Evidence That the Phospholipases A_2 of *Crotalus adamanteus* Venom Are Dimers*

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ABSTRACT: The α and β forms of phospholipase A_2 from *Crotalus adamanteus* venom were shown to be composed of 15,000 molecular weight subunits. Reversible dissociation occurs in 8 M urea or at low pH. Irreversible dissociation occurs at high pH or in 6 M guanidine hydrochloride containing 0.1 M mercaptoethanol. Each protein is composed of identical subunits, although the two proteins contain different subunits, as shown by electrophoresis under a variety of conditions. Carboxypeptidase hydrolysis of the proteins suggests that each subunit has a C-terminal sequence, Leu-Cys-Gly-Ser-. Although reassociation of monomers to dimers with concomitant recovery of enzymatic activity is readily achieved after dissociation at low pH or in 8 M urea, no evidence could be obtained for the formation of hybrid enzymes. A correlation between decrease in molecular weight and loss of enzymatic activity in solutions of varying urea concentrations, and the inability to demonstrate dissociation of the protein at concentration used for assay suggest that the dimer is the enzymatically active form of these proteins.

Recently phospholipase A_2 has been purified from several sources (de Haas *et al.*, 1968; Currie *et al.*, 1968; Wells and Hanahan, 1969; Wu and Tinker, 1969; Tu *et al.*, 1970; Augustyn and Elliott, 1970; Sakhibov *et al.*, 1970; Wahlström, 1971; Salach *et al.*, 1971). One striking feature of these various preparations is the range of molecular weights which have been reported: porcine pancreas, 15,000 (de Haas *et al.*, 1968); *Naja naja* (cobra), 24,000 (Currie *et al.*, 1968); middle eastern cobra, 14,000–15,000 (Sakhibov *et al.*, 1970); *Agkistrodon piscivorus* (water moccasin), 14,000 (Augustyn and Elliott, 1970); *Laticanda semifasciata* (sea snake), 11,000 (Tu *et al.*, 1970); *Crotalus atrox* (Western diamondback rattlesnake), 15,000 (Wu and Tinker, 1969); *Naja nigricollis* (black-necked cobra), two enzymes 13,000 and 14,500 (Wahlström, 1971); *N. naja*, several enzymes ranging from 8500 to 20,200 (Salach *et al.*, 1971); *Vipera russelli* (Russell's viper), several enzymes ranging from 15,000 to 23,800 (Salach *et al.*, 1971); *Crotalus adamanteus* (Eastern diamondback rattlesnake), two enzymes each 30,000 (Wells and Hanahan, 1969).

Although most of the venom phospholipases A_2 appear to have molecular weights about one-half that found for *Crotalus adamanteus*, it should be noted that most of these are from the Elipidae family, not the Crotalidae family. Of the Crotalidae reported one, *Agkistrodon piscivorus*, is from a different species, and recent data (Hachimori *et al.*, 1971) indicates that the *Crotalus atrox* enzyme also has a molecular weight of 30,000. The current study had two purposes: (1) to determine whether the phospholipases A_2 from *C. adamanteus* are composed of subunits, and, if so, whether the subunits were identical or nonidentical, (2) to determine whether the active form of the enzymes is the protomer or monomer.

Materials and Methods

The α and β forms of *C. adamanteus* phospholipase A_2 were prepared and assayed as previously described (Wells and Hanahan, 1969). The preparations were identical in all respects to those reported before. In some cases the activity was assayed using 1,2-dibutyryl-*sn*-glycero-3-phosphorylcholine as a substrate in aqueous solution.

Preparation of 1,2-Dibutyryl-sn-glycero-3-phosphorylcholine. *sn*-Glycero-3-phosphorylcholine was prepared from hen's egg yolk phosphatidylcholine (Wells and Hanahan, 1969) by the method of Brockerhoff and Yurkowski (1965), and its cadmium chloride complex reacted with butyryl chloride (Baer and Buchnea, 1959). The product was purified on a column of silicic acid (Silicic AR, CC-4 Mallinckrodt Chemical Works, St. Louis, Mo.) using a loading factor of 1 mg of P/g of absorbent. The column was packed in and the sample applied in chloroform. The column was eluted with five column volumes of chloroform and then with methanol which was collected in 20-ml fractions. The pure product showed a single spot on thin layers of silica gel G (Brinkmann Instruments, Westbury, N. Y.) in the solvent system chloroform–methanol–water (95:35:6, v/v), analyzed per cent P = 7.59 (theory 7.50), and $[\alpha]_{546}^{25}$ +13.1° (*c* 1.8, chloroform–methanol, 1:1, v/v).

