Hixson, H. F., Jr., Finkenstadt, W. R., and Laskowski, M., Jr. (1967), *Federation Proc. 26*, 276.
Kenner, G. W., and Laird, A. H. (1965), *Chem. Commun.*, 305.
Kozlov, L. V., Ginodman, L. M., Orekhovich, V. N., and Valueva, T. A. (1966), *Biokhimiya 31*, 315.
Kunitz, M. (1947), *J. Gen. Physiol. 30*, 291.
Laskowski, M., Jr., Ehrenpreis, S., Donnelly, T. H., and Scheraga, H. A. (1960), *J. Am. Chem. Soc. 82*, 1340.
Laskowski, M., Jr., and Scheraga, H. A. (1956), *J. Am. Chem. Soc. 78*, 5793.
Lebowitz, J., and Laskowski, M., Jr. (1962), *Biochemistry 1*, 1044.
Melamed, M. D. (1967), *Biochem. J. 103*, 805.
Ohno, M., and Izumiya, N. (1965), *Bull. Chem. Soc. Japan 38*, 1831.
Ornstein, L. (1964), *Ann. N. Y. Acad. Sci. 121*, 321.
Ozawa, K., and Laskowski, M., Jr. (1966), *J. Biol. Chem. 241*, 3955.
Peller, L., and Alberty, R. A. (1959), *J. Am. Chem. Soc. 81*, 5907.
Raymond, S. (1962), *Clin. Chem. 8*, 455.
Rigbi, M., and Greene, L. J. (1968), *J. Biol. Chem. 243*, 5457.
Wentworth, W. E. (1965), *J. Chem. Educ. 42*, 96.
Williams, D. E., and Reisfeld, R. A. (1964), *Ann. N. Y. Acad. Sci. 121*, 373.
Worthington Biochemical Corp. (1967), Enzyme Data Sheet.
Wu, Y. V., and Scheraga, H. A. (1962), *Biochemistry 1*, 698.

On the Conformation of Denatured Proteins*

Jan Hermans, Jr.,† David Puett,‡ and Giuseppe Acampora§

ABSTRACT: The effect of urea and ethanol on the denaturation of sperm whale myoglobin at low pH has been investigated. Both agents are found to increase the pH at which denaturation occurs, and are therefore denaturants. The molar free energies of denaturation at neutral pH ($\Delta G°_{den}$) in the various solvents have been calculated from these data. It is pointed out that for the reaction: native myoglobin to perfect random coil, quite different changes in $\Delta G°_{den}$ are predicted on the basis of model compound experiments. In fact, the prediction is that ethanol should increase the stability of native myoglobin with respect to the random coil. Optical rotation studies of denatured myoglobin in the presence of urea and ethanol reveal that the conformation of denatured myoglobin is not a random coil, except at high urea molarity. At 70% ethanol concentration, denatured myoglobin is as helical as the native protein, although potentiometric titration indicates that all the histidine side chains have a normal pK, and the sedimentation constant is typical of a swollen (or asymmetric) molecule. Thus the conformation of the denatured protein varies with the concentration and nature of the denaturant, and it is suggested that this can explain the discrepancy between predicted and observed effect of the denaturants on $\Delta G°_{den}$. The optical rotation of denatured lysozyme (oxidized or reduced) indicates the presence of less helix than in denatured myoglobin and also the induction of helix in reduced lysozyme by the addition of ethanol takes place at a higher ethanol concentration. Thus, it is possible that the secondary structure of each denatured protein is similar to the secondary structure of its native conformation. This structure may, in turn, provide a nucleus in the folding process.

We have recently shown how measurements of the reversible denaturation of myoglobin (Acampora and Hermans, 1967) at low pH can be used to calculate molar free energies of denaturation for the neutral molecule (Hermans and Acampora, 1967). These measurements can also be used to obtain a criterion to see how well the equilibrium can be described by the two-state model (Brandts, 1964, 1965; Lumry *et al.*, 1966). This study showed that a two-state equilibrium is closely approached, and yielded values of $\Delta G°_{den}$ = 16 kcal/mole, $\Delta H°_{den}$ = 40 kcal/mole, $(\Delta C_p°)_{den}$ = 1.40 kcal/(mole deg) for the changes in molar free energy, enthalpy, and heat capacity upon denaturation at 25° in 0.1 M KCl. The large value of $\Delta H°$ is attribu-

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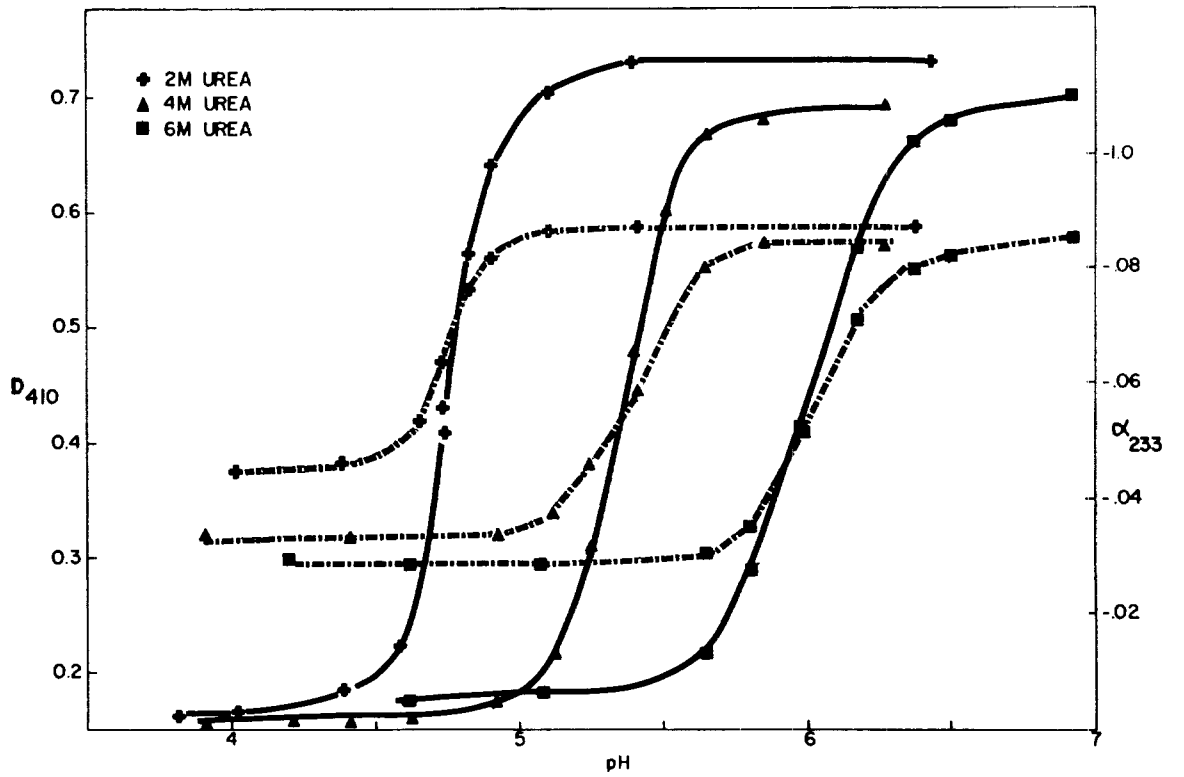

