CIRCULAR DICHROISM OF TWO PHOSPHOLIPASES A FROM *VIPERA AMMODYTES* VENOM

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Received 1 July 1974

1. Introduction

Two phospholipases A, one toxic (k) and the other non-toxic (i) [1] have been isolated in electrophoretically pure form. These two proteins from *Vipera ammodytes* venom differ in electrophoretic mobility but have almost identical molecular weights of 14 500 as determined by sodium dodecyl sulphate gel electrophoresis. Both were found to be highly resistant towards temperature and urea which reflects their compactness resulting from the number of disulphide bonds [2]. In this communication we report the results of preliminary structural studies of both phospholipases by circular dichroism (CD). Considering their different biological behaviour it seems likely that the two proteins have different conformation. In addition denaturation studies using guanidine hydrochloride and mercaptoethanol were also performed.

2. Materials and methods

Both phospholipases A were isolated by preparative electrophoresis from fractions D_2 and F obtained by column chromatography of crude venom on CM-cellulose [3]. Ultrapure guanidine hydrochloride was purchased from Schwarz/Mann. All other reagents were of analytical grade.

CD spectra were obtained at 23° ± 1°C with a JASCO Model J-20 CD/ORD spectropolarimeter. The instrument was calibrated with solutions of *d*-10-camphor sulphonic acid. Protein concentrations ranged from 0.3 to 2.0 mg/ml in the cell of 1.0 cm pathlength and from 0.15 to 0.5 mg/ml in the cell with 0.05 cm pathlength. The concentrations were determined spectrophotometrically at 280 nm using the value 12.5 for $A^{1\%}_{1\,cm}$ obtained on the basis of dry weight determinations. In all experiments the solvent was 0.1 M Tris–HCl buffer, pH 7.0, with or without 6 M guanidine hydrochloride. For reducing disulphide bonds, enough β-mercaptoethanol was added to make solutions 0.1 M.

The CD data are presented as mean residue ellipticity, $[\theta]$, degree · cm^2 · decimole^{-1}, at each wavelength using the following relationship:

$$[\theta]_\lambda = \frac{M_0\,\theta}{100\,c\,l}$$

where θ is the ellipticity, M_0 is the estimated mean residue weight taken to be 110, c is the concentration in g/cm^3 and l is the pathlength in dm. In the far ultraviolet the main contribution to error originated from high signal-to-noise ratio particularly in solutions containing guanidine hydrochloride and mercaptoethanol. The estimated error increases from ± 1 above 250 nm to ± 500 degree · cm^2 · decimole^{-1} at the lowest wavelengths.

3. Results and discussion

The ultraviolet CD spectra, between 320 and 205 nm, of the two proteins are shown in fig. 1 and fig. 2: they are clearly distinct. The spectrum of the non-toxic form below 250 nm shows two minima, which are characteristic of helical structures, at 222 nm and 209 nm, respectively, the latter being weakly expressed. Above 250 nm the spectrum is complex and reflects

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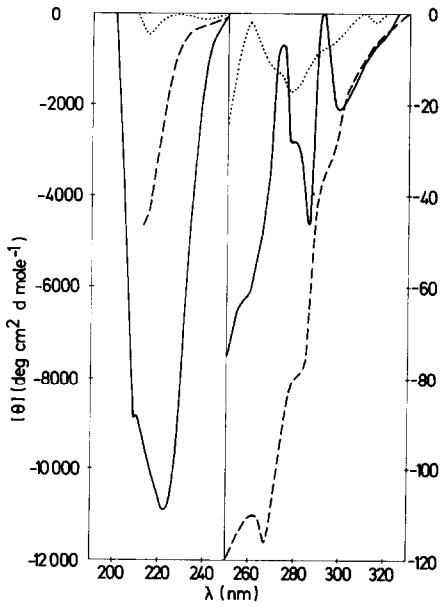

Fig. 1. CD spectra of non-toxic phospholipase A (protein i) from *Vipera ammodytes* venom: enzyme dissolved in 0.1 M Tris–HCl buffer, pH 7.0 (——); enzyme in the same buffer and 6 M guanidine hydrochloride with (–·–·–·) or without (– – – –) 0.1 M mercaptoethanol.

