Acknowledgments

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

Michael A. Wells

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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Solvent perturbation studies were performed as described by Herskovits (1967) and Herskovits and Sorensen (1968a) using a Cary Model 15 spectrophotometer. A linear response for difference spectra was obtained with samples having an absorbance up to 1.5 and all experiments were performed in this linear range. In all spectral measurements, corrections for solvent blanks were made. Fluorescence emission spectra were obtained with a Perkin-Elmer fluorescence spectrophotometer Model MPF-2A. Circular dichroism was measured in a Cary Model 60 spectropolarimeter. Mean residue ellipticity was calculated using a mean residue weight of 112, which was calculated from the amino acid composition (Wells and Hanahan, 1969). Concentration difference spectra (Fisher and Cross, 1965; Rupley *et al.*, 1967) were measured in a Cary Model 15 spectrophotometer. I am indebted to Dr. Richard Lord and Dr. John Rupley for making these measurements.

Ultracentrifugation was carried out as described before (Wells, 1971).

Results

Although there are two forms of phospholipase A_2 in *C. adamanteus* venom (Saito and Hanahan, 1962; Wells and Hanahan, 1969), their behavior in all the experiments reported in this paper were identical. Therefore, no distinction will be made as to which data presented were collected on which form of the enzyme.

Solvent Perturbation Studies. During attempts to obtain some data which might be useful in explaining the lack of hybridization of the monomeric forms of these enzymes (Wells, 1971), solvent perturbation spectra were measured. Even though these data did not show any difference between the two forms of the enzyme, they did point out some peculiar features of the structure of the enzymes.

Figure 1 shows the difference spectra obtained with the following perturbants: 20% methanol (v/v), 20% dimethyl sulfoxide (v/v), 20% glycerol (v/v), and 20% sucrose (w/w). All solutions were prepared in 0.01 M Tris-HCl (pH 8.0).

There are several striking features of these spectra: (1) The most unusual aspect is the pronounced blue shift in the 298- to 302-nm region caused by the perturbants. This effect is most prominent in the presence of methanol (curve 1) with decreasing effects in the presence of dimethyl sulfoxide (curve 2) and glycerol (curve 3) and no effect in the presence of sucrose (curve 4). (2) The magnitude of the absorbance change in the 298- to 302-nm region is also most remarkable, especially in the case of methanol, where the molar absorbancy difference in the 298- to 302-nm region exceeds that at 292 nm, which is the usual wavelength maximum for tryptophan difference spectra (Herskovits and Sorensen, 1968a). (3) Although these proteins contain 2.28 times as much tryosine as tryptophan (Wells and Hanahan, 1969), the solvent perturbation spectra do not reflect this in all cases. For example, in methanol and dimethyl sulfoxide the spectra are more characteristic of a tryptophan-rich protein, whereas in glycerol and sucrose the more expected spectra are obtained (Herskovits and Sorensen, 1968b).

Table I contains the calculated molar absorptivity changes at four wavelengths for the perturbants shown in Figure 1. Also included in Table I are data collected in 90% D_2O. In D_2O there is an unexpected red shift in the 300-nm region.

Figure 2 shows solvent perturbation spectra obtained in the presence of 8 M urea using the same solvents and buffer as described above. There are several changes when these

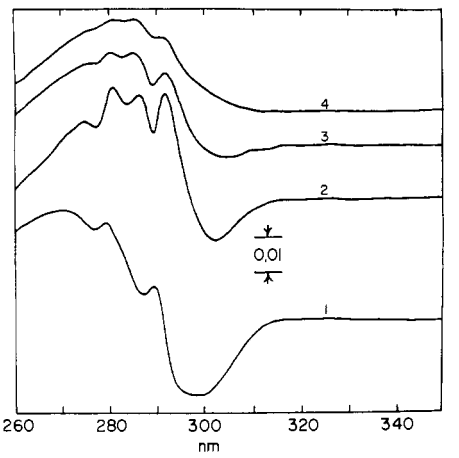

FIGURE 1: Solvent perturbation difference spectra of phospholipase A_2. All solutions contained 0.01 M Tris·HCl (pH 8.0) and 0.54 mg/ml of protein. The protein in buffer alone served as the reference and the protein in the presence of the perturbant was the sample. Curve 1 was obtained with 20% methanol as perturbant; curve 2 with 20% dimethyl sulfoxide; curve 3 with 20% glycerol and; curve 4 with 20% sucrose. A change of 0.01 *A* is shown between the arrows. The unperturbed sample had an absorbance of 1.27 at 280 nm.

