A CONFORMATIONAL STUDY OF NORMAL AND DICOUMAROL-INDUCED PROTHROMBIN

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1. Introduction

An abnormal prothrombin induced by dicoumarol administration has been purified from bovine plasma [1, 2]. This abnormal prothrombin has the same main antigenic determinants as normal prothrombin, but no prothrombin activity [1, 2]. While normal prothrombin has three to four high-affinity binding sites for calcium ions per molecule, the abnormal protein binds at most one calcium ion [3, 4]. Furthermore Ca^{2+} induces a conformational change in normal, but not in abnormal, prothrombin, as shown with immunochemical methods and suggested by equilibrium dialysis [3, 5]. Since normal Ca^{2+} binding is necessary for the physiological activation of prothrombin, a more detailed investigation of the conformation of the two prothrombins in the absence and presence of Ca^{2+} seemed warranted. This paper reports such a study with the use of optical rotatory dispersion (ORD) and circular dichroism (CD).

2. Materials and methods

Normal as well as dicoumarol-induced prothrombin was purified as described earlier [1]. Protein concentrations were measured from the absorbances at 280 nm with an extinction coefficient ($E^{1\%}_{1\,cm}$ at 280 nm) of 14.5 for both prothrombins [5].

Optical rotatory dispersion and circular dichroism spectra were measured with a Jasco Model J 20 spectropolarimeter (Japan Spectroscopic Company, Tokyo, Japan). The proteins studied were dissolved in 0.02 M Tris-HCl buffer, pH 7.2, containing 0.1 M NaCl and 0.002 M EDTA. They were then dialysed against the same buffer without EDTA for about 16 hr before use. This was necessary to reduce the light absorption at lower wavelengths caused by the EDTA. In some experiments $CaCl_2$ was added to a final concentration of 0.005 M. The spectra were recorded at room temp. (25°–27°), with protein concentrations of 0.4–0.8 mg per ml and cells having 10–0.5 mm pathlength. The results are given as plots of reduced mean residue rotation, $[m']$, or reduced mean residue ellipticity, $[\theta']$, against wavelength. The parameters $[m']$ and $[\theta']$ were computed in the usual manner [6, 7]. The mean residue weight of the two prothrombins was calculated from the amino acid composition [5] and was found to be 114 for both of them.

3. Results

The conformation of normal and dicoumarol-induced prothrombin in the absence and presence of calcium ions was investigated by optical rotatory dispersion and circular dichroism. It is clear from fig. 1 that, in the absence of Ca^{2+}, the ORD spectra showed no conformational differences between the two proteins that cannot be accounted for by experimental error. Both curves have minima at 232 nm with $[m']$ values about −2000, and both have a shoulder around 220 nm. The two spectra agree well with the spectrum of normal prothrombin measured by Ingwall and Scheraga [8], but only down to a wavelength of about 230 nm; for unknown reasons some differences are evident at lower wavelengths. Notably, the point of zero rotation is at about 220 nm in the spectrum of Ingwall

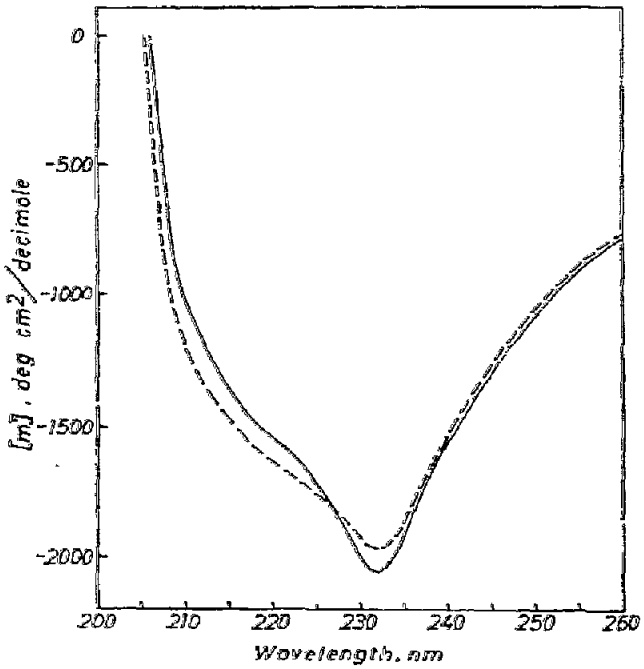

Fig. 1. Optical rotatory dispersion spectra of normal (——) and dicoumarol-induced (- - -) prothrombin in the absence of calcium ions.

and Scheraga [8], but at 206 nm in our curves. The through at 232 nm is indicative of some α-helix in both normal and abnormal prothrombin [9], but the low magnitude means that the α-helix content must be very small, and calculations of percentage are therefore meaningless. A low α-helix content of normal prothrombin was also suggested by Ingwall and Scheraga [8] on the basis of analyses of their ORD-data by the Moffitt-Yang equation.

Like the ORD results, studies by CD in the absence of Ca^{2+} did not show any conformational differences outside experimental error between normal and abnormal prothrombin, either in the aromatic region (fig. 2A) or in the peptide bond region (fig. 2B). In the latter region the two curves have minima between 202 and 204 nm with reduced mean residue ellipticities of around −5000, and also have broad shoulders between 220 and 230 nm. These features are not compatible with any of the spectra of the three model conformations for protein structure or combinations thereof [9, 10]; specifically no minima characteristic of the α-helix were found, which also suggests that the content of this structure in prothrombin is very low.

