Influence of Metal Ions on Prothrombin Self-association

DEMONSTRATION OF DIMER FORMATION BY INTERMOLECULAR CROSS-LINKING WITH DITHIOBIS(SUCCINIMIDYLPROPIONATE)*

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Interaction of certain metal ions with prothrombin and prothrombin fragment 1 has been shown to result in conformational change(s). Self-association of prothrombin and prothrombin fragment 1 in the presence of divalent cations has been reported. The present study has made use of a covalent cross-linking reagent, dithiobis(succinimidylpropionate), to study the self-association of prothrombin in the presence of divalent cations. In the presence of certain divalent cations, prothrombin dimer is the product of such cross-linking. Optimal dimerization of bovine prothrombin requires preincubation with calcium ions prior to the addition of cross-linking reagent. Calcium ions are also required for dimerization after this preincubation period. A similar time dependence is not seen with human prothrombin. The dimerization of bovine prothrombin is also supported by strontium and gadolinium to an extent comparable to that of calcium and to a lesser extent by barium or manganese. Magnesium ions do not support dimerization. The results suggest that certain divalent cations either induce or stabilize a conformation of prothrombin which can self-associate to form dimers. The results further suggest that divalent cations are also necessary for the actual cross-linking process subsequent to this conformational change.

Prothrombin is converted to thrombin by factor Xa in a reaction which requires phospholipid, factor V, and calcium ions. A number of laboratories have reported on various aspects of this reaction (cf. Refs. 1-15). Of particular interest in this area has been the study of the interaction of metal ions with the prothrombin molecule. Although the effect of calcium ions on factor Xa-catalyzed prothrombin activation has been known for some time (16-18), definitive evidence of calcium binding by prothrombin did not appear until some time later (19). Since that time, reports of various aspects of the binding of calcium ions to prothrombin have been studied in a number of laboratories (5-15, 20-27).

At the present time, it would appear that prothrombin has two classes of calcium binding sites and that the binding shows positive cooperativity (2, 3, 25). The fragment 1 region of the prothrombin molecule, which contains γ-carboxyglutamic acid, has been shown to have a major role in the binding of calcium ions and has been used as a model for metal-ion binding by prothrombin (cf. Refs. 9, 10, 22, and 25). Conformational changes occur in prothrombin and prothrombin fragment 1 as a result of calcium binding as assessed by changes in intrinsic fluorescence (10, 19, 26-27), development of neogenetic sites (24), changes in circular dichroism spectra (10, 26), susceptibility to proteolysis (14), changes in low angle x-ray scattering (15), and light scattering (27).

Together with these studies, there have been several investigations suggesting that prothrombin and/or prothrombin fragment 1 undergo self-association and that this self-association is increased in the presence of calcium ions (12, 20, 26, 27). The majority of the studies agree that prothrombin fragment 1 does undergo self-association in the presence of divalent cations (12, 20, 26). Evidence as to whether intact prothrombin undergoes self-association in the presence or absence of divalent cations is somewhat less convincing (12, 20, 27). The work described in the present communication was designed to explore the self-association of prothrombin using covalent cross-linking as a measure of spatial proximity in solution. The results suggest that the self-association of prothrombin results in dimer formation which involves the fragment 1 region of the molecule and that there may be two distinct calcium-dependent processes involved in the self-association of bovine prothrombin.

MATERIALS AND METHODS

Bovine prothrombin was purified from bovine plasma by barium citrate adsorption, ammonium sulfate fractionation, and chromatography on either DRAE-cellulose (28) or dextran sulfate-agarose (29). Human prothrombin was prepared from Cohn III paste by adsorption to barium citrate, ammonium sulfate fractionation, and chromatography on dextran sulfate-agarose (29). Both the human and bovine proteins were homogeneous by electrophoretic analysis on polyacrylamide gel in the presence of sodium dodecyl sulfate. Bovine e-thrombin was purified by the methods developed in this laboratory (30, 31). DSP* was obtained from Pierce. Protein standards for electrophoresis were obtained from Bio-Rad. All other chemicals used were of at least reagent grade.