spectra are compared to those obtained in the absence of urea (Figure 1). The most interesting change is again seen in the 300-nm region, where the prominent blue shift noted above is no longer evident. All of the spectra now resemble a tryosine-rich protein, and the enhanced tryptophan contribution to the difference spectra has been abolished, especially in the case of methanol and dimethyl sulfoxide. Table I also contains the calculated molar absorptivity changes in the presence of 8 M urea.

Spectral Perturbations Caused by Urea. The profound changes in the solvent perturbation spectra caused by the presence of 8 M urea prompted an investigation of possible spectral changes caused by urea itself. Figure 3 presents some results of these studies. Curve 1 was obtained with 0.45 mg/ml of protein dissolved in 0.01 M Tris·HCl (pH 8.0)

TABLE I: Spectral Changes by Solvent Pertubations in the Presence and Absence of 8 M Urea.[a]

Perturbant	Molar Absorptivity Differences 298–302 nm	290–293 nm	286–288 nm	278–280 nm
D_2O (90%)	200	−430	−270	−320
Methanol (20%)	−1125	535	535[b]	1435
Methanol (20%) in 8 M urea	0	695	1225	1180
Dimethyl sulfoxide (20%)	−640	1600	1545	1650
Dimethyl sulfoxide (20%) in 8 M urea	0	1385	1810	1330[b]
Glycerol (20%)	−160	1065	1385	1330
Glycerol (20%) in 8 M urea	0	1170	1435	1225[b]
Sucrose (20%)	0	635	845	800
Sucrose (20%) in 8 M urea	0	795	920	830[b]

[a] All solutions contained 0.01 M Tris·HCl (pH 8.0) and 0.54 mg of protein/ml. [b] No discernable peak.

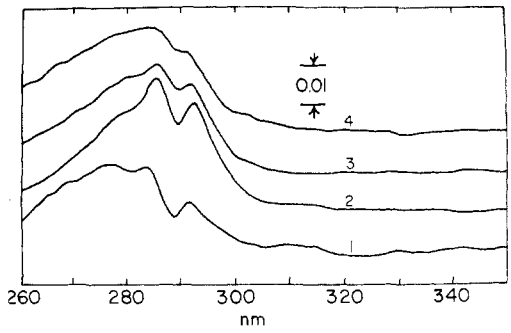

FIGURE 2: Solvent perturbation difference spectra of phospholipase A_2 in the presence of 8 M urea. All conditions are the same as for Figure 1. Curve 1 was obtained with 20% methanol as perturbant; curve 2 with 20% dimethyl sulfoxide; curve 3 with 20% glycerol and; curve 4 with 20% sucrose. A change of 0.01 A is shown between the arrows. The unperturbed sample had an absorbance of 1.25 at 280 nm.

in the sample position, and the same concentration of protein dissolved in 4 M urea in 0.01 M Tris·HCl (pH 8.0) in the reference position. In the absence of urea the protein showed a higher absorbance in the 295- to 305-nm region, with peaks at 293 and 285 nm, and a shoulder near 278 nm. The changes in molar absorptivity in the presence of 4 M urea were 300 nm, −1850; 293 nm, −3840; and 285 nm, −2670.