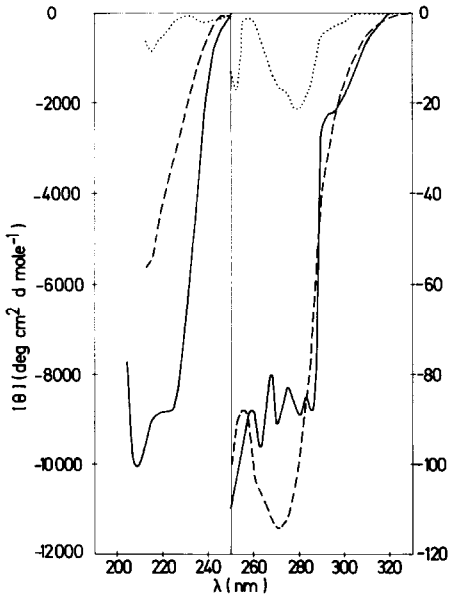

Fig. 2. CD spectra of toxic phospholipase A (protein k) from *Vipera ammodytes* venom. The experimental conditions were the same as in fig. 1.

the presence of several overlapping side-chain transitions. Since the amino acid composition of the protein has not been completed yet, a detailed discussion of this part of the spectrum is not yet possible. However, it is assumed that the molecule contains several disulphide bonds; because, in order to completely destroy the enzymatic activity the addition of β-mercaptoethanol to 8 M urea [2] and guanidine hydrochloride solutions (see below) is necessary.

The CD spectrum of the toxic form displays a minimum at 209 nm and a shoulder at 223 nm. Above 250 nm the spectrum is less complex than that of the non-toxic enzyme.

In recent yeats, several methods have been developed for estimating the helical content of proteins [4–6]. We have used the method of Chen et al. [4] which is in principle identical to that of Saxena and Wetlaufer [5]. The mean residue ellipticity is assumed to be the sum of the fractions of the helix, β and unordered forms. Using their data for H, β and R and eliminating f_β we obtained a system of two equations. Two-variable linear regression was made on Hewlet–Packard 9810A calculator using Model 10 STAT PAC III-1 program. The correlation coefficient was better than 0.95. For both phospholipases A, designated as i and k we found 24 ± 2% and 23 ± 2% helix and 74 ± 5% and 73 ± 5% random structure, respectively. The part of the spectrum between 204 and 243 nm was used at 3 nm intervals for these calculations.

A simplified equation [4] was also used for the calculation of helix content from $[\theta]_{222}$.

$$[\theta]_{222} = -30\,300\, f_H - 2340$$

Slightly different values i.e. 28% and 21% of helix were obtained for proteins i and k.

These results are to be taken cautiously, however, since the three-parameter fitting may not be satisfactory as was pointed out by Dalgleish [7].

The CD spectra of the two proteins in 6 M guanidine hydrochloride display several characteristic features. Below 250 nm smaller negative ellipticities than in the native form are observed. Furthermore, proteins with intact disulphide bonds have larger negative ellipticities than those with reduced bonds. This has also been found for several other proteins by Cortijo et al. [8]. In the near ultraviolet, above 250 nm the fine structure is lost but more negative ellipticities

are observed. Only the addition of the reducing β-mercaptoethanol reduced the negative ellipticities to values assumed for random coils [4]. Takagi and Izutsu [9] observed the same phenomenon after reducing the disulphide bonds of ribonuclease in 6 M guanidine hydrochloride. In order to elucidate this behaviour further CD studies of these and other proteins are necessary.

The snake venom neurotoxins are, like phospholipases, known to be resistant towards denaturation apparently due to abundant disulphide cross-linking. The spectrum of β-bungarotoxin which contains 31–34% helix and at least 9 disulphide bridges in the molecule of 28 500 molecular weight [10] is comparable.

We can say in conclusion that the CD spectra of the two phospholipases A confirmed the assumption of their structural difference which in turn could be responsible for their different biological activity, though their helix content is relatively close.

Acknowledgements

This work was supported by Boris Kidrič Foundation of SR Slovenia. The authors are indebted to Dr S. Maričić from Institute of Biology, University of Zagreb, who kindly allowed us to use their spectropolarimeter and to Dr C. Klofutar from this Institute for the use of the calculator.

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