Vol. 111, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1983 Pages 213-218

CALCIUM EFFECTS ON PROTHROMBIN AND ITS REACTION WITH BIFUNCTIONAL ALKYLATING REAGENTS

Ruth E. Silversmith, G. Jason Wei and Gary L. Nelsestuen
Department of Biochemistry, University of Minnesota, 1479 Gortner Ave., St.Paul, MN 55108

Received January 11, 1983

Monodisperse bovine prothrombin was prepared and its molecular states under several conditions examined. The protein showed no tendency to self-associate in the absence of calcium. Calcium (4 mM) caused small increases in the apparent molecular weight of the protein which may or may not represent protein dimerization with very low affinity. The allowed conclusion was that calcium-induced prothrombin dimerization is minimal up to protein concentrations of many mg/ml. Calcium-induced protein shape changes did not measurably alter the protein diffusion constant. A bifunctional alkylating reagent did produce extensive calcium-dependent prothrombin crosslinking. Prothrombin dimers formed by the crosslinking agent were not a measure of the state of native prothrombin.

Prothrombin, a vitamin K-dependent plasma protein, binds calcium and interacts with certain phospholipid membranes as a part of the blood coagulation process. The exact structures of the calcium binding sites are not known. Some results have suggested that prothrombin dimerizes in the presence and absence of calcium (see ref. 1 and 2 for discussion) and that this has a major impact on the calcium binding sites. Other studies of protein molecular weights showed minimal protein self-association under conditions of up to several mg prothrombin per ml (1,3). Tarvers et al (2,4) recently showed that bifunctional alkylating reagents crosslink prothrombin (0.5 mg/ml) in the presence but not the absence of calcium. These latter results suggest a calcium-dependent dimerization process but other explanations are possible. Direct examination of protein molecular weights demonstrate here that the degree of crosslinking is not indicative of the amount of native protein dimer in solution.

Materials and Methods

Most sources of materials and experimental methods were the same as in a previous study (1). The final step in preparation of monodisperse bovine

0006-291X/83/040213-06$01.50/0
Copyright © 1983 by Academic Press, Inc.
All rights of reproduction in any form reserved.

prothrombin consisted of gel filtration on a 1.6 x 75 cm column of Bio-Gel A 1.5 m (Fig. 1). To avoid the physical agitation of drop action the fractions were collected by placing the column outlet at the bottom of each tube. The molecular weights of the product were consistent with a homogeneous prothrombin protein (see below). Molecular weight and diffusion constant measurements were obtained using the light scattering and quasielastic light scattering techniques and apparatus described previously (1). The bifunctional reagent, Dithiobis-(succinimidyl propionate) (5) (Pierce Chemical Co.) was dissolved in dimethyl-sulfoxide at a final concentration of 15 mg/ml and added to buffered solutions of protein. The reagent did not alter the pH of the solution. The buffer used throughout was 0.05 M Tris (pH 7.5) - 0.1 MNaCl.

Sedimentation velocity studies were conducted in a Beckman Model E ultracentrifuge equipped with u.v. scanning optics. The wavelength of analysis was adjusted to obtain a maximum absorbance on scale. The rotor velocity was 50,740 rpm and samples ± calcium were run immediately after one another.

Results and Discussion

The gel elution profiles for prothrombin were the same in the presence and absence of 4 mM calcium (Figure 1). The peak elution position varied by only 1 fraction which was largely due to different volumes of applied sample and was therefore within experimental error. The elution profile showed no indication of skewing which would be expected for a self-associating system. The molecular weights of the prothrombin were correct (Table I) indicating no contamination by dust or protein aggregates which was observed previously (1).

Addition of calcium caused small increases in the weight average molecular weights, determined by light scattering measurements. The accumulated data did not allow us to determine if the changes were due to a calcium-dependent equilibrium dimerization with low affinity or if they represented a background due to other factors. The concentration dependence of the increased molecular weight did not appear to conform to an equilibrium dimerization (1) and