FIGURE 1: Optical rotation at 233 mμ (dashed curves) and optical density at 410 mμ (solid curves) of sperm whale myoglobin solutions of varying urea concentrations as a function of pH. Protein concentration is 0.1 mg/ml (see Figure 4 for the curve in the absence of urea).

table to the breaking of hydrogen bonds (Schellman, 1956; Hermans, 1966a), while the large value of $\Delta C_p°$ is evidence of the breaking of hydrophobic bonds which is accompanied by a *negative* change in ethalpy (Brandts, 1964, 1965; Némethy and Scheraga, 1962).

We thought that it might be possible to obtain some quantitative information regarding the relative importance of these classes of interaction by considering also the effect of the denaturants urea and ethanol on the stability, since quantitative experimental data on the effects of these denaturants on model systems are available for comparison (Cohn and Edsall, 1943; Nozaki and Tanford, 1963; Hermans, 1966b). Specifically both ethanol and urea are expected to weaken hydrophobic interactions, whereas urea probably weakens and ethanol should strengthen the hydrogen bond. Finally, one may try to compare such estimates of the importance of these interactions with predictions based on the crystal structure (Kendrew *et al.*, 1961).

Experimental Section

Reagents were: KCl (Mallinckrodt Reagent), urea (Baker and Adamson Reagent, once recrystallized from ethanol), mercaptoethanol (Eastman product 4196), citric acid (Baker and Adamson Reagent), and KOH (Baker and Adamson Reagent).

Crystalline sperm whale myoglobin was obtained from Pierce Chemical Co. (lot no. 24001). Crystalline chicken egg white lysozyme was obtained from Worthington Biochemical Corp. (lot no. LYSF 7CB). Denaturation measurements using the Zeiss PMQII spectrophotometer and the Cary 60 spectropolarimeter were performed as described before (Acampora and Hermans, 1967). The sensitivity of the Zeiss spectrophotometer is sufficient that in these measurements the slit width is so small that the spectra do not depend upon the slit width within experimental error. In our measurements with the Cary 60 polarimeter we have used the automatic slit control to provide a constant spectral band pass of 10 mμ. Use of a lower band pass will not noticeably affect the values of the extrema of the rotation. The response was damped sufficiently to decrease the noise to at most 1% of the full range (0.1°). (At wavelengths > 220 mμ a 1-cm cell was used, at lower wavelengths a 1-mm cell.) The scan speed was sufficiently low not to affect the results. Solutions for these measurements were made by mixing protein, KCl, and buffer stock solutions, adding 95% ethanol or urea stock solution, and adjusting the volume to 10 ml. Thus solutions referred to below as containing 10% ethanol were made as described above, using 1 ml of 95% ethanol. The solutions contained approximately 0.1 mg/ml of protein, 0.1 M KCl, and 0.01 M citrate. Some measurements at higher wavelength were carried out with solutions of concentration 1 mg/ml. The refractive index dispersion (used in calculating the reduced mean molar rotation) was accounted for by using the Sellmeier equation. The constants of this equation were calculated from the refractive index tables reported by

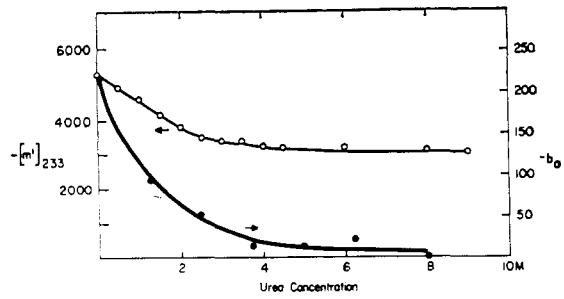

FIGURE 2: Reduced rotation at 233 mμ and b_0 for globin solutions at low pH as a function of urea concentration.

Fasman (1963) or from the International Critical Tables (1930).

The reversibility of the reactions studied was not investigated extensively. Rather, we have relied on the observation of reversibility at low pH in the absence of urea and ethanol (Acampora and Hermans, 1967) and in neutral solutions in the presence of high concentrations of guanidine or urea (Litman, 1966; Khalifah, 1968).

Potentiometric titrations were carried out as described before for ionizable polypeptides (Hermans, 1966a) using 50 mg of myoglobin in 10 ml of water, in a mixture of 3 ml of 95% ethanol and 7 ml of water, and in a mixture of 5 ml of ethanol and 5 ml of water, always in the presence of 0.1 M KCl. Precise concentrations were calculated on the basis of an extinction coefficient of 17.1×10^4 l./(mole cm) at 408.5 mμ (Litman, 1966).

