The Nature of the Slow Metal Ion-Dependent Conformational Transition in Bovine Prothrombin

By Henry C. MARSH,* Mary E. SCOTT,* Richard G. HISKEY* and Karl A. KOEHLER†

*Department of Chemistry, University of North Carolina at Chapel Hill, Chapel Hill, NC 27514, U.S.A., and †Department of Pathology, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27514, U.S.A.

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Kinetic parameters characterizing the slow structural isomerization observed via metal ion-dependent intrinsic fluorescence quenching of bovine prothrombin Fragment 1 have been determined. From forward and reverse rate constants, an equilibrium constant of approx. 0.25 is calculated. This result is consistent with the hypothesis that there exists, in the absence of metal ions, an equilibrium between two forms of bovine Fragment 1, one of which can interact rapidly with Ca^{2+} and subsequently with phospholipid. The other form of Fragment 1 cannot interact with Ca^{2+} in a manner that yields a phospholipid-binding form of the protein. Interconversion of these two forms of Fragment 1 occurs and may involve the isomerization of a proline residue.

In 1976, Nelsestuen reported the fluorescence behaviour of bovine prothrombin Fragment 1 in the presence of Ca^{2+} (Nelsestuen, 1976). At equilibrium, Fragment 1 fluorescence is 40 % quenched in the presence of Ca^{2+} at neutral pH. As a function of time, after addition of Ca^{2+} , it was observed that, after an initial rapid fluorescence decrease of 25 % of the total equilibrium quenching, a relatively slow first-order decay of fluorescence to its equilibrium value occurs. The magnitude of the 25 % fast fluorescence decrease is unaffected by the Ca^{2+} concentration. At 0°C the rate constant, k_{obs} , for this process is approx. 0.007 min^{-1} (calculated from the reported half-time). The k_{obs} is independent of the Ca^{2+} concentration over the range 0.2–20 mM- CaCl_2 (the concentration at half-maximal quenching is 0.4 mM). An Arrhenius plot yields an activation energy, E_a , of 87.9 kJ (21 kcal)/mol for this slow-fluorescence process.

Nelsestuen (1976) further demonstrated that the phospholipid-binding behaviour of bovine prothrombin and Fragment 1 paralleled the observed fluorescence behaviour of fragment 1. Immediately on addition of Ca^{2+} , approx. 25 % of the Fragment 1 binds to phospholipid; the remaining protein bound to phospholipid in a manner described by precisely the same kinetic and thermodynamic parameters as those that described slow Fragment 1 fluorescence quenching by Ca^{2+} .

The rate of this presumed conformational relaxation, identified by slow fluorescence quenching,

Abbreviations used: Fragment 1, N-terminal portion of prothrombin released on treatment of prothrombin with thrombin; SDS, sodium dodecyl sulphate.

was independent of the nature of the group IIA metal ion that triggers the transition. The k_{obs} and E_a for the slow process, determined in the presence of Mg^{2+} , Ca^{2+} , Sr^{2+} and Ba^{2+} , were all identical within the probable experimental error (Nelsestuen *et al.*, 1976). Nelsestuen (1977) has hypothesized that this clearly biphasic fluorescence rate behaviour is due either to two sequential protein transitions or to the presence of two populations of Fragment 1 or prothrombin molecules.

Prendergast & Mann (1977) confirmed the observations of Nelsestuen (1976) with bovine Fragment 1 and then extended them in several important regards; they presented a detailed model based on the premise of a sequential transitional process. The fast fluorescence-quenching process was postulated to be an initial conformational response of Fragment 1 to the electrostatic co-ordination of Ca^{2+} . Subsequent slow conformational adjustments are reflected in the slow transition that was postulated to be necessary for phospholipid binding.

In the present paper we provide data supporting the hypothesis of Nelsestuen (1977), namely that, at equilibrium in the absence of Ca^{2+} , two species of bovine prothrombin exist. We further demonstrate that these populations are in equilibrium with one another and that the rates of their interconversion are consistent with the kinetic and equilibrium data discussed above.

Experimental

EDTA (disodium salt) and KCl were Fisher Certified grade (Fisher Scientific Co., Fair Lawn,

NJ 07410, U.S.A.). Tris was Ultrol grade from Calbiochem, La Jolla, CA 92037, U.S.A. CaCl_2 was Ultrapure grade from Alfa Products, Danvers, MA 01923, U.S.A. All water used was distilled and deionized.