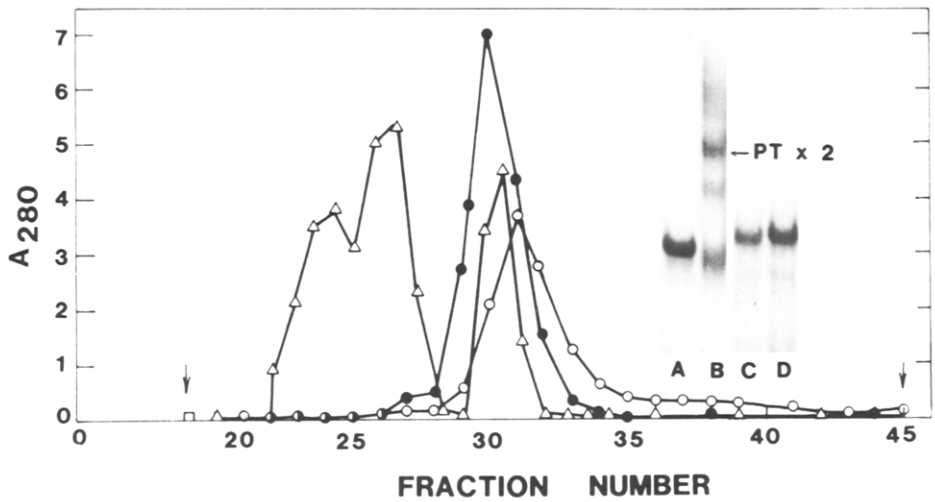

Figure 1. Gel filtration of prothrombin on Biogel A-1.5m. The elution profiles shown include prothrombin in buffer (●), prothrombin in buffer containing 4 mM Ca^{2+} (o) and chemicallly crosslinked prothrombin (see text) (Δ). The absorbance scale of the latter profile is X100. Fractions of 3.3 ml were collected and samples were loaded in 1.0 to 4 ml volumes. The exclusion and inclusion volumes of the column are indicated by the two arrows. The gels shown are A, prothrombin; B, covalently crosslinked protein; C, covalently crosslinked prothrombin reduced with mercaptoethanol; D, prothrombin reduced with mercaptoethanol. The band corresponding to a prothrombin dimer is identified (PTX2).

reversibility with EDTA was not always observed (Table I). We previously observed that the small changes in apparent molecular weight determined by light scattering varied depending on the technique used for metal ion addition. The standard deviation for the small changes obtained (Table I) indicated a similar difficulty in these studies. In any case, the increases in molecular weight observed when calcium was added represent upper limits for calcium-dependent protein dimerization. These values indicate that fifty percent calcium-induced dimer would occur at greater than 20 mg prothrombin per ml. This is a lower limit for the dissociation constant and indicates that dimerization will not be a significant factor in data interpretations under most experimental conditions.

The diffusion coefficient of prothrombin varied by only about two to four percent when calcium was added (Table I). This change was due primarily to the small change in apparent molecular weight. We conclude that shape changes associated with the calcium-dependent conformational change (3) have negligible

Table I

Molecular Weights of Prothrombin

Treatment	[Protein] mg/ml	M_r	M_{r_2}/M_{r_1} [a]	n [b]	$D_{20,w}$ $cm^2 \cdot s^{-1} \times 10^7$	$S_{20,w}$
none	.5	73,500	-	1	-	-
	2.5	71,300	-	1	5.0	-
	5.0	69,800	-	1	5.0	-
+4 mM Ca^{++}	1.8	-	1.06 ± .05	7	5.1 (n=1)	-
	5.0	-	1.14	1	4.8	-
+4 mM Ca^{++} + 5 mM EDTA	5.0	-	1.21	1	4.6	-
none	5	-	-	1	-	5.22
	21	-	-	1	-	4.53
2 mM Ca^{2+}	5	-	-	1	-	5.38 [c]
	21	-	-	1	-	4.29 [c]

a) The molecular weight ratio in the presence (M_{r_2}) to the absence (M_{r_1}) of calcium is given.

b) The number of independent determinations made to obtain the standard deviation reported for M_{r_2}/M_{r_1}.

c) These Sedimentation Coefficients were obtained from the bottom two thirds of the cell. Optical interference from a steep protein gradient inferfered with boundary measurements in the top third of the cell.

effects on the hydrodynamic radius of prothrombin. A similar conclusion was reached for prothrombin fragment 1 (1).

Sedimentation velocity measurements were also made and are shown in Table I. In this case, calcium was introduced by dialysis against buffer containing 2 mM calcium. The presence of calcium caused a three to five percent increase in the sedimentation coefficient which is similar to that observed by Jackson et. al. (6). Formation of a protein dimer should cause about a 50 pecent change in the sedimentation coefficient (7) unless unusual changes occur in the diffusion constant. This is clearly not the case (Table I). Sedimentation analysis therefore places the minimum protein concentration required for fifty percent prothrombin dimer much above 20 mg of protein per ml.