Sedimentation studies were performed using a Spinco Model E ultracentrifuge with schlieren optics. For sedimentation velocity experiments, a synthetic boundary cell was used and solution and solvent were equilibrated by dialysis prior to the experiment. The runs were made at three different protein concentrations. It was found that the sedimentation constant was not dependent on the protein concentration in the three solvents used. Molecular weights were determined by the Archibald method in a standard cell.

Lysozyme was oxidized following the procedure of Hirs (1956). The ultraviolet spectrum of the oxidized protein indicated extensive destruction of tryptophan. However, peptide maps of tryptic digests gave no evidence of additional chain cleavage at the (modified) tryptophan residues. Reduction of lysozyme with mercaptoethanol in 8 M urea (pH 8.6) was carried out as described by Anfinsen and Haber (1961). The reduced lysozyme was separated from the urea by passing the solution through a Sephadex G-25 column (2.5 × 100 cm) eluted with 0.1 M acetic acid. Mercaptoethanol was then immediately added to the protein fraction (1 μl/mg) and this mixture constituted the stock solution of reduced lysozyme.

Myoglobin was dehemed by precipitation in cold acetone containing 1% 1 N HCl.

Results

Myoglobin. OPTICAL STUDIES. Urea Solutions. In Figure 1 are shown the measured optical densities and optical rotations of myoglobin solutions containing urea, as a function of pH. These results were obtained at 25°; data at other temperatures from 5 to 55° are not much different, and the effects of temperature (Acampora and Hermans, 1967) and urea concentration on the pH at the midpoint are approximately additive. One will note that the transitions as followed by the two measurements occur at the same pH. The low pH limiting value of α_{233} depends upon the urea concentration.

TABLE I: Sedimentation Constants and Archibald Molecular Weights for Denatured Myoglobin at Low pH in Different Solvents.

Solvent	$s_w \times 10^{13}$	Mol Wt
Water	1.9	48,000
50% ethanol	0.46	21,000
6 M urea	0.38	20,000

Figure 2 shows the value of the reduced residue rotation of globin solutions as a function of urea concentration at pH 3.5. Dispersion curves in the presence and absence of 8 M urea are shown in Figure 3. We have also determined dispersion curves at a higher concentration (1 mg/ml) in urea and guanidine solution. From Moffitt–Yang plots of these curves, values of b_0 (b_0 is the slope of the plot $[m'](\lambda^2 - \lambda_0^2)/\lambda_0^2$ *vs.* $\lambda_0^2/(\lambda^2 - \lambda_0^2)$ with $\lambda_0 = 212$ mμ) were calculated to be −2 in 6.5 M urea and −10 in 6 M guanidine hydrochloride. Values at this and lower urea molarities are included in Figure 2. These results are in agreement with those of Harrison and Blout (1965) who found that 8 M urea (presumably at neutral pH) destroys all the helical structure of globin.

OPTICAL STUDIES. Ethanol Solutions. Denaturation curves in ethanol–water mixtures are shown in Figure 4. Again, these curves are similar at different temperatures. The behavior of the protein when measured with optical density (optical density transition) appears to be quite standard. The optical rotation measurements (optical rotatory dispersion transition) are similar to the optical density transition in 10% ethanol. In 20 and 30% ethanol the molecule appears to be more helical at low pH than near the midpoint of the optical density transition. (Since the native molecule is 70% α helix (Urnes *et al.*, 1961; Beychok and Blout, 1961), the observed changes correspond to large changes in calculated per cent helix and are highly significant.) The value of α_{233} of globin solutions of low pH is strongly dependent upon the ethanol concentration (Figure 5).

Very clearly, the pH transition in 20 and 30% ethanol is not a two-state transition, and one can even specify that the (or a) stable intermediate is a form with less helix than either native myoglobin or the end product of the denaturation. The pH range where the minimum value of $-\alpha_{233}$ is observed is also a region of precipitation, and the value of $-\alpha_{233}$ decreases with time as the turbidity increases. When more ethanol is present,