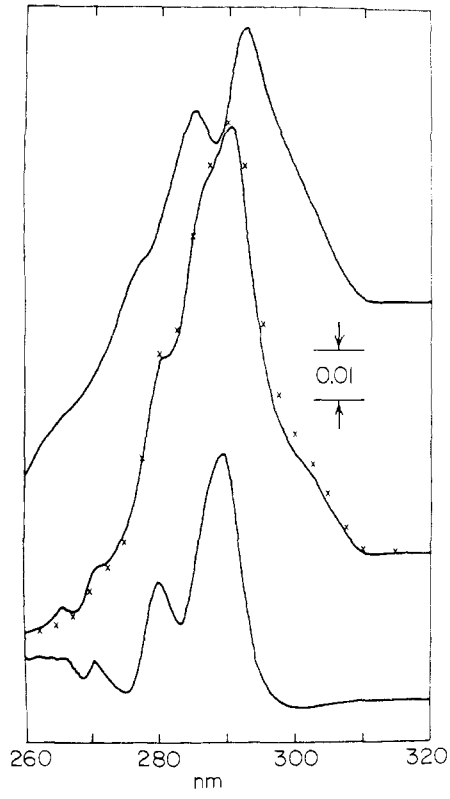

FIGURE 3: Spectral perturbations caused by urea. All solutions contained 0.01 M Tris·HCl (pH 8.0) and 0.45 mg/ml of protein. Curve 1 was obtained with the protein in buffer as the sample and in 4 M urea as the reference. Curve 2 was obtained with protein in buffer as the sample and in 8 M urea as the reference. Curve 3 was obtained with the protein in 4 M urea as the sample and in 8 M urea as the reference. A change of 0.01 A is shown between the arrows. The unperturbed sample had an absorbance of 1.03 at 280 nm. The X's on curve 2 represent the summation of curves 1 and 3.

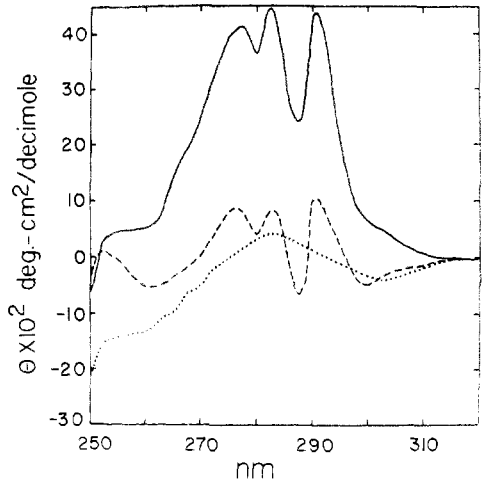

FIGURE 4: Circular dichroism of phospholipase A_2 in the 250-to 320-nm region. The solid line was obtained in 0.01 M Tris·HCl (pH 8.0). The dashed line in 4 M urea in 0.01 M Tris·HCl (pH 8.0). The dotted line in 0.01 N HCl or 8 M urea in 0.01 M Tris·HCl (pH 8.0).

Similar results were obtained when the protein in 0.01 M Tris·HCl (pH 8.0) was compared to solutions containing 1, 2, and 3 M urea in the same buffer. The shape of the curves were identical, only the magnitude of the differences increased as the urea concentration increased to 4 M.

The character of the difference spectra changed as the urea concentration was increased to 8 M. Curve 2 in Figure 3 shows the results when 0.45 mg/ml of protein in 0.01 M Tris·HCl (pH 8.0) was compared to the same concentration of protein in 8 M urea in 0.01 M Tris·HCl (pH 8.0). In this case there is a definite shoulder near 302 nm and a peak at 291 nm with shoulders near 278 nm and 281 nm. The molar absorptivity changes in 8 M urea are 302 nm, −1000; 291 nm, −5660; 287 nm, −4930; and 281 nm, −2600.

Curve 3 in Figure 3 represents a difference spectrum taken with 0.45 mg/ml of protein in 4 M urea in the sample position and the same concentration of protein in 8 M urea in the reference position. Both solutions also contained 0.01 M Tris·HCl (pH 8.0). The protein has a higher absorbance in 4 M urea with peaks at 289, 280, and 271 nm. The molar absorptivity changes between 8 M and 4 M urea are 289 nm, −3410; 280 nm, −1570; and 271 nm, −480. The X's on curve 2 in Figure 3 represent the summation of curves 1 and 3. It can be seen that there is close agreement.

Circular Dichroism Spectra. In order to help in distinguishing which absorption bands might be due to tryptophan, circular dichroism was measured in the following solutions: (1) 0.01 M Tris·HCl (pH 8.0); (2) 1, 2, 3, 4, and 8 M urea in the same buffer; and (3) 0.01 M HCl. Some of the results are shown in Figure 4.