Bovine prothrombin Fragment 1 was prepared by the method of Mann (1976) and stored in 50 % (v/v) glycerol at -20°C . The Fragment 1 obtained was single-banded by SDS/polyacrylamide-gel electrophoresis and yielded satisfactory amino acid compositions after either acid or base hydrolyses (γ -carboxyglutamic acid determination). Fragment 1 was prepared for fluorescence experiments as follows. An initial dialysis against 0.01 M-EDTA/0.05 M-Tris/0.1 M-KCl, pH 7.50, was followed by exhaustive dialysis against 0.05 M-Tris/0.1 M-KCl, pH 7.5 (the buffer for all runs reported here). The Fragment 1 solution was centrifuged immediately after dialysis.

pH values were obtained by using a Radiometer type-PHM 26c pH-meter equipped with a Radiometer GK2322c semimicro combination pH-electrode. Protein absorbance at 290 nm was measured on a Unicam SP.1800 spectrophotometer. Protein samples were diluted in buffer in order to obtain a final A_{290} of 0.05.

Fluorescence measurements were made on an Hitachi/Perkin-Elmer MPF-2A fluorescence spectrophotometer equipped with a thermostatically controlled compartment and strip chart recorder. The sample compartment was purged with dry argon during the low-temperature runs. An excitation wavelength of 290 nm and an emission wavelength of 340 nm were used. Runs were carried out at 5.0, 10.0, 15.0 and 20.0°C .

Single and double Ca^{2+} -addition runs were organized as follows.

Single addition: to buffered Fragment 1 samples, equilibrated at one of the four temperatures specified above, concentrated buffered CaCl_2 solution was added at zero time and the time-dependence of the observed Fragment 1 fluorescence decrease recorded. The final Ca^{2+} concentration in each experiment was 1.8 mM.

Double addition: buffered Fragment 1 samples containing 1.8 mM- CaCl_2 were equilibrated at 25°C for at least 1 h. These samples were then re-equilibrated at one of the four specified temperatures. The fluorescence intensity of the Fragment 1 Ca^{2+} sample was measured, and, at zero time, sufficient concentrated buffered EDTA was added to make the final total EDTA concentration 2.7 mM. On addition of EDTA the Fragment 1 fluorescence intensity increased rapidly to its original value. At a time interval, Δt , after addition of EDTA, CaCl_2 was again added such that the added total CaCl_2 concentration was 9.4 mM. Negligible volume changes accompanied these additions. Immediately after the second addition of CaCl_2 , the observed fluorescence intensity decreased

rapidly, followed by a much slower first-order decrease in intensity. The amount of the fast initial fluorescence decrease was the parameter of interest. A fresh Fragment 1 sample was used for each Δt value. In the presence of added Ca^{2+} , the equilibrium Fragment 1 fluorescence was approx. 55 % quenched. This value is somewhat higher than values reported from this (Scott *et al.*, 1979) and other (Nelsestuen, 1976; Prendergast & Mann, 1977) laboratories and may be due to our centrifuging the sample before the fluorescence experiments to decrease scattering.

The single- Ca^{2+} -addition method yields a first-order rate constant, $k_{\text{obs}}^{\text{fwd}}$ (min^{-1}), at each temperature, characterizing the rate of quenching of Fragment 1 fluorescence on addition of Ca^{2+} . The double- Ca^{2+} -addition method yields a first-order rate constant, $k_{\text{obs}}^{\text{rev}}$ (min^{-1}), at each temperature, characterizing the rate of recovery of the initial metal ion-free state of Fragment 1 after removal of Ca^{2+} . $k_{\text{obs}}^{\text{fwd}}$ values were obtained by treating the data by the method of Guggenheim (Frost & Pearson, 1965) at each of four temperatures. $k_{\text{obs}}^{\text{rev}}$ values were determined from plots of $\ln(\% \text{ state B})$ against time (min), where B is that form of Fragment 1 rapidly quenched by addition of Ca^{2+} . Kinetic data was regressed with statistical-analysis program SAS (A. J. Barr, J. H. Goodnight, J. P. Sall and J. T. Helwing, SAS Institute, PO Box 10066, Raleigh, NC 27605, U.S.A.).