In the presence of the bifunctional reagent a dramatic calcium-dependent aggregation of prothrombin was observed (Fig. 2). Gel filtration (Fig. 1) and SDS polyacrylamide slab gel electrophoresis (8) of the crosslinked product (Fig.

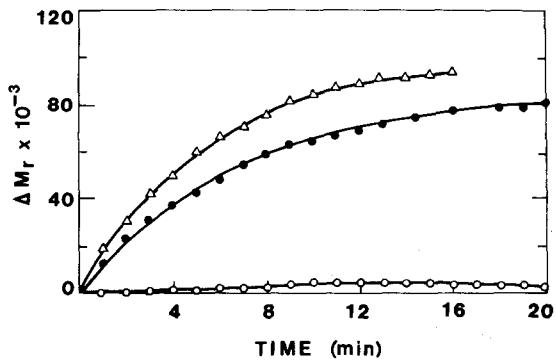

Figure 2. Covalent crosslinking of prothrombin by bifunctional reagent. Bifunctional reagent (10 µl of 15 mg/ml in dimethyl sulfoxide) was added to a solution of prothrombin (5.0 mg/ml) at room temperature (pH 7.5). The weight average molecular weight was monitored by light scattering and the change in molecular weight (M_r) after addition of bifunctional reagent is plotted. The three conditions shown were no further additions (o); addition of 4 mM calcium at time zero (●); reequilibration with 4 mM calcium for 15 minutes prior to addition of reagent (Δ). Protein from this latter experiment was used for chromatography in figure 1.

1) revealed a substantial amount of protein dimer with some larger aggregates. These were reduced to monomeric prothrombin upon disulfide reduction. The initial rate of aggregation was lower when calcium and bifunctional reagent were added simultaneously than when protein was preequilibrated with calcium (Fig. 2). This result was consistent with crosslinking of the membrane-binding conformation of prothrombin which forms slowly upon the addition of calcium (9). This feature of the covalent crosslinking was thoroughly documented by Tarvers et al (2,4). The results presented in figure 2 therefore corroborate the findings of Tarvers et al (2,4) but demonstrate that the degree of crosslinking is not indicative of the level of native prothrombin dimer.

There are several potential explanations for the action of the bifunctional reagent. It may irreversibly trap and thereby greatly enhance a low level of prothrombin dimer. Alternatively, covalent modification of lysine residues may produce an altered protein which does undergo calcium-dependent dimerization. Previous studies have indicated that even subtle modifications of lysine residues in prothrombin fragment 1 produce substantial differences in the proteins interaction with calcium (10). These changes must be carefully

considered when interpreting results obtained with chemically modified prothrombin.

Acknowledgements

The authors are indebted to Dr. Victor Bloomfield for the quasielastic light scattering apparatus used to measure diffusion constants and molecular weights. This apparatus is maintained by grant PCM 81 18107 from the National Science Foundation. This work was supported by grant HL 15728 from the National Institutes of Health.

References

1. Nelsestuen, G. L., Resnick, R. M., Wei, G. J., Pletcher, C. H. and Bloomfield, V. A. (1981) Biochemistry 20, 351-358.
2. Tarvers, R. C., Noyes, C. M., Roberts, H. R. and Lundblad, R. L. (1982) J. Biol. Chem. 257, 10708-10714.
3. Osterberg, R., Sjoberg, B., Osterberg, P. and Stenflo, S. (1980) Biochemistry 19, 2283-2286.
4. Tarvers, R. C., Roberts, H. R. and Lundblad, R. L. (1981) Fed. Proc. 40, 1586 (abst.).
5. Lamant, A. J. and Fairbnks, G. (1976) J. Mol. Biol. 104, 243-261.
6. Jackson, C. M., Peng, C-W, Brenckle, G. M., Jonas, A. and Stenflo, J. (1979) 254, 5020-5026.
7. Van Holde, K. E. (1975) The Proteins (Neurath, H. and Hill, R. I., eds.), Third Ed., Vol. 1, p. 235 Academic Press, New York.
8. Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.
9. Nelsestuen, G. L. (1976) J. Biol. Chem. 251, 5648-5656.
10. Pletcher, C. H. (1981) Fed. Proc. 40, 1585 (abst.).