The solid line in Figure 4 represents the spectrum obtained in 0.01 M Tris·HCl (pH 8.0). The peaks and mean residue ellipticities are: (a) shoulder near 302 nm, +500; (b) 291 nm, +4400; (c) 282 nm, +4450; (d) 277 nm, +4100; and (3) shoulder near 267 nm, +1750. In 4 M urea, dashed line in Figure 4, (a) 300 nm, −500; (b) 291 nm, +1005; (c) 282 nm, +1800; and (d) 277 nm, +1850. In 8 M urea and 0.01 M HCl identical results were obtained, dotted line in Figure 4. There are only vague indications of peaks near 302 nm, −450 and 282 nm, +400. As the urea concentration was increased to 4 M there was a progressive change from the solid curve to the dashed curve.

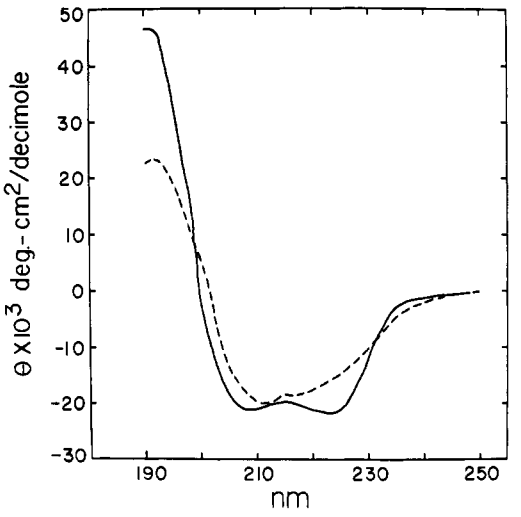

FIGURE 5: Circular dichroism of phospholipase A_2 in the 190- to 250-nm region. The solid line was obtained in 0.01 M Tris·HCl (pH 8.0). The dashed line was obtained in 0.01 N HCl.

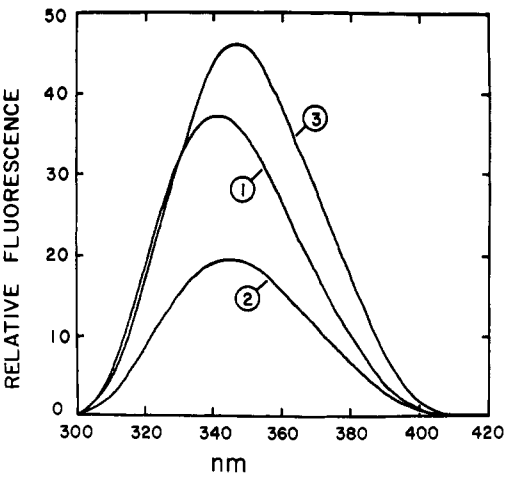

FIGURE 6: Fluorescence emission spectra of phospholipase A_2. The emission spectra was measured while activating at 292 nm. Curve 1 was obtained in 0.01 M Tris·HCl (pH 8.0). Curve 2 was obtained in 0.01 N HCl or in 4 M urea in 0.01 M Tris·HCl (pH 8.0). Curve 3 was obtained in 8 M urea in 0.01 M Tris·HCl (pH 8.0). All solutions contained 0.08 mg of protein/ml.

In the far-ultraviolet region, spectra were obtained in 0.01 M Tris·HCl (pH 8.0) and 0.01 M HCl only, due to difficulties caused by the absorbance of urea. Figure 5 presents these data. The solid line was obtained in 0.01 M Tris·HCl (pH 8.0), with peaks at 223 nm, −21,500; 208 nm, −21,000; 191 nm, +46,500. The dashed line represents the spectrum in 0.01 M HCl with a shoulder near 218 nm, −18,500, and peaks at 212 nm, −20,000 and 191 nm, +23,500. The data for the enzyme at pH 8.0 would indicate a high α-helix content, which would be estimated to be approximately 70% based on the ellipticity observed at 223 and 208 nm (Timasheff and Gorgunoff, 1967).

Fluorescence Measurements. All emission spectra were obtained while activating at 292 nm. In 0.01 M Tris·HCl (pH 8.0) the spectrum shown in Figure 6, curve 1 was obtained. The emission maximum was at 340 nm. Curve 2 in Figure 6 shows the emission spectrum obtained either in 0.01 N HCl or 4 M urea in 0.01 M Tris·HCl (pH 8.0). There is marked quenching of the fluorescence and a shift of the maximum to about 345 nm. Curve 3 is the emission spectrum observed in 8 M urea in 0.01 M Tris·HCl (pH 8.0). There is marked enhancement of fluorescence and a further shift in the maximum to 350 nm.