Results and Discussion

The results reported here and the data of Nelsestuen (1976, 1977) and of Prendergast & Mann (1977) are consistent with Scheme 1:

Scheme 1

where A and B are conformational isomers of bovine prothrombin Fragment 1 in the absence of Ca^{2+} . Ca^{2+} interacts only with the B-isomer to produce the conformation *in vivo*, C. In conformational state C, intrinsic protein fluorescence is substantially quenched. Conformation C is induced or stabilized by Ca^{2+} ; this form of the protein is capable of binding to a phospholipid surface.

In view of the absence of a rate-dependence on the Ca^{2+} concentration (Nelsestuen, 1976), the first step of the process illustrated in Scheme 1 ($\text{A} \rightarrow \text{B}$) is rate-limiting. Further supporting this contention are our preliminary stopped-flow studies, which indicate that the half-time for the rapid initial fluorescence-quenching process (conversion of B into C, Scheme 1) is less than 10 ms at 10°C .

On addition of Ca^{2+} to metal ion-free bovine prothrombin Fragment 1, the slow first-order decay of intrinsic protein fluorescence yields k_{+1} (Nelsestuen, 1976). The rate-limiting isomerization of A to B in the presence of Ca^{2+} is thus observed directly as quenching of fluorescence due to the high rate of conversion of B into C by Ca^{2+} . The immediate decrease of approx. 20 % of the total fluorescence change corresponds to quenching arising from the equilibrium concentration of state B in the absence of Ca^{2+} . The rate of this initial quenching process is considerably more rapid than the process under investigation here, and is thus not reflected in the determination of the forward rate constant.

The method utilized for the determination of the reverse-isomerization rate constant, $k_{\text{obs}}^{\text{rev}}$ ($k_{\text{obs}}^{\text{rev}} = k_{+1} + k_{-1}$) presumes that the concentration of the B-state is reflected in the magnitude of the immediate decrease in fluorescence on addition of Ca^{2+} to Fragment 1. Thus Fragment 1 is converted into state C by equilibration at room temperature in the presence of Ca^{2+} . At zero time Ca^{2+} ions are effectively removed by the addition of an excess of EDTA and the protein rapidly attains state B. The rate of loss of state B (isomerization to state A) can be monitored by making a second addition of Ca^{2+} at various times, Δt , after addition of EDTA and measuring the resulting immediate decrease in fluorescence intensity. Thus:

$$\% \text{ State B} = \frac{[I_{\text{EDTA}} - I_{\text{Ca}^{2+}}(\Delta t)] \cdot 100}{(I_{\text{EDTA}} - I_{\text{Ca}^{2+}})}$$

(Scheme 2)

where $I_{\text{Ca}^{2+}}$ is the fluorescence intensity of Fragment 1 equilibrated with Ca^{2+} , I_{EDTA} is the fluorescence intensity of Fragment 1 after removal of Ca^{2+} by EDTA (added at zero time), and $I_{\text{Ca}^{2+}}(\Delta t)$ is the fluorescence intensity of Fragment 1 on a second addition of Ca^{2+} at time Δt .

Fig. 1 shows the dependence of the disappearance of isomer B on the delay time between addition of EDTA to a Ca^{2+} -containing Fragment 1 sample and re-addition of Ca^{2+} . Thus at 20°C it is evident that, after 10 min in the absence of significant concentrations of free metal ions, an equilibrium distribution of the A- and B-isomers of Fragment 1 exists consisting of 20 % of isomer B and 80 % of isomer A. After longer periods of time (30 min) the equilibrium percentage of isomer B appears to increase slightly with temperature.

The rate of disappearance of isomer B with time closely follows first-order kinetics and yields observed first-order rate constants, $k_{\text{obs}}^{\text{rev}}$, which are collected in Table 1. An Arrhenius plot can be constructed from the temperature-dependence of $k_{\text{obs}}^{\text{rev}}$ ($= k_{+1} + k_{-1}$) (insert to Fig. 1; Hammes, 1978). Table 1 contains a compilation of determined and calculated

Fig. 1. Dependence of the disappearance of Fragment 1 isomer B on the delay time between addition of EDTA to a Ca^{2+} -containing Fragment 1 sample and re-addition of Ca^{2+} .