The proteins were transferred into 0.05 M triethanolamine-HCl, 0.15 M NaCl, pH 7.4, by dialysis. Unless otherwise noted, all experiments were performed in this solvent. In the experiments designed to study the effect of different cations in promoting cross-linkage by DSP, the proteins were first dialyzed against 0.005 M EDTA, pH 7.4, to chelate any cations which might be bound to the prothrombin. The EDTA was removed by dialysis against the triethanolamine buffer and the prothrombin was equilibrated with the desired ion.

The chemical cross-linking reactions with DSP were performed essentially according to the method of Lomant and Fairbanks (32). DSP was dissolved in dimethyl sulfoxide at a concentration of 10-15 mg/ml. This solution was then diluted 50-100-fold into the buffer for the experiment. Portions of this DSP were added to the protein...
solution in an ice/water bath at 4 °C to start the cross-linking reaction. At various times thereafter, portions were taken from the protein solution and excess DSP was quenched with 0.1 M lysine, pH 8.6. All experiments were set up such that the DSP was added to all of the samples within 15 min of the time it was diluted into the trichloroacetic acid buffer. This procedure eliminated any variations between samples due to reagent instability, as Lamont and Fairbanks have shown the half-life of DSP in buffer at pH 7.0 to be at least 4 h (32).

Electrophoretic analysis of these samples on polyacrylamide gel was performed as described by Laemmli (33). Unless otherwise indicated, the experiments were performed using a stacking gel of 4.75% acrylamide and a separation gel of 7.5% acrylamide. Gels were stained for protein with a staining solution of 0.125% Coomassie brilliant blue R-250, 10% acetic acid, 9% water for 15 min at 85 °C; they were then destained by diffusion against a destaining solution of 15% methanol, 7.5% acetic acid, 77.5% water. Gels were stained for carbohydrate using the periodic acid-Schiff reagent according to the protocol of Zacharius et al. (34), with the exception that the gels were oxidized with 1% periodic acid for 2 h instead of 50 min and were not treated with metabisulfite.

Following destaining, gels were scanned at 640 nm for protein or 525 nm for carbohydrate using a Zeinex soft laser scanning densitometer. The relative amount of protein in a given band was estimated by determining the area under the peak in the corresponding gel scan. The amount of prothrombin dimer formed was expressed either as the per cent of total prothrombin initially present in the reaction mixture or as the per cent of the maximum amount of prothrombin dimer formed during a specific experiment; in this latter case, to allow for ease of comparison between different experiments, the absolute yield of prothrombin dimer obtained is also shown.

For purposes of chemical characterization, prothrombin dimer was isolated by the following procedure. In a representative experiment, 25 mg of prothrombin (0.5 mg/ml in 0.05 M triethanolamine hydrochloride, 0.15 M NaCl, pH 7.5) was reacted with a 20-fold molar excess of DSP in the presence of 7.5 mM calcium chloride for 30 min. After quenching with lysine as above, the reaction mixture was gel-filtered on Sephadex G-200 to isolate prothrombin dimer. Prothrombin dimer thus obtained was subjected to sequence analysis (below) and before treatment with trypsin (1:100 weight ratio, 6 h at 37 °C).

Automated Edman degradations (35) were performed in a Beckman 890C Sequencer. The 0.1 mM Quadrol program of Brauer et al. (36) was used, with modification of the cleavage portion to minimize blowing of heptfluorobutyric acid vapors through the effluent valve. The phenylthiohydantoinines produced by the Edman degradation were identified by high performance liquid chromatography using a UtraspHERE-ODS (Beckman) column (4.6-mm internal diameter × 250 mm) with a Beckman Model 324 gradient liquid chromatograph and Model 114B variable wavelength detector operated at 254 nm. Absorbance at 323 nm (for detection of phenylthiohydantoin-dehydrothreonine) was monitored by an Isco Model 1840 variable wavelength detector. A guard column (2.1-mm internal diameter × 7 mm) filled with C8/Pell ODS (Whatman) was connected ahead of the analytical column. Gradient elution was performed at 55 °C with an acetic buffer/acetoniitile solvent system.