The lower curve in Figure 7 shows the effect of pH on the fluorescence of the proteins. There is an abrupt quenching of fluorescence between pH 4.0 and 3.0. This is the same pH region in which the dimer is converted to monomer (Wells, 1971). Assuming that the quenching can most simply be represented by the following equilibrium

$$A + nH^+ \rightleftharpoons B \qquad (1)$$

where A represents the form of the protein present at pH greater than 4.0 and B represents the form of the protein present at pH less than 3.0. If we let α represent the fraction of the protein in form A, and $1 - \alpha$ represents the fraction in form B, and further assuming that the quenching is a measure of the conversion of A to B, then a plot of log $[\alpha/(1 - \alpha)]$ *vs.* pH should be straight line with a slope equal to n, the number of protons involved in eq 1. Such a plot is shown in the upper portion of Figure 7 and has a slope of 2.

If we further assume that the equilibrium represented in eq 1 actually represents the conversion of dimer to monomer, as represented in eq 2, then the slope of the log $[\alpha/(1 - \alpha)]$

$$\text{dimer} + nH^+ \rightleftharpoons 2\text{monomer} \qquad (2)$$

plot is equal to $n/2$ or in other words, gives a value for the number of protons taken up by each subunit. Thus, it would appear that at least two protons per subunit are somehow involved in the conversion of dimer to monomer at low pH.

Concentration Difference Spectra. In order to obtain further evidence that the spectral changes which were observed in the presence of urea were actually an effect of the dimer–monomer transition, and not merely an effect of urea, con-

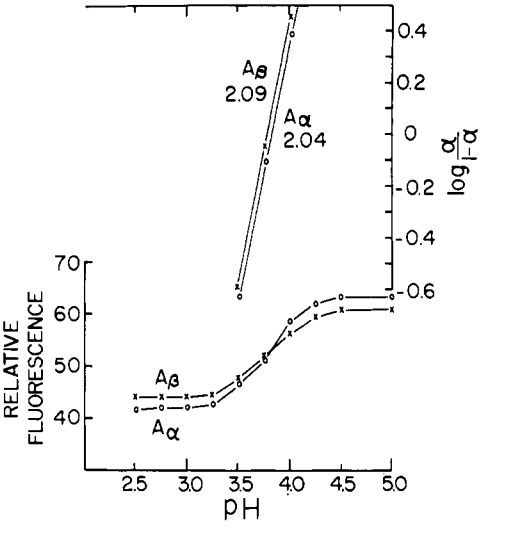

FIGURE 7: Lower portion: effect of pH on Fluorescence emission at 340 nm. Relative peak heights measured with constant excitation slit (2.5 mμ) and emission slit (5 mμ). Each solution contained 0.1 mg/ml of protein in citrate buffer at the indicated pH. A_α and A_β refer to the two forms of phospholipase A_2. The upper portion of the figure is a plot of the log of the fraction of dimer, α, over fraction of monomer, $1 - \alpha$, *vs.* pH. The numbers (2.09, 2.04) are the calculated slopes. See text for details of this calculation.

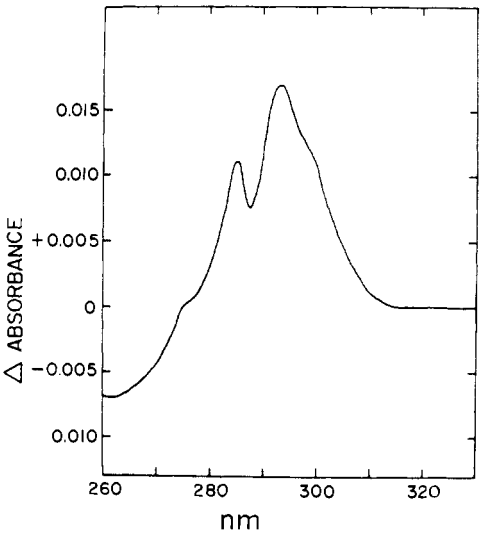

FIGURE 8: Concentration difference spectrum of phospholipase A_2 at pH 3.75. The sample cuvet contained 2.7 mg/ml of protein in a 0.1-cm cell and the reference 0.054 mg/ml of protein in a 5.0-cm cell. Samples were in 0.01 M sodium acetate buffer (pH 3.75).