Runs were carried out at: ●, 5.0°C; ○, 10.0°C; ■, 15.0°C and □, 20.0°C. The insert is an Arrhenius plot constructed from the rate constants, $k_{\text{obs}}^{\text{rev}}$ (min^{-1}), characterizing the curves drawn in this Figure and the corresponding temperatures. The line drawn represents a linear least-squares fit to the data. State B is that form of bovine Fragment 1 whose intrinsic fluorescence is rapidly quenched on addition of Ca^{2+} .

rate constants as well as calculated equilibrium constants (defined as $K_{\text{eq}} = \text{B/A}$) for the slow Fragment 1 fluorescence transition. The equilibrium constants reported were obtained in three distinct ways. First, via the kinetic and thermodynamic definitions of equilibrium, it is a useful approximation that $K_{\text{eq}} = k_{+1}/k_{-1}$. Second, the immediate fast decrease in Fragment 1 fluorescence intensity observed at the beginning of $k_{\text{obs}}^{\text{wd}}$ determinations yields the zero-time distribution of isomers A and B and, hence, $K_{\text{eq}}^{\text{wd}}$. Third, the measurement of $k_{\text{obs}}^{\text{rev}}$, which involves observation of the approach to equilibrium from an initially high concentration of isomer B, yields $K_{\text{eq}}^{\text{rev}}$. Within the probable experimental error of these methods, approx. 20–25 % of Fragment 1 is present as the B-isomer and the remainder as the A-isomer at equilibrium. The equilibrium constants in Table 1 may show some temperature-dependence.

Since $k_{+1} = k_{\text{obs}}^{\text{rev}} - k_{\text{obs}}^{\text{wd}}$, it is possible to calculate k_{-1} values at each temperature and construct an Arrhenius plot. Such a plot (not shown) yields an E_a value of 82.6 kJ/mol (S.D. = 0.12; correlation coefficient, $r = 0.993$). Fig. 2 shows the Arrhenius plot for k_{+1} , which yields an E_a value of 84.2 kJ/mol (S.D. = 0.12, $r = 0.992$). The Arrhenius plot shown as an insert to Fig. 1 for $k_{\text{obs}}^{\text{rev}}$, which equals $k_{+1} + k_{-1}$, yields an E_a value of 82.4 kJ/mol (S.D. = 0.084, $r = 0.997$). From the intercepts of the Arrhenius plots involving k_{+1} and k_{-1} , an entropy of activation of

Table 1. Kinetic and equilibrium parameters characterizing the slow metal ion-induced conformational process in bovine prothrombin Fragment 1

$T(^{\circ}\text{C})$	$k_{\text{obs.}}^{\text{fwd.}} (\text{min}^{-1})^*$	$k_{\text{obs.}}^{\text{rev.}} (\text{min}^{-1})^{\dagger}$	$k_{-1}^{\text{calc.}} (\text{min}^{-1})^{\ddagger}$	
5	0.023 (0.0006)	0.10 (0.0011)	0.077	
10	0.038 (0.0003)	0.22 (0.0050)	0.182	
15	0.067 (0.0022)	0.36 (0.0174)	0.293	
20	0.15 (0.0167)	0.66 (0.0494)	0.510	
$T(^{\circ}\text{C})$	$K_{\text{eq.}}^{\text{calc.}} \S$	$K_{\text{eq.}}^{\text{rev.}} \P$	$K_{\text{eq.}}^{\text{fwd.}} \P$	$\Delta G (\text{kJ/mol}) \parallel$
5	0.30	0.36	0.20	2.88
10	0.21	0.35	0.18	3.31
15	0.23	0.33	0.18	3.39
20	0.29	0.24	0.14	3.67

* $k_{\text{obs.}}^{\text{fwd.}} = k_{+1}$, first-order rate constant for the forward reaction, conversion of A into B (Scheme 1). Calculated by the method of Guggenheim (Frost & Pearson, 1965). Values in parentheses indicate the s.e. of the estimate. Correlation coefficients characterizing the linear regressions were all 0.99 or greater.