The assay was conducted at 37° in a Radiometer pH-Stat. The substrate was used at a concentration of 25 mM in 10^{-3} M $CaCl_2$. The pH was maintained at 8.0 with 0.025 M NaOH. A detailed analysis of this assay system will be reported later.

Urea was crystallized from ethanol and only freshly prepared solutions were used. Guanidine hydrochloride was purified by the method of Nozaki and Tanford (1967). Bovine hemoglobin, ovalbumin, β-lactoglobulin, and carboxypeptidase were from Pentex Inc. (Kankakee, Ill.); cytochrome *c* (equine heart) and α-chymotrypsinogen (bovine pancreas) were from Calbiochem (Los Angeles, Calif.). All other reagents were reagent grade and used without purification.

Sephadex gel filtration and ion-exchange chromatography were carried out as before (Wells and Hanahan, 1969). Gel filtration in 0.1 M mercaptoethanol–6 M guanidine hydrochloride on agarose beads was performed by the method of Fish *et al.* (1969). Disc gel electrophoresis was carried out by the method of Ornstein (1964) and Davis (1964). Cellulose ace-

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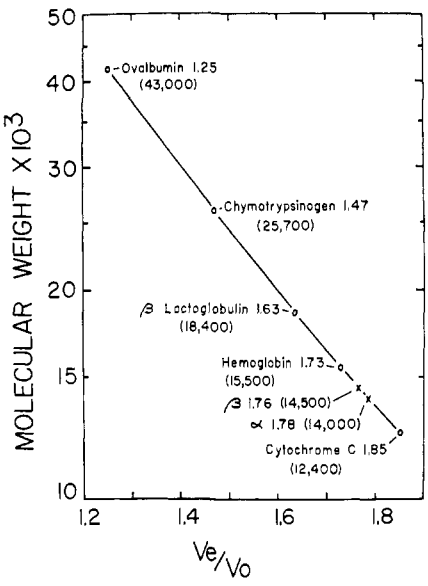

FIGURE 1: Log A_{280} *vs.* r^2 plots at sedimentation equilibrium at various pH's. Initial protein concentration 0.15 mg/ml of α form of phospholipase A_2. Runs carried out at 18,000 rpm and 20.0°

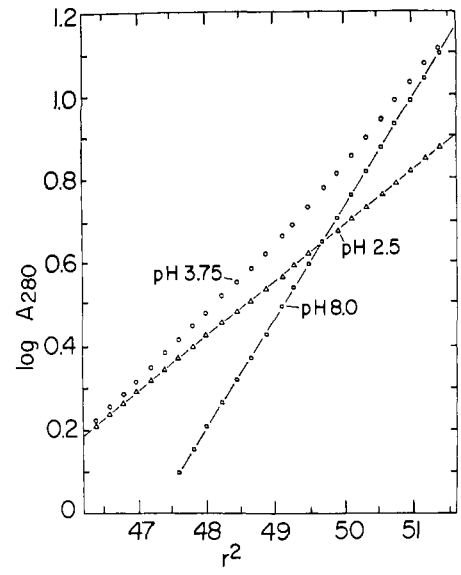

FIGURE 2: Semilogarithmic plot of molecular weight *vs.* V_e/V_0. The elution volume (V_e/V_0) and molecular weights of protein standards are included. α and β refer to elution volume of the phospholipases A_2 of *C. adamanteus*.

tate electrophoresis was conducted in a Phoroslide apparatus (Millipore Corp., Bedford, Mass.) using 0.01 M buffers.

Carboxypeptidase A hydrolysis was done on performic acid oxidized protein. The performic acid oxidation was performed as described by Hirs (1956). Performic acid oxidized protein (5 mg) was dissolved in 1 ml of 0.2 M *N*-ethylmorpholine acetate buffer (pH 8.5) (Ambler, 1967). The reaction was carried out at 37° using 0.1 mg of diisopropyl fluorophosphate treated carboxypeptidase A. At 20, 40, 60, 90, and 120 min, 100-μl samples were removed, added to 1 ml of 0.01 N HCl, centrifuged, and the supernatant solution dried *in vacuo*. The dried sample was then analyzed for released amino acids on a Beckman Model 120C by the accelerated method of Spackman *et al.* (1958). Controls were run without added carboxypeptidase, and corrections were made for self-hydrolysis of carboxypeptidase in the absence of added phospholipase A_2.

