermediates. (A) Prothrombin (10.4 mg/mL) in the absence of metal ions. (B) Isolated prethrombin 1 (4.32 mg/mL) in the absence of Ca^{2+} or in the presence of 5 mM Ca^{2+}. (C) Prothrombin fragment 2 (3.74 mg/mL) in the absence of Ca^{2+} or in the presence of 5 mM Ca^{2+}. In all cases, the heating rate was 20 °C/h.

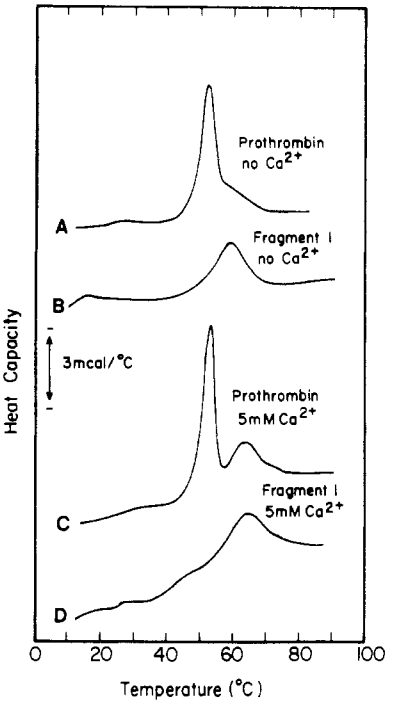

FIGURE 4: Thermal denaturation endotherms of bovine prothrombin and prothrombin fragment 1. (A) Prothrombin (10.4 mg/mL) in the absence of Ca^{2+}. (B) Prothrombin fragment 1 (4.63 mg/mL) in the absence of Ca^{2+}. (C) Prothrombin (12.6 mg/mL) in the presence of 5 mM Ca^{2+}. (D) Prothrombin fragment 1 (4.38 mg/mL) in the presence of 5 mM Ca^{2+}. In all cases, the heating rate was 20 °C/h.

Prethrombin 1 possesses a single endotherm (Figure 3) with a T_m of 52.6 ± 1.5 °C and a ΔH of 200 ± 10 kcal mol^{-1}, in the absence of added metal ions. Fragment 1 (Figure 4) possesses a T_m of 58.5 ± 1.5 °C and a ΔH of 134 ± 10 kcal mol^{-1}. The total ΔH for prethrombin 1 and fragment 1, isolated regions of prothrombin which represent its entire structure, is 334 kcal mol^{-1}, a value nearly identical with the total ΔH of the intact prothrombin transitions. Thus, it appears that, in the absence of metal ions, the prothrombin thermal transitions can be represented by the sum of those of fragment 1 and prethrombin 1. Also, it seems very likely that

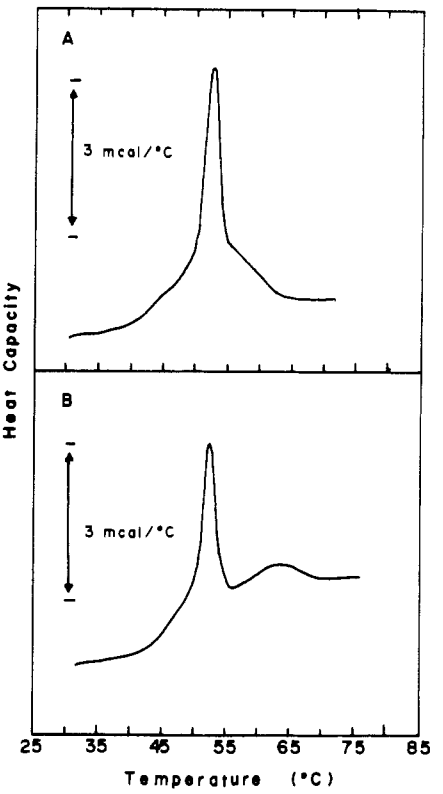

FIGURE 5: Thermal denaturation endotherms of an equimolar mixture of bovine prothrombin fragment 1 and bovine prethrombin 1. (A) Prothrombin fragment 1 (2.3 mg/mL) plus prethrombin 1 (5.0 mg/mL) in the absence of Ca^{2+}. (B) Prothrombin fragment 1 (2.2 mg/mL) plus prethrombin 1 (4.8 mg/mL) in the presence of 5 mM Ca^{2+}. The heating rate was 20 °C/h.