**RESULTS**

The initial experiments were designed to determine whether the putative self-association of prothrombin could be studied using chemical cross-linkage with DSP. Bovine prothrombin was allowed to react with DSP in the presence of calcium ions. Fig. 1A shows the results of the electrophoretic analysis of these reaction mixtures on polyacrylamide gel in the presence of sodium dodecyl sulfate. In the presence of calcium ions there is a time-dependent formation of a species with electrophoretic mobility on the sodium dodecyl sulfate gels slower than the prothrombin monomer. Molecular weight calibration plots showed that the apparent molecular weight of this cross-linked protein is about 150,000, while the monomer under the same conditions has an apparent Mr = 78,000-80,000. Thus, the size of the cross-linked species is consistent with a dimer of prothrombin. Fig. 1B shows the densitometric scans of the samples shown in Fig. 1A. The amount of cross-

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*Fig. 1. Analyses of prothrombin dimer formation. A, electrophoretic analysis of dimer formation with dihydrobiotin (succinimidyldiiminate) in the presence of calcium ions. Bovine prothrombin (0.5 mg/ml in 0.05 M triethanolamine hydrochloride, 0.15 M NaCl, pH 7.5) was allowed to react with a 20-fold molar excess of DSP at 4 °C in the presence of 5 mM CaCl2. Gel 1 is the unmodified prothrombin. Gels 2 through 6 represent samples taken from the above reaction mixture at 5, 10, 15, 30, and 60 min. Gel 7 is the 60-min sample incubated with mercaptoethanol. B, densitometric analysis of prothrombin dimer formation in the presence of calcium chloride. The experiment was conducted as indicated in A. Samples subjected to electrophoretic analysis were then scanned on a Zeinex soft laser gel scanner. Sample 1 is unmodified prothrombin. Samples through 6 were obtained at 5, 10, 15, 30, and 60 min of reaction time with DSP. Sample 7 is the 60-min sample plus mercaptoethanol.*

linked dimer formed was determined by integration of the monomer and dimer peaks from the gel scans, and the amount of dimer formed as a function of time is shown in Fig. 2. Detectable dimer was not observed in the absence of added calcium ion. The effect of calcium ion concentration on this reaction was then examined; the results of this experiment are shown in Fig. 3. The concentration dependence observed in this experiment is similar to that previously reported for the dimerization of bovine prothrombin fragment 1 in the presence of calcium ions (12, 25-27).

There is substantial evidence to suggest that prothrombin fragment 1 does undergo self-association in the presence of divalent cations. Therefore, it would be expected that the fragment 1 region of the prothrombin molecule would be involved in the formation of the prothrombin dimer described above. If this is the case, then the treatment of isolated prothrombin dimer as prepared above in the presence of calcium ions with thrombin, which cleaves Arg 156-Ser 157 in bovine prothrombin between the fragment 1 and prothrombin 1 portion of the molecule (2), should yield fragment 1 dimer and monomer prothrombin 1. The presumed dimer species formed in the presence of calcium ions and DSP was isolated by gel filtration on Sephadex G-200. As shown in Table II, the NH2-terminal sequence of the isolated prothrombin dimer is that which would be expected based on the published primary structure for prothrombin (2, 37). Treatment of the isolated prothrombin dimer with bovine a-thrombin generates the expected new NH2-terminal sequence of prothrombin 1 (Table I). The reaction products from the thrombin cleavage of