Ultracentrifugation was carried out in a Spinco Model E equipped with electronic speed control. All data were collected with the photoelectric scanner and multiplexer accessory. All runs were carried out at 20.0°. Generally low-speed equilibrium was used, but in some cases high-speed equilibrium was used. Equilibrium was hastened by overspeeding. After 24 hr, scans were made every 2 hr until three scans were identical. The data were calculated from the average of four scans made on each cell. Solvent densities were determined in a 10.0-ml pyncnometer at 20.0°. A partial specific volume of 0.718 (Wells and Hanahan, 1969) was used in all calculations. It was assumed that $\bar{v}$ was the same for the native and dissociated forms of the enzyme. Further, it was assumed that the same $\bar{v}$ could used for the studies carried out in urea.

Results

Molecular Weight Studies. Low-speed sedimentation equilibrium was carried out in the following solutions: (A) 0.01 M Tris–0.1 M NaCl (pH 8.0), (B) 0.01 M Tris–8 M urea (pH 8.0), (C) 0.01 M HCl–0.1 M NaCl, (D) 0.01 M citrate–0.1 M NaCl (pH 2.5), (E) 0.01 M citrate–0.1 M NaCl (pH 3.75), (F) 0.01 M citrate–0.1 M NaCl (pH 4.5), and (G) 0.01 M NaOH–0.1 M NaCl. In 8 M urea (pH 8.0), 0.01 M HCl, and citrate (pH 2.5) a homogeneous distribution was observed with an average molecular weight of 15,000. In citrate (pH 4.5) and Tris (pH 8.0) a homogeneous distribution was observed with an average molecular weight of 30,000. In citrate (pH 3.75) and in 0.01 M NaOH a heterogeneous distribution was observed. Typical log A_{280} *vs.* r^2 plots are shown in Figure 1. In more than 30 experiments no significant difference between the two proteins was observed.

Several studies were carried out at pH 8.0 to determine whether any dissociation occurs at low protein concentration. Using a combination of high-speed and low-speed sedimentation equilibrium over a concentration range of approximately 0.05–10 mg/ml, no evidence could be obtained for dissociation. Due to the very high specific activity of the enzymes, it was possible to carry out gel filtration studies over a wider concentration range. Using Sephadex G-100 and a solvent of 0.1 M NaCl in 0.01 M $CaCl_2$ and a concentration range of 2 μg/ml to 2 mg/ml, the elution volume (V_e/V_0) was 1.80 ± 0.02. Even at very low protein concentrations complete recovery of enzymatic activity was obtained. An elution volume of 1.80 corresponds to a molecular weight of 30,000.

Further evidence for the molecular weight of the monomer was obtained by gel filtration in 0.1 M mercaptoethanol in guanidine hydrochloride on agarose beads. Figure 2 shows the results of the experiments. For both proteins an average molecular weight of 14,750 ± 500 (two experiments) was found.

Sedimentation equilibrium was carried out in different concentrations of urea in 0.01 M Tris (pH 8.0) to determine at what molarity dissociation occurs. The results of experiments in 1, 2, 4, 6, and 8 M urea using the β form of phospholipase A_2 are presented in Table I. The data are whole cell weight-average molecular weights. No attempt was made to

TABLE I: Molecular Weights and Enzymatic Activity of Phospholipase A_2 in Various Concentrations of Urea.[a]

Urea Concn (M)	Whole Cell $\bar{M}_w$[b]	Sp Act.[c] (μmoles/min per mg of Protein)
0 (containing 0.1 M NaCl)	30,000 ± 750	5.6
1	26,000 ± 1000	3.5
2	20,500 ± 1000	2.0
4	16,000 ± 800	0.6
6	14,750 ± 500	0.0
8	15,000 ± 500	0.0

[a] This represents data collected on the β form of phospholipase A_2. [b] The initial protein concentration was 0.15 mg/ml. Runs were carried out at 18,000 rpm and 20.0° in 0.01 M Tris (pH 8.0) and the indicated concentration of urea. [c] Assays were carried out at 37° in 10^{-3} M $CaCl_2$ using 25 mM 1,2-dibutyryl-*sn*-glycero-3-phosphorylcholine and the indicated concentration of urea. The pH was maintained at pH 8.0 with 0.025 M NaOH using a pH-Stat.