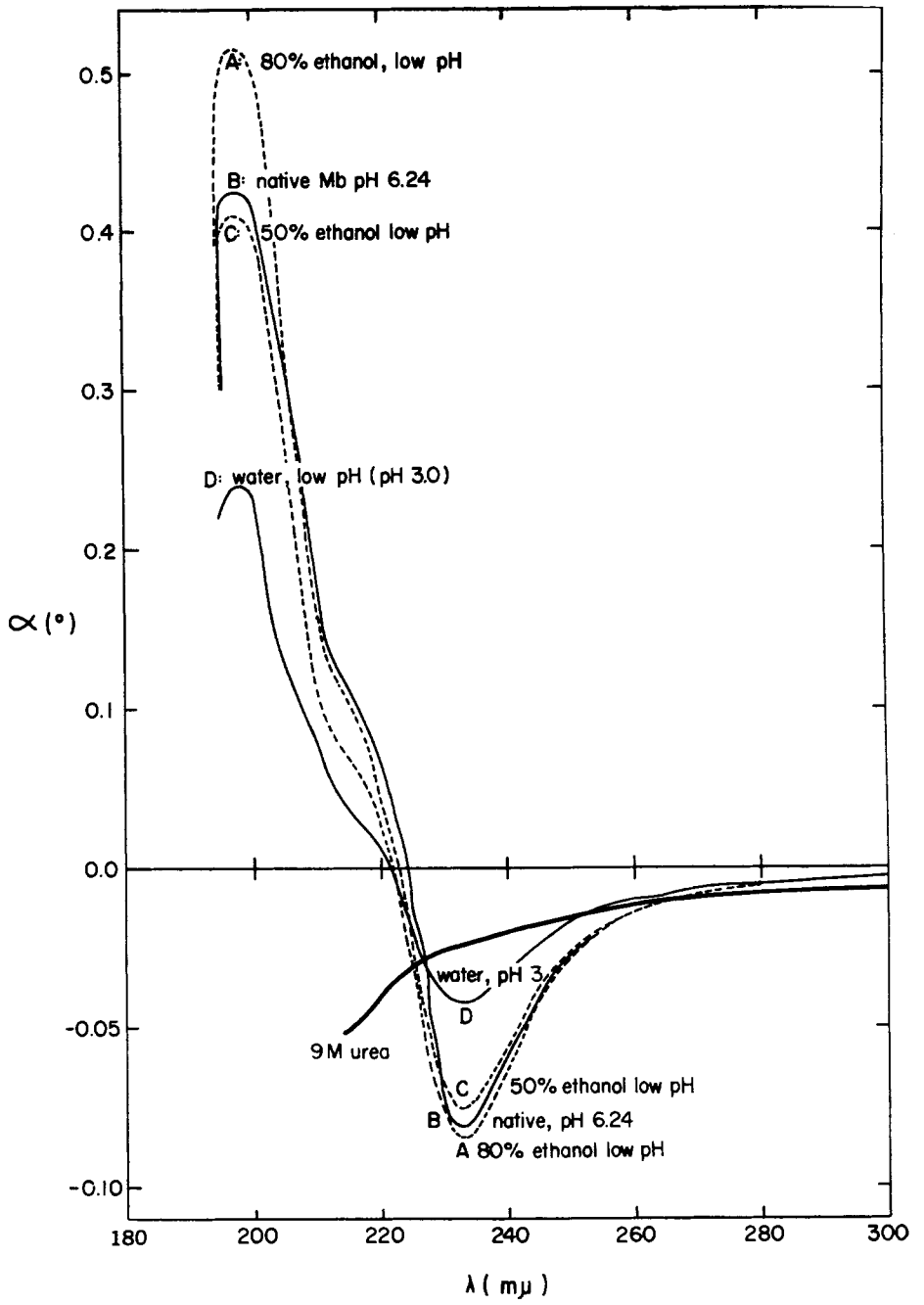

FIGURE 3: Optical rotatory dispersion curves for myoglobin in various solvents. Note difference in scale for positive and negative values of α. Protein concentration is 0.1 mg/ml.

the protein precipitates in neutral solution. This material can be solubilized by adjusting the pH to about 3. The optical rotation spectrum in 70% ethanol in the range 300–225 mμ is almost superimposable on that of native myoglobin in water at neutral pH (Figure 3). The ultraviolet absorption spectrum near 410 mμ appears to be similar to that of native myoglobin (sharp maximum), but is actually that of hemin micelles in this solvent, as can be shown by centrifuging the solution at, say, 15,000g for 1 hr. A precipitate of hemin is obtained at the bottom of a yellowish solution, which contains all the protein. The addition of more ethanol causes a further increase in apparent helix content (Figure 5).

SEDIMENTATION STUDIES. Table I shows the sedimentation constants and molecular weights obtained for denatured myoglobin at low pH in water, in 50% ethanol, and in 6 M urea. In water, the molecules are aggregated, but in the presence of either of the two denaturants, the aggregation is suppressed. From the sedimentation constants it is clear that the molecule is either much swollen or asymmetric in the latter two cases. Considering also the optical rotatory dispersion

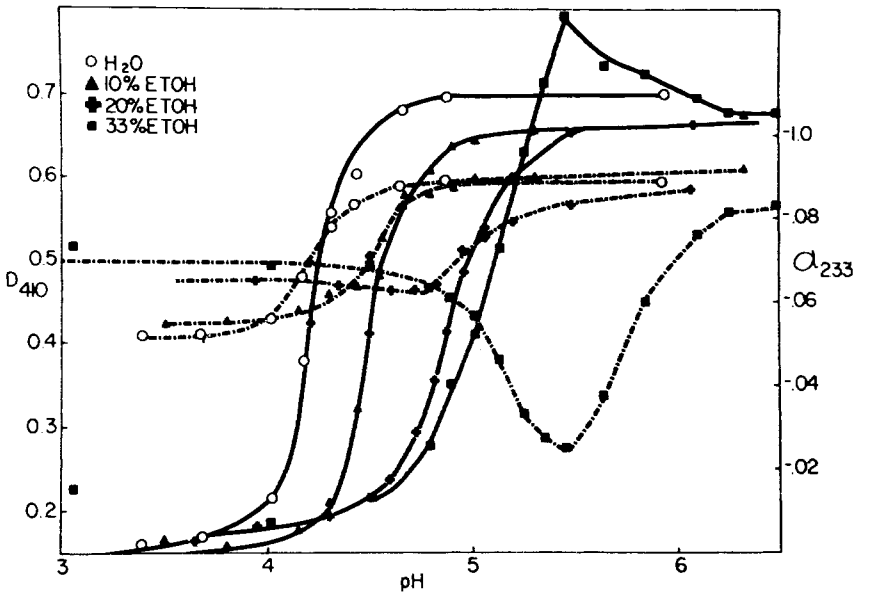

FIGURE 4: Optical rotation at 233 mμ (dashed curves) and optical density at 410 mμ (solid curves) of sperm whale myoglobin solutions of varying ethanol concentrations as a function of pH. Protein concentration is 0.1 mg/ml. The midpoints of the curves obtained in water and in 20% ethanol are marked (+). The unexpectedly high optical densities observed near pH 5.5 in 33% ethanol solutions are caused by precipitation.

measurements, we can conclude that the conformation in 6 M urea is close to a random coil (Tanford *et al.*, 1967a,b), while in 50% ethanol the molecule consists of helical segments which are not held together by noncovalent bonds as they are in the native molecule. (The sedimentation constant of native myoglobin is 1.98; Breslow, 1964.)