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*2 C. M. Noyes, manuscript in preparation (1982).
the thrombin cleavage. Analysis of the same reaction mixture with periodic acid-Schiff reagent. As others have previously shown, prothrombin fragment 1 stains very well with PAS and poorly with Coomassie blue, whereas prethrombin 1 stains poorly with PAS and very well with Coomassie blue. As expected, thrombin treatment of control prothrombin produces prethrombin 1 and fragment 1. When the prothrombin dimer is treated with thrombin, no protein is observed in the position of fragment 1 (No. 7, unreduced). As seen from the PAS staining intensity, most of this fragment 1 migrates at a position consistent with a fragment 1 dimer, just anodally of the Coomassie blue peak for the prethrombin 1 produced by the thrombin cleavage. Analysis of the same reaction mixture after reduction with mercaptoethanol shows the disappearance of the fragment 1 dimer and appearance of the normal fragment 1 band. Thus, it would appear that the sites of cross-linkage seen in our study is consistent with expectation.

**TABLE I**

<table>
<thead>
<tr>
<th>Known amino acid sequences from bovine prothrombin (36)</th>
<th>NH₂-terminal sequence of prothrombin dimer</th>
</tr>
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<tbody>
<tr>
<td>Fragment 1 NH₂-Ala-Asn-Lys-Gly-Phe-Leu-</td>
<td>NH₂-terminal sequence of prothrombin dimer</td>
</tr>
<tr>
<td>Fragment 2 NH₂-Ser-Gly-Gly-Ser-Thr-Thr-</td>
<td>NH₂-Ala-Asn-Lys-Gly-Phe-Leu-</td>
</tr>
<tr>
<td>Amino acid sequences determined on the isolated prothrombin dimer</td>
<td>Dimer</td>
</tr>
<tr>
<td></td>
<td>NH₂-Ala-Asn-Lys-Gly-Phe-Leu-</td>
</tr>
<tr>
<td></td>
<td>Dimer treated with NH₂-Ala-Asn-Lys-Gly-Phe-Leu-thrombin</td>
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<td>1.9 2.7</td>
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<table>
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<tr>
<th>[CaCl₂]mM</th>
<th>% Maximal Dimer Formation</th>
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<tr>
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<td>10</td>
</tr>
<tr>
<td>1</td>
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<td>30</td>
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<tr>
<td>5</td>
<td>80</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
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</table>

**Fig. 2 (left).** Time dependence of prothrombin dimer formation in the presence or absence of calcium chloride. Bovine prothrombin (0.5 mg/ml in 0.05 M triethanolamine hydrochloride, pH 7.5) was allowed to react with a 20-fold molar excess of DSP at 4 °C in the presence of either 5 mM CaCl₂ (O—O) or 5 mM EDTA (●—●). Samples were removed at the indicated times, quenched with 0.1 M lysine, and subjected to electrophoretic analysis as described under Fig. 1. The percentage of dimer for each time point was determined as described under “Methods.”

**Fig. 3 (right).** The effect of calcium chloride concentration on prothrombin dimer formation. The reactions were performed as described under Fig. 1 in the presence of the indicated final concentration of calcium chloride. Reaction mixtures were incubated for 30 min at 4 °C before the addition of 0.1 M lysine, pH 8.6. The extent of prothrombin dimer formation was determined as described above. The 100% level at 10 mM calcium chloride was 25% prothrombin dimer.
Metal Ion Effects on Prothrombin Dimerization

Fig. 4. Electrophoretic analysis of the reaction between prothrombin dimer and thrombin. Prothrombin dimer was formed by cross-linkage with DSP in the presence of calcium ions. It was isolated by gel filtration as described under "Methods." The polyacrylamide gels contained a stacking gel of 4.75% acrylamide and a running gel of 10% acrylamide. Duplicate gels were fixed and stained with either periodic acid-Schiff reagent (---) or Coomassie (----) and scanned using a Zeineh laser scanning densitometer. Gel I is native prothrombin, gel 2 is reduced prothrombin, gel 3 is the isolated prothrombin dimer, gel 4 is the reduced prothrombin dimer, gel 5 is native prothrombin treated with thrombin, gel 6 is native prothrombin treated with thrombin + mercaptoethanol, gel 7 is prothrombin dimer treated with thrombin, and gel 8 is prothrombin dimer treated with thrombin + mercaptoethanol.