Table II: T_m and ΔH Values for Bovine Prothrombin Fragments in the Presence and Absence of Added Metal Ions

material studied	T_m (°C)	ΔH total (kcal mol^{-1})
prethrombin 1	52.6 ± 1.5	200 ± 10[a]
prethrombin 1 + 5 mM Ca^{2+}	52.4 ± 1.5	196 ± 10
fragment 1	58.5 ± 1.5	134 ± 10
fragment 1 + 5 mM Ca^{2+}	64.5 ± 1.5	102 ± 10
fragment 1 + 5 mM Mg^{2+}	63.5 ± 1.5	106 ± 10
fragment 1 + 5 mM Mn^{2+}	65.5 ± 1.5	106 ± 10
fragment 2	43.0 ± 1.5	36 ± 5
fragment 2 + 5 mM Ca^{2+}	43.5 ± 1.5	37 ± 5

[a] Error estimate is based on triplicate measurements in all cases.

the low-temperature transition seen for bovine prothrombin in Figure 1 can be identified as the prethrombin 1 region of the molecule and that the higher temperature shoulder can be identified as the fragment 1 region of the molecule. This latter point is further documented by analysis of the results of Figure 5. Here, a DSC thermogram is shown for an experiment in which an equimolar mixture of prethrombin 1 and fragment 1 was mixed and then placed in the calorimeter. In Figure 5A, in the absence of Ca^{2+}, it is clear that the thermogram of the mixture is virtually identical with that of intact prothrombin, except for the presence of a leading shoulder not seen in intact prothrombin.

In the presence of 5 mM (saturating) Ca^{2+}, the DSC thermogram of isolated prethrombin 1 (Figure 3) is essentially unchanged from that obtained in the absence of Ca^{2+} (Figure 3). As is summarized in Table II, a T_m of 52.4 ± 1.5 °C and a ΔH of 196 ± 10 kcal mol^{-1} is obtained for the single prethrombin 1 endotherm, in the presence of 5 mM Ca^{2+}. On the other hand, Figure 4 shows that the T_m of the isolated fragment 1 transition is shifted from 58.5 ± 1.5 °C, in the

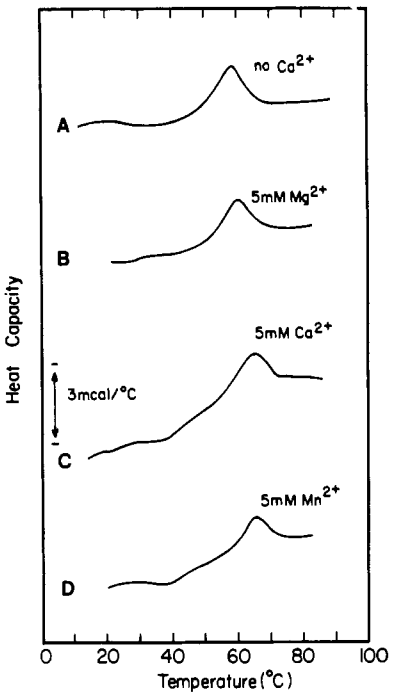

FIGURE 6: Thermal denaturation endotherms of bovine prothrombin fragment 1 in the presence of various divalent cations. (A) Prothrombin fragment 1 (4.63 mg/mL) in the absence of metal ions. (B) Prothrombin fragment 1 (3.60 mg/mL) in the presence of 5 mM Mg^{2+}. (C) Prothrombin fragment 1 (4.38 mg/mL) in the presence of 5 mM Ca^{2+}. (D) Prothrombin fragment 1 (3.45 mg/mL) in the presence of 5 mM Mn^{2+}. In all cases, the heating rate was 20 °C/h.

metal-free fragment, to 64.5 ± 1.5 °C, in the presence of 5 mM Ca^{2+}. This temperature shift is identical with the shift that occurs upon addition of 5 mM Ca^{2+} to intact prothrombin. As is summarized in Table II, the isolated fragment 1 transition of 134 ± 10 kcal mol^{-1} is reduced to 102 ± 10 kcal mol^{-1} in the presence of 5 mM Ca^{2+}. Thus, the T_m values and the total ΔH of 280 ± 12 kcal mol^{-1} of Ca^{2+}-bound prothrombin are completely represented by the T_m values and the total ΔH of 298 kcal mol^{-1} of isolated prethrombin 1 and fragment 1. This result is fortified by the data of Figure 5B, in which the thermogram of an equimolar mixture of prethrombin 1 and fragment 1, in the presence of 5 mM Ca^{2+}, appears virtually identical with that of intact prothrombin, in the presence of 5 mM Ca^{2+}. As can be observed from Figure 6, other metal ions, such as Mg^{2+} and Mn^{2+}, appear to interact with isolated fragment 1. This interaction results in a shift of the T_m to a higher temperature, in a fashion similar to that of Ca^{2+}. Also, as listed in Table II, all the above metal ions reduce the ΔH of the thermal denaturation of isolated fragment 1, similar to the changes produced by the interaction of Ca^{2+} with isolated fragment 1.