TITRATION STUDIES. Titration curves of mygolobin in ethanol–water mixtures, shown partly in Figure 6, provide further evidence in support of these conclusions. In 0.15 M KCl the unfolding is accompanied by the uptake of six protons in a narrow range and this is seen as a step in the titration curve (Breslow and Gurd, 1962). In 50% ethanol, the step is absent, and the six groups are found to titrate with a pK of about 6, which is typical of histidine side chains. (The precipitation of the protein in 50% ethanol at neutral pH does not appear to affect the shape of the curve in any clear-cut manner.)

Lysozyme. In Figure 5 are shown $[m']_{233}$ values for native, performic acid oxidized, and reduced lysozyme at pH ~3 in ethanol–water mixtures. (Most of these values are quite low, and one should not calculate precise helix contents on their basis.) It is obvious that the helix content is low and that the induction of helix by the addition of ethanol in reduced lysozyme takes place at a higher concentration of ethanol than with myoglobin. It is interesting that with both native and oxidized lysozyme the amount of helix produced with addition of ethanol is much smaller than with reduced lysozyme. One would draw the reasonable conclusion that the SH groups in reduced lysozyme fit better in helical regions than the SO_3^- groups in the oxidized protein. (Besides, the destruction of tryptophan residues noted above may have lowered the tendency to helix formation.) The data for native lysozyme suggest that the constraints imposed by the disulfide bridges hinder helix formation at high EtOH concentration. (The small *decrease* in the optical rotation of native lysozyme at about 25% EtOH probably corresponds to denaturation without a significant loss in helix content. This conclusion is consistent with work by Hamaguchi and Kurono (1963) who found that at neutral pH the reduced viscosity of lysozyme increases significantly between 25 and 35% EtOH, whereas the optical rotatory dispersion b_0 parameter is independent of EtOH concentration up to 80%.)

Discussion

The most striking point in the observations reported here is the large helicity of denatured myoglobin and the low helicity of reduced lysozyme, and the way the structure of these denatured proteins can be altered by the addition of protein denaturants. In contrast, the helicity of oxidized lysozyme is very low and cannot be increased very much with ethanol.

Of course, the work of Weber and Tanford (1959) and Tanford and De (1961) has already shown that apolar solvents can cause helix formation in nonhelical proteins (ribonuclease and β-lactoglobulin). (More recent work by Inoue and Timasheff, 1968, and Herskovits, 1965, applies these principles as well.) Also, the strengthening of the α helix in poly-L-glutamic acid by ethanol was known. The higher helix-forming tendency of denatured myoglobin gives us a measure (if one sided) of the structure present in the denatured molecule in water which is largely absent when one studies molecules such as ribonuclease and lysozyme which have very little helix in the native state (Kartha *et al.*, 1967; Blake *et al.*, 1967) and whose chains, one may conclude, do not have any inherent tendency (by

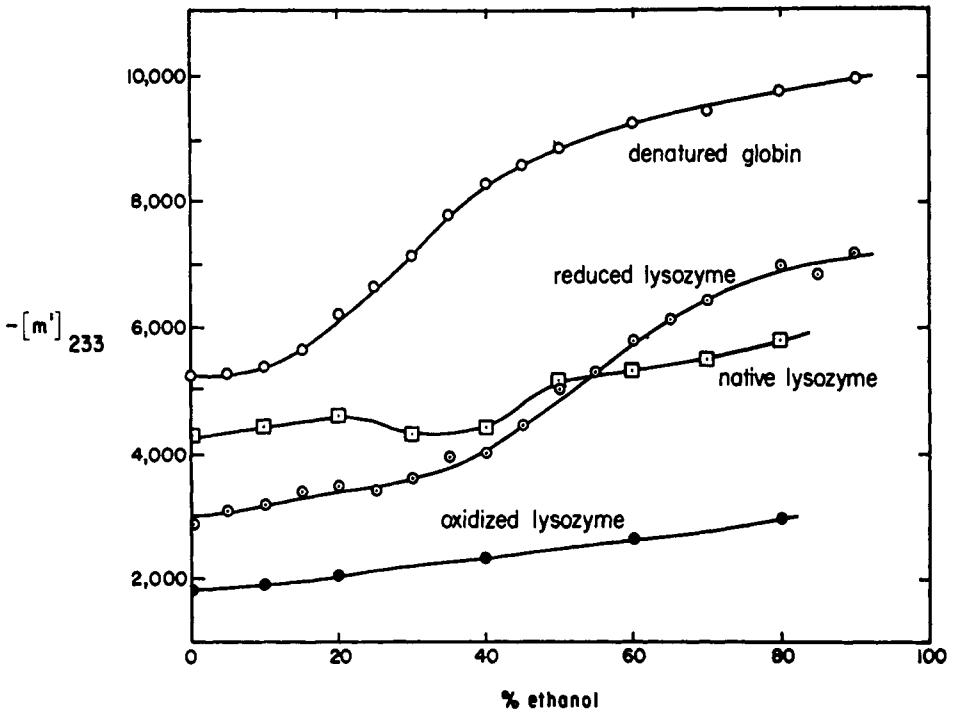

FIGURE 5: Reduced residue rotation of solutions of denatured globin and of native, reduced, and oxidized lysozyme as a function of ethanol concentration, pH ~3.

virtue of amino acid composition and sequence) to form *helices* when made random by denaturation. However, the existence of secondary structure in denatured proteins in general has been recognized by studies of ultraviolet absorption spectra (Bigelow, 1960), optical rotation (Aune *et al.*, 1967), and viscosity (Brandts and Lumry, 1963). The observation of so much helix in denatured myoglobin is experimental evidence for the possible existence of regions which can function as structural nuclei which guide the folding of the protein chain, with a resulting increase in the folding rate,[1] and suggests the possible existence of similar, and *ipso facto* nonhelical, nuclei in randomly coiled proteins such as ribonuclease and lysozyme. Perhaps careful kinetic studies of chain refolding can shed some light on this last point.