Table II

<table>
<thead>
<tr>
<th>Metal ion effect on prothrombin cross-linkage</th>
<th>Incubation time</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>15 min</td>
</tr>
<tr>
<td>Calcium</td>
<td>6</td>
</tr>
<tr>
<td>Strontium</td>
<td>8</td>
</tr>
<tr>
<td>Barium</td>
<td>10</td>
</tr>
<tr>
<td>Manganese</td>
<td>4</td>
</tr>
<tr>
<td>Magnesium</td>
<td>&lt;1</td>
</tr>
<tr>
<td>None</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

* Percentage of total prothrombin dimerized during reaction with DSP.

The experiments were performed at a prothrombin concentration of 0.5 mg/ml in 0.05 M triethanolamine-hydrochloride, 0.15 M NaCl, pH 7.5. The protein was preincubated with the indicated metal ions for 4 h at 4°C. The concentration of metal ion was 7.5 mM. DSP was added at zero time in 20-fold molar excess. Reaction at 4°C was allowed to proceed for the indicated time before quenching with lysine. Electrophoretic analysis of the samples was as indicated under "Methods."

The mechanism of the calcium ion effect, however, is not clear from the above results. Calcium could possibly be involved either in a "conformational" change necessary for dimerization or in the indirect "bridging" between two prothrombin molecules (cf. Ref. 12) or perhaps both. In order to explore these possibilities, the effect of a calcium chelating agent (EDTA) on the cross-linkage reaction was studied. Fig. 7 shows the effect of EDTA when added to prothrombin previously incubated with calcium ions. In this experiment, bovine prothrombin was preincubated with calcium ions as shown in Fig. 5. After the protein was preincubated at 4°C with calcium chloride for 4 h, EDTA was added in slight excess of the calcium chloride concentration. These samples were incubated for different amounts of time, after which DSP and calcium ions were added. The samples were reacted in the presence of DSP and calcium ions for 10 min before quenching with lysine. As shown in Fig. 7, as a function of time after the addition of EDTA, there is a decrease in the formation of cross-linked prothrombin dimer upon subsequent reaction with calcium ions and DSP. An apparent half-life for this change of 10-12 min can be estimated from Fig. 7; this is consistent with the time dependence expected for the "reverse-conformational change" previously studied in other laboratories by fluorescence (22) and conformation-specific antibodies to bovine fragment 1 (39). In the experiment shown in Fig. 7, it is critical to emphasize that the portions removed from the EDTA-protein mixtures were subsequently added to a reaction mixture which contained calcium ions in excess of the EDTA carried over. If calcium ions are not present during the cross-linkage step (Fig. 8), there will be no cross-linked product formed.

Finally, the specificity of metal ions in this reaction was investigated. The effect of various metal ions on the extent of bovine prothrombin dimer formation is shown in Table II. Calcium ions and strontium ions have a great effect on dimer formation while barium and manganese have a somewhat lesser effect. No dimerization is observed in the presence of magnesium. Gadolinium ions were also effective in promoting dimerization, as seen in Fig. 9. This experiment was performed at pH 6.8; although not shown here, at this pH, gadolinium was somewhat more effective than calcium in promoting the formation of prothrombin dimer. Gadolinium also has this effect at much lower concentrations than calcium (micro instead millimolarity).