Prethrombin 1 has been digested, by factor Xa, into two other relevant peptides, fragment 2 and prethrombin 2. We have examined the DSC thermograms of each of these peptides in order to assess whether these regions also existed as separate domains in the prethrombin 1 (and therefore, prothrombin) molecule. The DSC thermogram of fragment 2 is illustrated in Figure 3. The T_m of 43.0 ± 1.5 °C is representative of a peak which is not observable in native prothrombin or in prethrombin 1. As is noted from the data of Table II, the ΔH of the thermal transition is 36 ± 5 kcal mol^{-1}. In addition, neither the T_m nor the ΔH of fragment 2 is altered by the presence of Ca^{2+}. When prethrombin 2 was analyzed in a similar fashion, no observable heat changes occurred over a temperature range of 10–95 °C. In order to ascertain that this lack of an observable heat change was not due to prethrombin 2 being previously denatured during purification, we have analyzed the secondary structure of prethrombin 2 by circular dichroism (CD). The spectrum obtained was virtually identical with that recently published by Bloom & Mann (1979). Our results suggest that prethrombin 2 contains 15–20% α helix and 80–85% random structure. Therefore, the lack of a perceptible denaturation enthalpy change may be related to the high degree of disorder already present in the molecule.

Discussion

The presence of independent structural domains in protein molecules has been previously established. For example, the results of DSC experiments were consistent with the proposal that fibrinogen contained a three-nodular structure and that fibrinogen fragments D and E do not interact in the native molecule in a fashion which affects their denaturation behavior, when studied independently (Donovan & Mihalyi, 1974). In addition, Cathou et al. (1968) found that the Fab and Fc fragments of rabbit antidinitrophenyl antibody likely exist as independent domains.

During activation to thrombin, several important peptide fragments are released from the prothrombin molecule, and these purified fragments have been utilized to assess their individual contributions to several physicochemical aspects of the intact prothrombin molecule. For example, on the basis of examination of the mode of Ca^{2+} binding to bovine prothrombin, prothrombin fragments 1 and 2, and prethrombin 1 and 2, a model has evolved suggesting that the strong Ca^{2+} binding sites in bovine prothrombin reside in the fragment 1 portion of the molecule and that the weak class of Ca^{2+} binding sites of bovine prothrombin reside in the fragment 2 portion of the molecule (Bajaj et al., 1975). Further studies with the isolated fragments have led to other well-accepted conclusions: the fragment 1 region of bovine prothrombin is responsible for its phospholipid binding properties (Bajaj et al., 1975; Nelsestuen et al., 1975); the fragment 2 portion of bovine prothrombin is responsible for factor Va function (Esmon & Jackson, 1974b; Bajaj et al., 1975). In these, and several other similar studies, it has been tacitly assumed that the behavior of the isolated fragments regarding these properties can be extrapolated to the role of these regions in the intact molecule where their conformations can be quite different. In an attempt to resolve this problem, it has recently been observed that the CD spectra of each of these isolated fragments, when added, resemble the CD spectrum of intact prothrombin (Bloom & Mann, 1979). Thus, it has been proposed that fragment 1, fragment 2, and prethrombin 2 exist as separate domains in the prothrombin molecule.

We felt that CD, while valuable as an approach, is not well suited to the solution of questions of this nature. A major problem involves the relatively low sensitivity of CD to the subtle types of conformational alterations that can occur (and drastically influence the results) upon release of the fragments from the intact molecule. Indeed, it could be anticipated that large conformational alterations would not occur upon fragment release. We felt that another means of addressing this important question was necessary and chose a thermodynamic tool, DSC, for this purpose. The parameters obtained by DSC are, by their nature, additive properties and are best suited to the problem at hand.