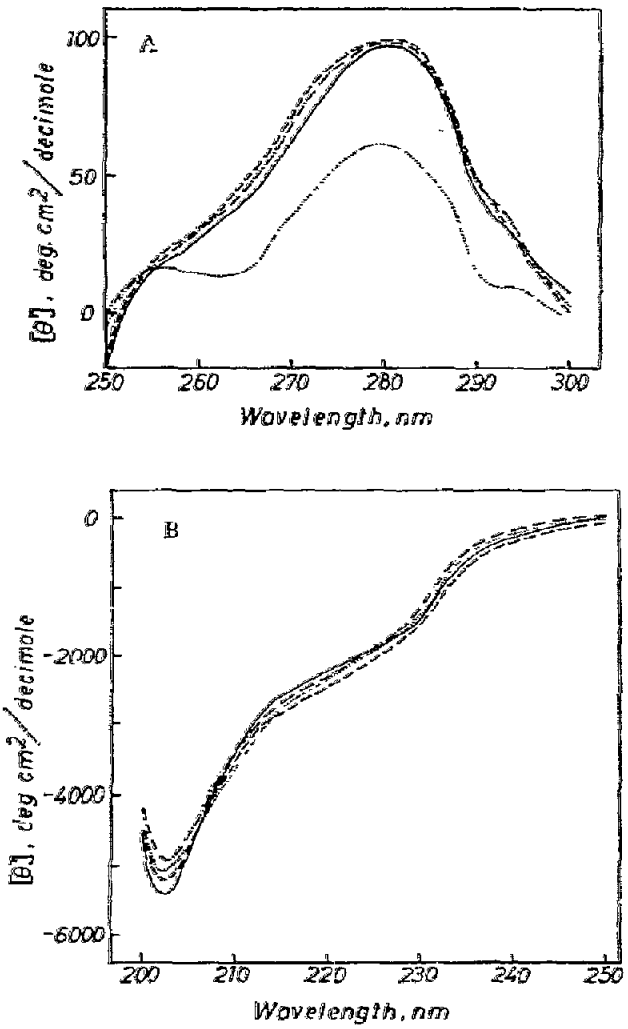

Fig. 2. Circular dichroism spectra of normal prothrombin in the absence (——) and presence (. . . . .) of calcium ions, and of dicoumarol-induced prothrombin also in the absence (- - -) and presence (- . - . -) of calcium ions. A) Aromatic region; B) peptide-bond region.

The conformation of the two proteins in the presence of Ca^{2+} was studied only by CD (fig. 2). Normal prothrombin exhibits a marked change in the aromatic region of the spectrum on binding of calcium ions (fig. 2A), whereas no such conformational change was found in the case of the dicoumarol-induced protein. In contrast, no difference in the peptide bond region of the spectra were found to accompany calcium-binding by neither protein (fig. 2B).

4. Discussion

Earlier investigations have shown that the properties of normal and dicoumarol-induced prothrombin in the absence of calcium ions are very similar, although not identical. The abnormal protein has a somewhat lower electrophoretic mobility than normal prothrombin [1, 11], and the two proteins can also be distinguished by quantitative immunoprecipitation [5, 2]. It has been suggested that these differences are due to a minor structural difference, e.g. in disulfide pairing, in a prosthetic group or in conformation. Attempts to reveal a conformational difference by titration of phenolic groups and by determination of fluorescence emission spectra failed in that identical results were obtained for both proteins [5]. Neither did the conformational probes used in the present investigation, i.e. ORD and CD analyses, produce evidence of any difference in the conformation of calcium-free, normal and abnormal prothrombin. The inference of these results is that the structural difference between the two proteins, of whatever kind it may be, must be so small that neither of the latter methods is capable of detecting it. A result pertinent to this conclusion is that recent experiments, where peptide maps were prepared from NH_2-terminal fragments, which constitute approximately one third of the intact prothrombins, revealed clear differences between the prothrombins. On the other hand the C-terminal fragments had identical electrophoretic and immunochemical properties [11]. Furthermore, Nelsestuen and Suttie have established that activation of normal and abnormal prothrombin by trypsin gives rise to the same amounts of thrombin [2], indicating that the major parts of the two molecules are identical.

When calcium is added, normal and dicoumarol-induced prothrombin behaves differently. The normal prothrombin molecule binds three to four calcium ions with high affinity, while the abnormal protein binds at most one calcium ion with comparable binding strength [3, 4]. Simultaneously, a change occurs in the antigenic structure of normal prothrombin but not in that of the abnormal protein [3, 5]. This has been interpreted as reflecting a conformational change induced by the binding of calcium, a conclusion further supported by the anomalous shape of the Scatchard plots obtained in equilibrium dialysis experiments [3]. Our circular dichroism data corroborrate these results. The CD spectra obtained with and without Ca^{2+} indicate that this ion induces a conformational transition in normal prothrombin but not in the abnormal protein. The change is expressed only in the aromatic region of the spectrum and is therefore presumably only a local change in the neighbourhood of some aromatic amino acid. This is in accordance with earlier results, which indicate that the calcium-binding groups of high affinity reside only in the NH_2-terminal part of the molecule [11]. However, the experiments do not permit any conclusion about the exact nature of the conformational change.

In conclusion, all available data indicate that the main functional difference between normal and dicoumarol-induced prothrombin is in their calcium-binding and in the properties induced by this ion. Normal prothrombin binds an optimal amount of calcium ions, which causes the protein to assume a conformation that allows its physiological acitvation to thrombin to take place. Abnormal prothrombin, however, lacks the ability to bind a comparable amount of calcium and therefore cannot be activated in the normal way. However, under physiological conditions the calcium level is such that the active conformation of normal prothrombin is presumably always maintained in the circulating blood

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