**DISCUSSION**

Although there is general agreement that prothrombin fragment 1 does undergo self-association in the presence of divalent cations (9-12, 20, 25), conflicting evidence has been presented in the literature regarding the effect of divalent cations on the self-association of prothrombin. Jackson et al. (12) found some self-association of bovine prothrombin in the absence of calcium ions and an increased extent of self-association in the presence of calcium ions. The conclusions of

*It has been shown that this quantity of EDTA does not change the pH of the buffered protein solution (R. C. Tarvers, unpublished observations, 1981).
Metal Ion Effects on Prothrombin Dimerization

**FIG. 5** (left). The effect of the preincubation of bovine prothrombin with calcium chloride on dimer formation with di-thiobis(succinimidylpropionate). Bovine prothrombin (0.5 mg/ml in 0.05 M triethanolamine hydrochloride, pH 7.5) was kept at 4 °C and calcium chloride was added to a final concentration of 10 mM at zero time. At the indicated times, a portion of the reaction mixture was removed and added to a 20-fold molar excess of DSP. The experiment was designed so that the addition to DSP occurred at the same time for each sample to exclude problems with reagent stability. This second incubation was allowed to proceed for 50 min and then quenched with 0.1 M lysine. Dimer formation was assessed by electrophoretic analysis as described under Fig. 1. Each experimental point is the average of three separate experiments. The maximum reached at 240 min of preincubation time was 30% dimer.

**FIG. 6** (right). The effect of the preincubation of human prothrombin with calcium chloride on dimer formation with di-thiobis(succinimidylpropionate). Human prothrombin (0.5 mg/ml in 0.05 M triethanolamine hydrochloride, 0.15 M NaCl, pH 8.5) was kept at 4 °C and calcium chloride was added to a final concentration of 10 mM at zero time. At the indicated times, a portion of this reaction mixture was removed and added to a 20-fold excess of DSP. The experiment was designed so that the addition of DSP occurred at the same time for each sample to exclude problems with reagent stability. This second incubation was allowed to proceed for 50 min and was then quenched with 0.1 M lysine. Dimer formation was assessed by electrophoretic analysis as described under Fig. 1. The 100% maximal dimer was 33%.

**FIG. 7** (left). Reversal of prothrombin dimer formation by chelation of calcium ions. Bovine prothrombin was incubated with calcium chloride as described under Fig. 5. After 4 h of incubation at 4 °C, EDTA was added in slight excess of the calcium chloride concentration. At the indicated times following the addition of EDTA, a portion of the reaction mixture was removed and added to a 20-fold molar excess of DSP in the presence of 7.5 mM CaCl₂. As indicated under Fig. 5, the addition to DSP occurred at the same time for each sample to exclude problems with reagent stability. This reaction was allowed to proceed for 10 min before quenching with 0.1 M lysine. Dimer formation was assessed by electrophoretic analysis as described under Figs. 1 and 2.

**FIG. 8** (center). The effect of calcium chelation on dimer formation. Bovine prothrombin (0.5 mg/ml in 0.05 M triethanolamine hydrochloride, 0.15 M NaCl, pH 7.5) was preincubated with 10 mM CaCl₂ for 4 h at 4 °C. At zero time, the preincubated protein was added to a 20-fold molar excess of DSP in the presence of 10 mM CaCl₂ (O--O) or 10 mM EDTA (●--●). Portions were removed from these reaction mixtures at the indicated times, quenched with lysine, and subjected to electrophoretic analysis as described under "Methods.

**FIG. 9** (right). The effect of gadolinium on prothrombin dimerization. The reaction was performed as described under Fig. 2 except in the absence of CaCl₂ and with the final indicated concentration of gadolinium in 0.05 M triethanolamine hydrochloride, 0.15 M NaCl, pH 6.8.