The addition of ethanol or urea to a solution of denatured myoglobin causes a change in the conformation of the protein. One may, of course, describe the observed curves of α_{233} *vs.* denaturant concentration (Figures 2 and 5) as transitions. However, these are certainly not cooperative transitions of the type of the denaturation reaction, nor may it be supposed that the conformation does not change when going from 0 to 10% ethanol because no change in α_{233} is found. Rather, it is to be concluded that the conformation of denatured myoglobin may be shifted easily by diluents or by a change in temperature (as is evidenced by the temperature dependence of α_{233}; Acampora and Hermans, 1967). Thus, one has to be somewhat cautious in interpreting the large value of $(\Delta C_p^\circ)_{den}$ as being due to the breaking of hydrophobic bonds alone (Brandts, 1964, 1965) since there is an undetermined contribution to ΔC_p° from the changes in conformation of the denatured species with temperature.

Free-Energy Changes. The measurements reported here allow one to calculate the exact dependence of the *free energy of denaturation* of the myoglobin molecule *at neutral pH* upon the concentration of the denaturants. The method has been discussed extensively in a previous paper (Hermans and Acampora, 1967). It consists of calculating the free energy as RT multiplied

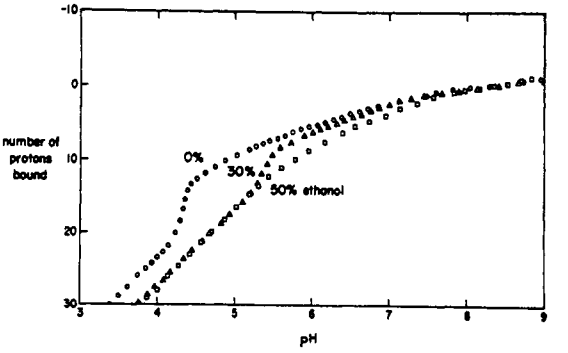

FIGURE 6: Potentiometric titration curves (high to low pH) of sperm whale myoglobin in 0.1 M KCl and 20 and 40% ethanol containing 0.1 M KCl. The zero of the ordinate for each curve corresponds arbitrarily to the least steep portion of the curve and not to the isoelectric point.

[1] It is, of course, possible that the high helix content of native myoglobin places this molecule sufficiently apart from other proteins of low helicity, that some of the conclusions reached in this article apply only to myoglobin. At the present, it is extremely difficult to confirm or disprove this experimentally.

TABLE II: Molar Free-Energy Changes for the Denaturation of Myoglobin ($\Delta G°_{den}$) at Neutrality in Various Solvents.

Solvent[a]	pK_{His}[b]	$\Delta G°_{den}$ (kcal/mole)
Water	6.48[c]	18,600
2 M urea	6.75	16,300
4 M urea	6.80	11,700
6 M urea	6.85	7,300
Water	6.48	18,600
10% ethanol	6.66	17,700
20% ethanol	6.57	14,000
30% ethanol	6.57	11,300

[a] All solutions contain 0.1 M KCl as well. [b] See footnote 2. [c] From Breslow and Gurd (1962).

by the integral of the difference in the titration curves for a native and a denatured molecule

$$\Delta G°_{den} = RT\int \Delta\nu_{H^+}° \, d(\ln a_{H^+}) \qquad (1)$$

This curve can be *calculated* quite easily on the basis of the well-established model with six buried histidine side chains (see above). The optical density (or optical rotatory dispersion) transition curves are needed to provide the reference pH in each solvent where the free energy difference is zero. This method has the advantage that the transition need not be two state.[2]

Table II shows calculated values of $\Delta G°_{den}$ at pH 8 for all solutions in which the optical rotatory dispersion and optical density transitions were reasonably coincident. The transition curves in 30% ethanol should be used with caution, since these do not clearly indicate at what value of the pH $\Delta G° = 0$. For this solvent, one may use the titration curve (Figure 6) to calculate directly an approximate value of 11,300 cal/mole for the free energy as given by eq 1. Since the low-pH denatured form of the molecule was found to be more or less helical, depending upon the ethanol or urea concentration, the values of Table II do not refer to the same conformation change in all solvents!

One would of course like to explain these data in terms of the structure of myoglobin in the crystal (Kendrew *et al.*, 1961) along the lines of our trial analysis for poly-L-glutamic acid (Hermans, 1966a), even if only to see how far such calculations can take one. However, an insurmountable obstacle to doing any such calculation in a realistic manner is presented by the fact that we do not know what the conformation of denatured myoglobin in water is. Or rather, we do know its (average) helical content from the optical rotatory dispersion curve, but not the extent of hydrophobic bonding, or of hydrogen bonding outside of the helical regions. We therefore wish to reverse the procedure and find out what the dependence of $\Delta G°_{den}$ upon denaturant concentration can tell us about the conformation of denatured myoglobin.

We would like to estimate the effect of the denaturants on the hypothetical transition of native myoglobin to randomly coiled myoglobin in water. We follow here a method used by Kauzmann (1959), Schellman (1956), and several others (Brandts, 1964, 1965; Scheraga, 1960; Tanford, 1962), in which hydrogen bonds, hydrophobic bonds, and frozen in rotations are considered to give the main contributions to the conformational free energy.

From an analysis of a model, one finds that there are about 100 hydrogen bonds in the native molecule. Hydrophobic bonding can be assessed in terms of water molecules which might be in contact with side chains, but are excluded from this contact by other parts of the protein molecule. The number of water molecules not adjacent to hydrocarbon side chains which would be adjacent in a fully extended conformation is about 1500. These are supposedly the two main contributions favoring the folded conformation. Opposing these is the effect of loss of freedom of rotation about single bonds. One estimates that about 400 bonds are frozen in myoglobin.[3]

Solubility studies of amino acids give one estimates of the effect of ethanol and urea on the stability of hydrophobic and of hydrogen bonds. Thus, in 20% ethanol the free energy of a dissolved leucine side chain is −140 cal/mole different from in water (Cohn and Edsall, 1943), which comes to about −6 cal/mole per adjacent water molecule. For going from water to 1 M urea the change is somewhere near −3 cal/mole (Nozaki and Tanford, 1963) per water molecule.