TABLE II: Carboxypeptidase A Hydrolysis of the Performic Acid Oxidized α and β Forms of Phospholipase A_2.[a]

Time (min)	Amino Acid Released (moles/30,000 g of Protein)							
	Leucine		Cysteic Acid		Glycine		Serine	
	α	β	α	β	α	β	α	β
20	0.70	0.63	0.25	0.22	0.12	0.16	0.06	0.10
40	1.15	1.20	0.56	0.50	0.26	0.38	0.16	0.21
60	1.65	1.50	0.80	0.70	0.47	0.56	0.36	0.31
90	1.86	1.96	1.04	0.94	0.63	0.71	0.47	0.41
120	1.92	2.02	1.34	1.49	0.94	0.83	0.59	0.67

[a] Performic acid oxidized protein was dissolved in 1.0 ml of 0.2 M *N*-ethylmorpholine acetate buffer (pH 8.5). The reaction was carried out at 37° using 0.1 mg of diisopropyl fluorophosphate treated carboxypeptidase A. At the indicated times 100-μl samples were removed, treated with 1.0 ml of 0.1 N HCl, centrifuged, and the supernatant solution dried *in vacuo*. The dried sample was then analyzed for amino acids.

analyze the molecular weight distributions further. Similar results were obtained with the α form of the enzyme.

Evidence That the Dimer is the Active Form of the Enzyme. No enzymatic activity could be detected when the enzymes were assayed in either 8 M urea pH 8.0 or at pH 2.0 in the absence of urea using the ether assay system. Since the nature of the reaction in this nonaqueous system is not understood, it was felt that an assay in aqueous solution should be used. In order to eliminate possible confusion introduced by using an aggregated substrate, an assay using 1,2-dibutyryl-*sn*-glycero-3-phosphorylcholine was developed. Although the activity of the enzyme is low, it can be easily measured. Further this substrate is monomeric in the concentration range used.[1] It was felt that any effect of urea on enzymatic activity would, therefore, be a reasonable reflection of the state of the enzyme. Table I shows the results of enzyme assays carried out in the presence of various concentrations of urea. It is clear that enzymatic activity is abolished in the presence of about 4 M urea. There is a rough correlation between the decrease in molecular weight and loss in enzymatic activity. As indicated above, no evidence for dissociation could be found at pH 8.0 in the protein concentration range used in this assay (35 μg/ml).

Nature of the Subunits. In order to investigate whether each protein was composed of identical ($\alpha\alpha$, $\beta\beta$) or nonidentical ($\alpha_1\alpha_2$, $\beta_1\beta_2$) subunits the following experiments were carried out.

Electrophoresis was carried out on cellulose acetate strips under conditions which are known to lead to dissociation. In 0.01 M Tris (pH 8.0) and 8 M urea in 0.01 M Tris (pH 8.0) and 0.01 M phosphate (pH 2.0) both α and β showed single protein bands. In 8 M urea (pH 8.0), the relative mobility of β to α was one-half that observed in the absence of urea. At pH 2.0 the mobilities were identical. Under no circumstances was it possible to demonstrate more than one protein band for either protein.

As noted previously (Wells and Hanahan, 1969) N-terminal amino acid analyses for both proteins were negative; however, it has been possible to obtain some information on the C-terminal amino acids of these proteins. The results of carboxypeptidase A hydrolysis of the performic acid oxidized proteins are presented in Table II. No amino acids other than those reported in Table II were detected in the hydrolysates. There is no significant difference between the rate of release of amino acids from the two proteins. Attempts to fingerprint the two proteins have been unsuccessful as noted previously (Wells and Hanahan, 1969). All derivatives of reduced enzyme are very insoluble, and difficult to purify or characterize.

Reassociation and Hybridization Studies. Preparations of either enzyme which had been treated with 8 M urea and shown to have a molecular weight of 15,000, and then dialyzed against 0.01 M Tris-chloride–0.1 M NaCl were shown to have a molecular weight of 30,000 without any indication of heterogeneity. After removal of the urea both enzymes recovered complete enzymatic activity and had specific activities which were identical to the untreated enzymes. Reassociation appeared to be very rapid as shown by the following experiment. When a concentrated enzyme solution in 8 M urea was diluted 1000-fold with 0.01 M $CaCl_2$ and immediately assayed in the ether assay system, the specific activity was identical with a control sample which had not been treated with urea. No enzymatic activity could be detected if the assays were carried out in the presence of 8 M urea.