centration difference spectra were obtained at pH 3.75. This pH was chosen since it appeared to represent the midpoint of the dimer–monomer conversion (Figure 7, Wells, 1971). Figure 8 represents the difference spectrum obtained when 2.7 mg/ml of protein in a 0.1-cm cell (in the sample position) was compared to 0.054 mg/ml of protein in a 5.0-cm cell (in the reference position). Samples were dissolved in 0.01 M sodium acetate buffer (pH 3.75). Corrections for path-length differences and solvent blanks were made. Compared to the monomer, the dimer has peaks at 293 and 285 nm, with a shoulder near 278 nm and a broad band near 300 nm. This spectrum is identical in all important aspects to dimer–monomer difference spectrum obtained in Figure 3, curve 1, where the monomer was formed in 4 M urea.

Ultracentrifugation. Low-speed sedimentation equilibrium studies were carried out in: (1) 0.01 M Tris (pH 8.0), (2) 20% methanol in 0.01 M Tris (pH 8.0), and (3) 20% dimethyl sulfoxide in 0.01 M Tris (pH 8.0). In all cases, the molecular weight was 30,000 ± 500. This indicates that these solvents do not cause the dimer to be converted to monomer.

Discussion

At this point, it is worth summarizing the unusual spectral properties of the proteins reported here. The blue shift in the 295- to 305-nm region caused by methanol, dimethyl sulfoxide, and glycerol, and the red shift caused by D_2O are unexpected based on published solvent perturbation studies (Herskovits and Sorensen, 1968a,b). The magnitude of the absorbance change does not correlate with the size of the perturbant or their relative effects on the shift of the tryptophan peak at 292 nm as reported for model compounds (Herskovits and Sorensen, 1968a). The relative diameters for D_2O–methanol–dimethyl sulfoxide–glycerol–sucrose are 1.0:1.4:2.4:2.6:4.7 (Herskovits, 1967). The relative molar absorbancy changes for the first tryptophan peak (292 nm) is 1.0:1.16:2.41:1.50:0.94. (Herskovits, 1967). In the case of phospholipase A_2 the relative molar absorbancy changes at 300 nm is 1.0:5.63:3.20:0.80:0.0. Therefore, the spectral changes in the 300-nm region do not appear to correlate with any published data on model compounds.

Furthermore, if one calculates the apparent number of exposed tryptophans and tyrosines for the native enzyme, one is lead to the anomalous result that as the size of the perturbant increases, the number of exposed groups also increases. For example, the number of exposed tryptophans calculated from data in D_2O, methanol, dimethyl sulfoxide, glycerol, and sucrose, is 2.1, 2.3, 3.3,3 .5, and 3.4, respectively, whereas the number of exposed tyrosines in the same solvents is 0.3, 3.0, 4.7, 8.8, and 9.9. This would indicate that the tryptophans in the native proteins are contributing an unexpectedly large proportion of the difference spectra in some solvents. Further, this shows that solvent perturbation studies are of little value in the study of these proteins.

Granting that the solvent perturbation studies do not yield any useful information concerning chromophore exposure, they do nonetheless point out a significant structural change when the proteins are placed in 8 M urea. In this situation all the anomalous effects noted above are eliminated. For example, the data in Table I suggest that the number of exposed tryptophans is three to four tyrosines eight to nine for all the perturbants studied. The data from the solvent perturbation studies would suggest that all the anomalous spectral observations could be attributed to some peculiar structural feature of the native dimeric state of the enzymes.

Additional data are available to support this hypothesis. First, the spectral changes observed when the dimer is compared to monomer formed in 4 M urea. In the dimeric state there is enhanced absorbance in the 300-nm region, which correlates with the region where anomalous behavior was observed in the solvent perturbation studies. In addition, the peak at 293 nm suggests the involvement of tryptophan. The peak at 285 nm would also suggest that tyrosine may be in a different state in the dimer than in the monomer. The fact that these spectral changes increase in intensity from 0 to 4 M urea would correlate with the previously reported conversion of dimer to monomer in this urea concentration range (Wells, 1971).