For the backbone, the free energy is changed by +200 cal/mole in 20% ethanol (Cohn and Edsall, 1943) and by from 0 to −300 cal/mole in 1 M urea (Nozaki and Tanford, 1963), both numbers being calculated per peptide unit. Thus, one calculates the following *changes* in $\Delta G°_{den}$ (native myoglobin to randomly coiled myoglobin): +11,000 cal/mole in 20% ethanol and −9500 cal/mole in 1 M urea. The experimental values (native myoglobin to denatured myoglobin) are −4500 cal/mole in 20% ethanol and −2300 cal/mole in 2 M urea. The interesting result of these calculations

[2] This calculation requires a knowledge of the pK of normal histidine side chains in the solvent employed. Using potentiometric titration, we have determined the following pK's for β-alanylhistidine in the presence of a 0.1 M KCl at 25°: 6.86 in water, 6.96 in 2 M urea, 7.00 in 4 M urea, 7.28 in 6 M urea (this determination is probably in error; instead we assumed an interpolated value of 7.07), 7.14 in 8 M urea, 6.79 in 20% ethanol, and 6.79 in 40% ethanol. We assume that the pK of normal histidine side chains in myoglobin varies by the same difference from the value found in 0.15 M KCl, 6.48 (Breslow and Gurd, 1962). To be exact, the values of $\Delta G°_{den}$ calculated in this manner refer to the transition of a molecule in which no changes in proton binding take place during the denaturation. This situation exists at a pH in the range 8–9.

[3] The authors are grateful to Drs. Kendrew and Watson for providing them with the opportunity to analyze a model of the myoglobin structure.

is of course the difference in *sign* for the effect on $\Delta G°_{den}$ obtained by changing the solvent from water to 20% ethanol.[4] This is such a pronounced effect that the following should apply to all proteins which have been found to be more readily denatured in the presence of ethanol.

While we had concluded above that part of the denatured molecule is not accessible to the solvent, we may now specify that a negative value of $\Delta(\Delta G°_{den})$ upon addition of ethanol can only be obtained if relatively many more hydrophobic groups than hydrogen bonding groups are exposed to the solvent upon denaturation.[5] At this point we cannot say if this is due to the formation of cooperative structured areas reminiscent of the native molecule in the denatured molecule, and therefore dependent on the amino acid sequence, or if such behavior is shown by any randomly coiled polypeptide chain with a sizable fraction of hydrophobic side chains. The high helical content of denatured myoglobin, which is not found in denatured lysozyme, would perhaps indicate the former.

A completely different possibility is of course that some of the model compound experiments bear no relation to the experiments reported here.

Two States vs. Many States. The steepness of the pH–transition curves may be used as evidence as to how closely the transition follows the two-state model. One find that the steepness is as great in 10% ethanol and 2 M urea as it is in water, appears somewhat lower in 4 and 6 M urea, and is definitely lower in 20% ethanol. Of course, in 20% ethanol and in 30% ethanol the presence of an intermediate is evident from the observation of a minimum in $-\alpha_{233}$, and this is in agreement with what one concludes from the decrease in slope. Since the minimum is found to become much more pronounced when precipitation is evident, it is to be concluded that the intermediate could well be similar to the precipitate observed in solutions containing denatured myoglobin at neutral pH and high temperature, for which we have tentatively suggested interchain cross-linking similar to a β structure. Therefore, the results obtained here with myoglobin in 30% ethanol do not reveal whether ethanol can lower the cooperativity in the transition from native to denatured protein, as it is often believed to do (Foss, 1961; Poland and Scheraga, 1965).

Acknowledgment

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[4] It should be pointed out that the model compound values do predict the right sign and order of magnitude of the effect of ethanol on $\Delta G°_{helix}$, the free energy of the coil to helix transition of poly-L-glutamic acid (Hermans, 1966b).

[5] Following this argument, one would expect that ethanol added to 6 M urea (in which denatured myoglobin is close to randomly coiled, as shown above) will favor the native state. However, we observed that the transition is shifted to higher pH, as it is in the absence of the urea. The difficulty in interpreting this experiment is that the model compound experiments have not been done in this system, and that the effect of ethanol in the presence of so much urea cannot be predicted.

References

Acampora, G., and Hermans, J. (1967), *J. Am. Chem. Soc. 89*, 1543.

Anfinsen, C. B., and Haber, E. (1961), *J. Biol. Chem. 236*, 1361.

Aune, K. C., Salahuddin, A., Zarlengo, M. H., and Tanford, C. (1967), *J. Biol. Chem. 242*, 4486.

Beychok, S., and Blout, E. R. (1961), *J. Mol. Biol. 3*, 769.

Bigelow, C. C. (1960), *Compt. Rend. Trav. Lab. Carlsberg 31*, 305.

Blake, C. C. F., Mair, G. A., North, A. C. T., Phillips, D. C., and Sarma, V. R. (1967), *Proc. Royal Soc. (London) B167*, 365.

Brandts, J. F. (1964), *J. Am. Chem. Soc. 86*, 4291, 4302.

Brandts, J. F. (1965), *J. Am. Chem. Soc. 87*, 2759.

Brandts, J. F., and Lumry, R. (1963), *J. Phys. Chem. 67*, 1484.

Breslow, E. (1964), *J. Biol. Chem. 239*, 486.

Breslow, E., and Gurd, F. R. N. (1962), *J. Biol. Chem. 237*, 372.

Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids and Peptides, New York, N. Y., Reinhold, Chapter 8.