Preparations which had been taken to pH 2.0 and then returned to pH 8.0 were indistinguishable from native enzyme both in enzymatic activity and molecular weight. No activity was detected if the assays were carried out at pH 2.0. In one study a sample which had been taken from pH 8.0 to 2.0 and then to pH 3.75 was compared in the ultracentrifuge to a sample which had been taken directly from pH 8.0 to 3.75. Both preparations were heterogeneous, but no significant difference in the distribution patterns could be detected. This was taken to indicate the ready reversibility of the monomer–dimer transformation.

Disc gel electrophoresis was carried out on preparations

which has been dissociated by either acid or 8 M urea and then reassociated at pH 8.0 in 0.01 M Tris. Each protein showed a single band which was indistinguishable from the starting material. The two proteins retained their difference in electrophoretic mobility. The patterns were identical with those reported previously (Wells and Hanahan, 1969).

Treatment of the enzymes with mercaptoethanol leads to irreversible denaturation. Several attempts to regenerate active enzymes by slow reoxidation in dilute solution, in the presence or absence of dilute mercaptoethanol, and at several different pH's were completely unsuccessful. Less than 0.01% active protein was produced. These reoxidized preparations were highly aggregated and insoluble.

As noted above, dissociation also occurs at a pH of 12. However, no enzymatic activity was found after preparations which had been treated with 0.01 M NaOH were returned to pH 8.0. Further studies on this apparent irreversible denaturation have not been carried out.

Several experiments were carried out to try to form hybrid enzymes. Mixtures of the enzymes in varying proportions were either treated with 8 M urea, pH 8.0 or at pH 2.0, in the absence of urea. After 24 hr the preparations were reassociated either by (1) dialysis against several changes of 0.01 M Tris (pH 8.0) or (2) by dialysis in decreasing concentrations of urea and finally 0.01 M Tris (pH 8.0) (for urea-treated samples), or (3) against solutions of increasing pH and finally pH 8.0 (for samples treated at pH 2.0). Complete recovery of enzymatic activity was found in all cases. The samples were analyzed by disc gel electrophoresis and ion-exchange chromatography on DEAE-cellulose. In no case was it possible to demonstrate a protein with intermediate electrophoretic mobility. No protein peak or enzymatic activity could be detected between the two native peaks after ion-exchange chromatography. Ninety-five per cent of the applied activity and protein was recovered in the two native enzyme peaks. The elution pattern was similar to that previously reported (Wells and Hanahan, 1969).

Discussion

The data presented above clearly show that the two forms of phospholipase A_2 of *C. adamanteus* venom are dimers in the native state. Sedimentation equilibrium in 8 M urea and at acid pH, and gel filtration in 6 M guanidine hydrochloride–0.1 M mercaptoethanol all suggest a monomer molecular weight of 15,000. It may be noted that this molecular weight corresponds to the molecular weight reported for the pancreatic enzyme (de Haas *et al.*, 1968), several snake venom enzymes (Sakhibov *et al.*, 1970; Augustyn and Elliott, 1970; Tu *et al.*, 1970; Wahlström, 1971; Salach *et al.*, 1970; Salach *et al.*, 1971), and the enzyme from *C. atrox* (Wu and Tinker, 1969). However, Hachimori *et al.* (1971) have found a single protein of mol wt 30,000 in this latter venom. The *C. atrox* enzyme also is a dimer of 15,000 molecular weight subunits.

Two lines of evidence suggest that each protein is composed of identical subunits, although each protein contains different subunits. Electrophoresis at a variety of pH's consistently shows only one protein band for each protein, whether carried out under conditions where the enzymes are dimeric or monomeric. At acid pH the α and β subunits have identical mobilities, but at alkaline pH the β subunit has a higher mobility. This would suggest that the β subunit contains a higher proportion of negatively charged groups.

The carboxypeptidase digestion would also suggest that each protein is composed of identical subunits. The finding of 2 moles of leucine released per 30,000 g of protein is consistent with two subunits which are identical. The data might also suggest a C-terminal sequence Leu-Cys-Gly-Ser- for each protein. This is in contrast to the C-terminal sequence reported for the pancreatic enzyme, Cys-Trp-Lys-Lys- (Maroux *et al.*, 1969).

The conversion of dimer to monomer is readily reversible, if carried out at low pH or in the presence of urea; however, dissociation at high pH appears to lead to irreversible denaturation. The inability to demonstrate hybrid proteins is particularly interesting. Attempts to form hybrid enzymes have been carried out under a wide variety of conditions, and it would appear that if a hybrid is formed it is unstable.