The circular dichroism data also strongly suggest that some tryptophans are in an unusual environment in the dimer. In Figure 4, the spectrum of the native enzyme is characterized by an unusually large ellipticity with a great deal of structure in the spectrum. As the protein is placed in increasingly stronger solutions of urea, up to 4 M, there is a diminution of the ellipticity, which also correlates with the conversion of dimer to monomer. At the least, these data would suggest that some tryptophans are in a highly constrained environment in the dimer, since this spectrum resembles low-temperature spectra obtained with model compounds (Strickland *et al.*, 1969). It is possible that some of the spectrum may be contributed by tyrosine, but the 300-nm peak must certainly arise from tryptophan (Strickland *et al.*, 1969). This would strongly implicate tryptophan as the cause of absorption anomalies in this region of the spectra.

The quenching of fluorescence in the pH range 4.0–3.0 would also indicate that there is an environmental change of some tryptophans on going from dimer to monomer. This quenching correlates with previous observations on the decrease in molecular weight in this pH range (Wells, 1971).

Finally, and perhaps most convincing, are the results of the concentration difference spectra obtained at pH 3.75. This pH was chosen on the basis of preliminary studies which suggest that this is the midpoint of the dimer–monomer conversion. The spectral properties of the dimer compared to monomer, Figure 8, are in complete accord with those discussed above. Thus, the dimer has higher absorbance near 300 nm, with peaks at 293 and 285 nm. The spectrum was identical with that obtained when dimer was compared to monomer in 4 M urea. All available data, therefore, strongly suggests that in the dimer form of these enzymes some tryptophans, and possibly tyrosines, are placed in an environment which leads to enhanced absorptivity, especially at longer wavelengths, striking circular dichroism spectra, and unusual sensitivity to certain solvents.

The red shift in the spectrum upon dimerization could either be attributed to placing the tryptophans in an environment of lower polarity than water or the juxtaposition of a negatively charged group (Wetlaufer, 1962; Donovan *et al.*, 1961; Leach and Sheraga, 1960). Even though it is not possible to distinguish between these possibilities on the basis of the available data, there are some indications that charged groups may be involved in these proteins. First, there are the unusual results with solvent perturbation studies. Inasmuch as these solvents did not lead to disaggregation of the dimer, it must be assumed that the spectral properties are a result of a conformational change in the protein. Although a small change might not alter the polarity of the environment appreciably, it could lead to a small movement of a negatively charged group away from a tryptophan. Donovan *et al.* (1961) have shown that charge effects on tryptophan are extremely sensitive to distance, therefore, it may be reasonable to ascribe some of the spectral peculiarities to the proximity of a charged group. Secondly, some comments on the circular dichroism spectra are in order. The spectra would be consistent with the tryptophans being in an environment which severely restricts conformational mobility (Strickland *et al.*, 1969). However, there have been no reports on charge effects on the circular dichroism of tryptophan and, therefore, one cannot ascribe any of the spectrum to this possible effect.

Three lines of evidence would suggest that in going from 4 M urea to 8 M urea there is a conformational change in the monomer. First, there are spectral changes (Figure 3) which indicate an additional change in environment of some tryptophans. Secondly, there is an almost complete loss of activity in the circular dichroism spectra (Figure 4). Finally, there is an enhanced fluorescence emission in 8 M urea when compared to 4 M urea (Figure 6). It would, therefore, appear that in urea there is first a conversion of dimer to monomer with concomitant loss in enzymatic activity (Wells, 1971), and then a further change in conformation of the monomer at higher urea concentrations. Although this process would appear complex it is readily reversible whether carried out in 4 or 8 M urea (Wells, 1971).

The circular dichroism of the dimer in the far-ultraviolet region (Figure 5) would suggest a high α-helix content. The circular dichroism spectrum of the monomer at pH 2.0 (Figure 5) would suggest a conformational change when compared to the dimer, although it is not obvious what the conformation might be. The presence of a shoulder near 218 nm (Figure 5) might be suggestive of some β structure. However, this conclusion is regarded as tenuous. A high α-helix content has also been reported for the phospholipase A_2 from porcine pancreas (Scanu *et al.*, 1969).

The fluorescence-quenching data (Figure 6) would suggest that the conversion of dimer to monomer proceeds with uptake of two protons per monomer, but there are not sufficient data at this time to warrant discussion of this point. A more detailed examination of the pH-dependent monomer–dimer equilibrium of these proteins is under investigation.

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