$\dagger k_{\text{obs.}}^{\text{rev.}} = k_{+1} + k_{-1}$, observed first-order rate constant for the reverse reaction, conversion of B into A (Scheme 1). Infinite-time values were obtained by checking fluorescence intensity at an estimated ten half-times and at appropriate intervals thereafter until the fluorescence intensity remained constant. Values in parentheses indicate the s.e. of the estimate. Correlation coefficients characterizing the linear regressions were all 0.98 or greater.

$\ddagger k_{-1}^{\text{calc.}} = k_{\text{obs.}}^{\text{rev.}} - k_{\text{obs.}}^{\text{fwd.}}$

$\S K_{\text{eq.}}^{\text{calc.}} = k_{+1}/k_{-1}$.

$\P$ Calculated by assuming that only the B-form of bovine Fragment 1 is rapidly quenched by added Ca^{2+} . Thus $\% \text{ A} = (\text{T} - \text{B})/\text{T}$, where T = the total Fragment 1 fluorescence quenching due to addition of Ca^{2+} , B = the magnitude of the fast fluorescence quenching immediately after addition of Ca^{2+} (determined by extrapolation of fluorescence-intensity-against-time plots to zero time). T was calculated from k_{+1} or by direct observation of approach to equilibrium for the conversion of B into A. See the text for further details.

$\parallel \Delta G = -RT \ln K_{\text{eq.}}$. The three $K_{\text{eq.}}$ values at each temperature were averaged.

8.21 kJ (1.96 kcal)/mol per K is calculated for the $\text{A} \rightleftharpoons \text{B}$ interconversion. The activation energy determined here for the $\text{A} \rightarrow \text{B}$ conversion is in good agreement with the value reported by Nelsestuen (1976). Although we are able to utilize Ca^{2+} in this system to measure the distribution of isomers A and B, the values reported reflect the behaviour of the system in the absence of metal ions.

The present observations and those of Nelsestuen (1976, 1977) and of Prendergast & Mann (1977) are consistent with the hypothesis that Fragment 1 exists as an equilibrium mixture of two conformations (A, B) and that Ca^{2+} binding converts conformation B into a species (conformation C) that can bind phospholipid. This view is strengthened by the observation that immediately on addition of Ca^{2+} ,

Fig. 2. Arrhenius plot constructed from the rate constants $[k_{+1} (\text{min}^{-1})]$ obtained at 5, 10, 15 and 20°C

The line drawn represents a linear least-squares fit to the data. $E_a = 84.2 \text{ kJ/mol}$.

approx. 25 % of Fragment 1 binds to phospholipid (Nelsestuen, 1976), the remainder binding in a manner involving the slow process previously described.

We suggest that the trigger for the $\text{A} \rightleftharpoons \text{B}$ interconversion involves proline-22 and the cystine loop (residues 18–23). The rates, equilibria and activation parameters for the *trans* $\rightarrow$ *cis* isomerization of proline residues in model peptides (Cheng & Bovey, 1977; Lin & Brandts, 1979) and proteins (Brandts *et al.*, 1975; Lin & Brandts, 1978; Bächinger *et al.*, 1978) are consistent with the data obtained for the $\text{A} \rightleftharpoons \text{B}$ conversion in Fragment 1. In the absence of metal ions, bovine Fragment 1 exists at equilibrium as approx. 80 % *trans*-proline-22 (A) and 20 % *cis*-proline-22 (B). Addition of Ca^{2+} leads to the conversion of the *trans* isomer to the *cis* isomer. When this process is monitored by fluorescence, addition of Ca^{2+} leads to the 20 % of *cis*-Fragment 1 becoming fully quenched, in a fast process, whereas the remaining *trans* isomer is isomerized in a rate-determining process to the *cis* isomer by mass action. Thus the *trans* $\rightarrow$ *cis* isomerization limits the rate of an otherwise very rapid Ca^{2+} -Fragment 1 interaction.

The fact that the process is first-order [the present work and Nelsestuen (1976)] suggests that only a single proline residue is involved. We speculate that proline-22 is implicated, since bovine Factor X and

human prothrombin Fragment 1, which do not show slow conformational changes when metal ion-induced fluorescence quenching is examined (Nelsestuen, 1977; Prendergast & Mann, 1977), contain substitutions of alanine and threonine respectively at position 22.

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