In the absence of metal ions, the DSC profile of bovine prothrombin (Figure 1) indicates that at least two endothermic transitions exist. On the basis of the data of Figures 3, 4, and 5, it appears as though the low-temperature prothrombin en-

dotherm is associated with the prethrombin 1 region of the molecule and that the high-temperature endotherm is essentially the fragment 1 region of the molecule. The fact that the sum of the ΔH of the thermal transitions of isolated fragment 1 and prethrombin 1 is approximately equal to the total ΔH for the thermal transitions of prothrombin and the fact that the T_m values of these isolated regions of the prothrombin molecule are observable in intact prothrombin lends strong support to the idea that the fragment 1 and prethrombin 1 regions of intact prothrombin exist as separate domains. However, when prethrombin 1 is digested into fragment 2 and prethrombin 2, results from DSC thermograms of these isolated regions lead to the conclusion that the fragments interact cooperatively in the prethrombin 1 and prothrombin molecule. The fragment 2 thermogram (Figure 3) indicates the presence of a single endothermic transition, with a T_m of 43 °C. This transition is not readily observed in the intact prothrombin or in the prethrombin 1 thermograms. Further, as previously indicated, no thermal transition could be observed in the temperature range of interest for prethrombin 2. Therefore, the total ΔH of the thermal transition of prethrombin 2 and fragment 2 is reflected solely by the fragment 2 contribution (Table II). The ΔH so obtained is much lower than that observed for prethrombin 1. This strongly suggests that prethrombin 1 exists in a conformation which relies on the cooperative interaction of the component regions, fragment 2 and prethrombin 2. Our observation may not be surprising in that it has been previously demonstrated that a strong noncovalent interaction exists between fragment 2 and prethrombin 2 (Myrmel et al., 1976).

When Ca^{2+} is added to bovine prothrombin (Figure 1), the fragment 1 endotherm is shifted to a higher T_m and the prethrombin 1 region is markedly sharpened when compared to the thermogram obtained in the absence of Ca^{2+}. These results are also found for divalent cations, such as Mn^{2+} and Mg^{2+}. Thus, it appears as though interaction of these metal ions with the fragment 1 region of prothrombin resulted in a stabilization of this domain of the molecule to thermal denaturation. The total ΔH of the thermal transitions of bovine prothrombin is lowered as a consequence of interaction with these cations (Table I). This may indicate that a conformational alteration takes place in bovine prothrombin upon interaction with certain cations, a conclusion which has been forwarded as a result of the cooperative binding isotherms previously observed for Ca^{2+} and prothrombin (Bajaj et al., 1975) and from the intrinsic fluorescence alterations which occur in prothrombin when it is bound to metal ions (Prendergast & Mann, 1977). When studying the isolated fragments, we find that the T_m and ΔH of the thermogram of isolated prethrombin 1 (Figure 3) are not changed upon addition of Ca^{2+}, although Ca^{2+} does interact with isolated prethrombin 1 (Bajaj et al., 1975), whereas the T_m of the isolated fragment 1 thermogram (Figure 4) is elevated to the same value found for this region of intact prothrombin, in the presence of Ca^{2+} (Figure 4) or other divalent cations (Figure 6). Also, the ΔH of the isolated fragment 1 transition is lowered upon addition of Ca^{2+}, Mn^{2+}, and Mg^{2+} (Table II), suggesting that a conformational alteration has occurred in this region in the presence of divalent metal ions. This conclusion is also supported by the observation that the binding of Ca^{2+} to fragment 1 displays cooperativity (Bajaj et al., 1975).

The question as to whether any interaction exists between the prethrombin 1 and fragment 1 domains in native prothrombin can be addressed. The fact that the ΔH and T_m values of isolated fragment 1 and prethrombin 1, in the presence and absence of Ca^{2+}, can be summed to produce the native prothrombin thermogram suggests that the domains do not interact. However, evaluation of the $\Delta H_{cal}/\Delta H_{vH}$ ratios leads to a different conclusion. For isolated prethrombin 1 and fragment 1, in the presence or absence of Ca^{2+}, this ratio is $\sim$1.0. On the other hand, the $\Delta H_{cal}/\Delta H_{vH}$ ratio for the prethrombin 1 domain of bovine prothrombin is 2.3 in the absence of Ca^{2+} and 1.6 in the presence of 5 mM Ca^{2+}. This suggests that the presence of the fragment 1 domain in native prothrombin affects thermal denaturation characteristics of prethrombin 1 and that this effect is markedly reduced in the presence of Ca^{2+}. Thus, interaction between the two domain regions may, in fact, occur. Due to the complex nature of the prothrombin thermograms, however, it is not prudent to attempt further elaboration of this point.

In conclusion, we have presented strong evidence for the existence of separate domains of fragment 1 and prethrombin 1 in the intact prothrombin structure. Fragment 2, however, appears to interact cooperatively with the prethrombin 2 structural region of intact prothrombin. We have also, for the first time, directly shown that Ca^{2+} interacts with the fragment 1 region of native intact prothrombin and appears to alter the conformation of this domain of the intact molecule.