Jackson and co-workers are based on sedimentation velocity experiments. Nelsestuen et al. (27), in experiments based on light-scattering intensity measurements, have presented evidence supporting the opposite; that is, prothrombin does not self-associate in either the presence or absence of divalent cations although a species consistent with a trimer of prothrombin was suggested to form in the presence of La³⁺ (27). The differences between these latter observations and those reported in our present study are not readily explained at the present time. Since previous studies using hydrodynamic approaches had yielded differing conclusions, we chose to study this interac-
tion using a chemical cross-linking reagent. The rationale for 
this approach was based on the previous use of such tech-
niques to explore spatial relationships such as those of the 
subunits of oligomeric proteins (40, 41), the hemoglobin-hap-
toglobin complex (42), the aggregation state of phospholipase 
A$_2$ (43), and the arrangement of the components of the pyru-
vate dehydrogenase complex (44). The reagent selected for 
this study, DSP, is a homobifunctional imidoester with spe-
cificity for reaction with primary amino groups in proteins (32).
It is assumed that the results obtained represent cross-linking 
resulting from spatial proximity rather than either random 
cross-linking or cross-linking caused by enhanced reactivity of 
specific lysine side chains. Since cleavage of cross-linked pro-
thrombin by thrombin produces fragment 1 dimer and pro-
thrombin 1 monomer, the cross-links are not randomly dis-
tributed. Moreover, we found that the formation of cross-
linked prothrombin dimer was absolutely dependent on the 
presence of a suitable cation, whereas in studies not reported 
in the present manuscript, cation dependence has not been 
observed for the reaction of prothrombin or prothrombin 
fragment 1 with DSP. The use of a chemical cross-linking agent has several ad-
advantages for this study as compared with the hydrodynamic 
measurements previously used (12, 20, 27). The main ad-
antage is that cation-dependent changes in conformation which 
occur in prothrombin and prothrombin fragment 1 (10, 15, 19, 
24, 26) tend not to confuse the interpretation of our results.
Using hydrodynamic techniques, cation-dependent changes in 
conformation will be superimposed upon the self-association 
process, making the contribution due to either process difficult 
to evaluate. A disadvantage of the cross-linking technique is 
that it is not possible to determine accurately the "absolute" 
percentage of the protein existing in solution as a dimer. It 
would be expected that reaction with the cross-linking agent 
would perturb the monomer-dimer equilibrium. However, it 
is a reasonable expectation that the amount of cross-linkage 
seen during reaction with DSP is directly related to the 
amount of protein which is self-associated under those reac-
tion conditions. By making this interpretation, our study 
allows us to make a number of suggestions which further the 
understanding of the self-association of prothrombin in solu-
tion. These include: 1) under the conditions of our experiments 
(0.5 mg/ml protein), the self-association process is totally 
dependent on the presence of a suitable cation. At higher 
protein concentrations it is quite possible that self-association 
could occur by cation-independent mechanisms (12, 2). The 
self-association involves the fragment 1 domain of prothrom-
bin. 3) There is a very slow time and cation-dependent change 
which must occur before optimal self-association and cross-
linkage of bovine prothrombin. The time dependence of this 
process (Fig. 5), as well as its reverse (Fig. 9), suggests that 
this is the same conformational transition which must be 
undergone before prothrombin can bind to phospholipid (9), 
or fragment 1 can bind to antibodies that are "specific for the 
calcium conformation" (39). 4) The calcium concentration 
dependence of the cross-linkage supports Prendergast and 
Mann's suggestion (20) that the dimerization sites are of lower 
affinity than those which cause the conformational change. 
Bloom and Mann (25) have suggested that no structural 
changes occur in fragment 1 during the filling of these “dimer” 
sites, and that these sites may involve a “calcium ion bridge” 
different between molecules. Our finding that the addition of 
EDTA will virtually instantaneously prevent the cross-linkage 
is consistent with this type of interaction. 5) The results shown 
in Table II and Fig. 9 suggest that there is a definite cation

5 R. C. Tarvers, unpublished observations, 1981.

specificity for the self-association. Tentatively we can suggest 
that the self-association is supported best by Gd$^{3+}$, Ca$^{2+}$, and 
Sr$^{2+}$ and less well by Ba$^{2+}$, Mn$^{2+}$, and Mg$^{2+}$.

Additional experiments will be designed to further charac-
terize the cation specificity of the self-association and to 
resolve some of the discrepancies among different laboratories 
concerning the self-association characteristics of fragment 1 
(compare Refs. 12, 20, and 27).

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