There are a large number of similarities between the two enzymes, including identical enzymatic activities, very similar amino acid compositions, similar C-terminal amino acids, identical molecular weights, and an apparent blocked N-terminal amino acid. In addition several attempts to cleave the enzyme with various enzymes have been unsuccessful, and both enzymes behave similarly. There are, however, two properties in which they differ. First is the difference in electrophoretic mobility, which may arise from a single replacement per subunit of an acidic amino acid by an uncharged amino acid or the amide of an acidic amino acid. Secondly empirical observations made during numerous preparations of crystalline enzymes suggest a difference in solubility. Thus the α form of the enzyme crystallizes optimally from 2.0 M ammonium sulfate, whereas the β enzyme requires 2.2 M ammonium sulfate. The β enzyme appears to be more soluble in the cold than at room temperature, but the α enzyme is more soluble at room temperature.

It should be noted that others have unknowingly demonstrated the lack of hybridization. For example, Saito and Hanahan (1962) used a heat treatment at pH 3.0 in their purification of the two phospholipases A_2 from *C. adamanteus* venom. Based upon data presented here this would lead to the formation of monomers. However, these authors found only two active enzymes after purification of this heat-treated venom.

The observations on these proteins are not unlike the observations on some of the hemoglobin variants (Antonini and Brunori, 1970) in which it is well established that single amino acid replacements can lead to altered subunit interactions. At this time there is no information which could be used to explain the lack of hybrid formation.

Although it is difficult to prove absolutely that the monomeric forms of these enzymes do not have enzymatic activity, all available data suggest that the dimer is the active form of these enzymes. Under no circumstance was it possible to demonstrate any appreciable concentration of monomer at neutral pH, even at concentrations used in the assay. In addition all treatments which lead to dissociation lead to loss of enzymatic activity. Of course treatment at acid pH may lead to protonation of groups which must be unprotonated for activity. The correlation between loss in enzymatic activity and conversion to monomer in various urea containing solutions (Table I) would appear to offer the best evidence for the dimer being the active form of the enzyme. One cannot rule out the possibility that urea causes alterations in the monomer which inactivate it without irreversible denaturation.

Acknowledgments

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Spectral Peculiarities of the Monomer–Dimer Transition of the Phospholipases A_2 of *Crotalus adamanteus* Venom*

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ABSTRACT: Solvent perturbation difference spectra of *Crotalus adamanteus* phospholipases A_2 gave several anomalous results. With 20% methanol and 20% dimethyl sulfoxide as perturbant, there was an unexpected blue shift in the 300-nm region. This blue shift was much less pronounced in 20% glycerol and not detected in 20% sucrose. In the presence of methanol and dimethyl sulfoxide the tryptophans contributed an unexpected large proportion of the difference spectra. When difference spectra were obtained in 8 M urea all the anomalous effects were abolished. Spectral examination of the dimer-to-monomer conversion caused either by urea or at low pH show that in the dimer there is higher absorbance at 293 and 285 nm with a broad band centered near 300 nm. Circular dichroism also suggests that tryptophans in the dimer are in an unusual environment. During conversion of dimer to monomer there is a quenching of tryptophan fluorescence. The pH dependence of this quenching suggests that two protons per subunit are involved in the dimer-to-monomer conversion. Circular dichroism of the native protein in the far ultraviolet suggests a high α-helix content of near 70%. In the monomeric state the conformation is uncertain. Spectral examination of the monomer in 8 M urea suggests the dimer-to-monomer conversion in urea is a two-step process: (1) conversion of dimer to monomer with loss of enzymatic activity in 4 M urea and (2) further conformational changes upon going from 4 to 8 M urea.

During a study of the monomer–dimer transition of the phospholipases A_2 from *Crotalus adamanteus* venom (Wells, 1971), several interesting spectral properties of the proteins were noted. In particular solvent perturbation difference spectra, spectral changes caused by urea, circular dichroism and fluorescence measurements, and concentration difference spectra all suggest that some tryptophans and tyrosines are in a peculiar environment in the dimeric forms of these enzymes.

This paper presents these spectral data and a discussion of their possible implications in the structure of the dimeric forms of these enzymes.

Materials and Methods

C. adamanteus phospholipases A_2 were isolated and assayed as previously described (Wells and Hanahan, 1969; Wells, 1971). Other reagents were prepared as in the previous paper (Wells, 1971).

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