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Purification and Biochemical Study of Viriditoxin, Tissue Damaging Toxin, from Prairie Rattlesnake Venom†

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ABSTRACT: Rattlesnake poisoning frequently produces a severe tissue damaging effect including myonecrosis and hemorrhage. In this investigation, viriditoxin was isolated from the venom of the prairie rattlesnake (*Crotalus viridis viridis*), and its chemical properties and biological activities associated with tissue damage were investigated. The toxin has a molecular weight of ~115 000 with an isoelectric point of 4.8. Amino acid composition analyses indicate that the toxin consists of ~1018 residues with high content of acidic amino acid residues. Circular dichroism was used to examine the conformation of viriditoxin. Although there is some indication of a high β-sheet content, it is not possible to identify the exact conformation due to the anomalous CD spectra. Viriditoxin has both myotoxic and hemorrhagic activities. High serum creatine kinase activity of mice injected with the toxin substantiates the destructive action of viriditoxin on muscle. Viriditoxin has an LD_{50} value of ~5.0 μg/g in mice indicating the protein is also moderately lethal in addition to its tissue damaging effect.

Rattlesnake bites in the United States, although rarely fatal, induce considerable damage to the tissues surrounding the site of envenomation. The most common of these local tissue effects are myonecrosis and hemorrhage. Since antivenin treatments may not prevent myonecrosis and hemorrhage unless treatment is administered immediately following envenomation (Minton, 1954; Stahnke et al., 1957), these effects remain a source of serious clinical concern. A number of toxins which induce hermorrhage, myonecrosis, or both have been isolated from pit viper (Crotalidae) and viper (Viperidae) venoms.

Five hemorrhagic toxins, which contain zinc, have been isolated from western diamondback rattlesnake (*Crotalus atrox*) venom (Bjarnason & Tu, 1978). One of these toxins, hemorrhagic toxin *b*, has been found to also induce myonecrosis in addition to its hemorrhagic activity (Ownby et al., 1978). Myotoxin *a*, a nonhemorrhagic muscle degenerating factor, was isolated from prairie rattlesnake (*Crotalus viridis viridis*) venom (Cameron & Tu, 1977). Myotoxin *a* has 42 amino acid residues, and its complete amino acid sequence and the position of the disulfide bonds have been identified (Fox et al., 1979). Despite myotoxin *a*'s small size, it is antigenic and an antiserum capable of neutralizing its myotoxic action has recently been produced (Ownby et al., 1979).

During the course of the isolation of myotoxin *a*, Cameron & Tu (1977) observed that a high molecular weight venom fraction (which eluted in the void volume of Sephadex G-50) induced considerable tissue damage following injection into experimental lab animals. A toxin has been purified from the venom of the prairie rattlesnake (*C. viridis viridis*) in order to study the specific high molecular weight toxin responsible for this tissue damage. This toxin has been designated viriditoxin. This study of viriditoxin is intended to provide for a more complete understanding of venom-induced tissue damage and of the overall toxic action of rattlesnake venoms.

In the present investigation, it has been shown that viriditoxin is a high molecular weight toxin with a molecular weight of approximately ~115 000. Presented in this report are some of the chemical properties of viriditoxin, such as amino acid composition, isoelectric point, secondary structure, and molecular weight. Several biological actions of viriditoxin are also discussed: myotoxicity, hemorrhagic activity, hemolytic activity, proteolytic activity, and the effect upon serum creatine kinase levels.

Materials and Methods

Crude venom was purchased as a lyophilized powder from Miami Serpentarium Laboratories. DEAE[1] Bio-Gel A was purchased from Bio-Rad Laboratories. Sephadex G-150-40, Sephadex G-150-120, insulin, Temed, and creatine kinase diagnostic kit 40-UV were purchased from Sigma Chemical Co. Spectrophor dialysis tubing was purchased from Fisher Chemical Co. Ampholytes over the pH ranges of 3.5-10 and

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[1] Abbreviations used: DEAE, diethylaminoethylagarose; Tris, tris(hydroxymethyl)aminomethane; Temed, *N,N,N',N'*-tetramethylethylenediamine; Bis, *N,N'*-methylenebis(acrylamide); Cl_3CCOOH, trichloroacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; ADP, adenine diphosphate; NADH, reduced nicotinamide adenine dinucleotide; LD_{50}, lethal dose for 50%; CD, circular dichroism; $[\theta]_{mrw}$, mean residue ellipticity; $[\theta]_M$, molar ellipticity.

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