Fasman, G. D. (1963), *Methods Enzymol. 6*, 928.

Foss, J. G. (1961), *Biochim. Biophys. Acta 47*, 569.

Hamaguchi, K., and Kurono, A. (1963), *J. Biochem. (Tokyo) 54*, 497.

Harrison, S. C., and Blout, E. R. (1965), *J. Biol. Chem. 240*, 299.

Hermans, J. (1966a), *J. Phys. Chem. 70*, 510.

Hermans, J. (1966b), *J. Am. Chem. Soc. 88*, 2418.

Hermans, J., and Acampora, G. (1967), *J. Am. Chem. Soc. 89*, 1547.

Herskovits, T. T. (1965), *J. Biol. Chem. 240*, 628.

Hirs, C. H. W. (1956), *J. Biol. Chem. 219*, 611.

Inoue, H., and Timasheff, S. N. (1968), *J. Am. Chem. Soc. 90*, 1890.

International Critical Tables (1930), Washburn, E. W., Ed., Vol. 7, New York, N. Y., McGraw-Hill, pp 63–94.

Kartha, G., Bello, J., and Harker, D. (1967), *Nature 213*, 862.

Kauzmann, W. (1959), *Advan. Protein Chem. 14*, 1.

Kendrew, J. C., Watson, H. C., Strandberg, B. E., Dickerson, R. E., Phillips, D. C., and Shore, V. C. (1961), *Nature 90*, 606.

Khalifah, R. G. (1968), Ph.D. Thesis, Princeton University, Princeton, N. J.

Litman, B. (1966), Ph.D. Thesis, University of Oregon, Eugene, Ore.

Lumry, R., Biltonen, R., and Brandts, J. F. (1966), *Biopolymers 4*, 917.

Némethy, G., and Scheraga, H. A. (1962), *J. Phys. Chem. 66*, 1773.

Nozaki, T., and Tanford, C. (1963), *J. Biol. Chem. 238*, 4074.

Poland, D. C., and Scheraga, H. A. (1965), *Biopolymers 3*, 401.
Schellman, J. A. (1956), *Compt. Rend. Trav. Lab. Carlsberg 29*, 230.
Scheraga, H. A. (1960), *J. Phys. Chem. 64*, 1917.
Tanford, C. (1962), *J. Am. Chem. Soc. 84*, 4240.
Tanford, C., and De, P. K. (1961), *J. Biol. Chem. 236*, 1777.
Tanford, C., Kawahara, K., and Lapanje, S. (1967a), *J. Am. Chem. Soc. 89*, 729.
Tanford, C., Kawahara, K., Lapanje, S., Hooker, T. M., Zarlengo, M. H., Salahuddin, A., Aune, K., and Takagi, T. (1967b), *J. Am. Chem. Soc. 89*, 5023.
Urnes, P., Imahori, K., and Doty, P. (1961), *Proc. Natl. Acad. Sci. U. S. 47*, 1635.
Weber, R. E., and Tanford, C. (1959), *J. Am. Chem. Soc. 81*, 3255.

Isolation and Characterization of the Peptides Derived from the α1 Chain of Chick Bone Collagen after Cyanogen Bromide Cleavage*

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ABSTRACT: Cleavage of the α1 chain of chick bone collagen with CNBr gives rise to ten peptides which have been separated by ion-exchange and molecular sieve chromatography. The peptides constitute unique portions of the α1 chain as demonstrated by chromatographic properties, amino acid composition, and molecular weight. They account for all of the amino acids in the α1 chain. The molecular weights of the peptides vary from 242 to 26,000 and total 92,000, in good agreement with the measured molecular weight of about 95,000 for α chains. The isolation of ten unique peptides in approximately equimolar amounts from a chain containing nine methionyl residues indicates that the two α1 chains of the bone collagen molecule have identical or very similar primary structures. Variation in the degree of hydroxylation of lysine is evident from the presence of nonintegral numbers of hydroxylysyl and lysyl residues in many of the peptides. The peptide from the cross-link region, α1-CB1, contains a lysyl residue (amino acid number 7 from the NH_2 terminus) which is about 50% hydroxylated. Cleavage of this peptide with trypsin demonstrated that hydroxylysine and lysine occupy the same position. Heterogeneity of this type cannot be the result of differences in the original primary structure, but arises after synthesis of the protein. The CNBr peptides derived from the α1 chain of chick bone collagen are clearly homologous to those obtained previously from the α1 chain of rat skin collagen. An apparent exception was noted in that a dipeptide (α1-CB0) was present in digests of the α1 chain of chick bone collagen that was not among the cleavage products of rat skin collagen α1. α1-CB0 was placed at the NH_2-terminal end of the α1 chain, preceding α1-CB1. These two residues, plus two additional residues at the NH_2-terminal end of α1-CB1 not found in rat skin collagen α1-CB1, placed the lysyl residue that is a precursor of cross-links at position 9 in the α1 chain of chick bone collagen. Other than the addition of these four residues, the cross-link region of the chick bone collagen α1 chain, as represented by α1-CB1, is identical with the same region in rat skin collagen α1 except for an alanine–serine substitution.

The COOH-terminal peptide from rat skin collagen α1 (α1-CB6) is represented by two peptides (α1-CB6A and α1-CB6B) from chick bone collagen α1 indicating the presence of an extra methionyl residue in the COOH-terminal region.

Cleavage of the α1 and α2 chains of collagen with CNBr has proved to be a highly useful procedure in the study of these large polypeptide chains of about 95,000 molecular weight (Bornstein and Piez, 1965, 1966; Bornstein *et al.*, 1966; Butler *et al.*, 1967). Cleavage of the seven methionyl residues of rat skin collagen α1 led to the isolation of eight unique peptides accounting for all the amino acids and the molecular weight of the chain (Butler *et al.*, 1967). These peptides are suitable for sequence studies, some of which have been reported (Kang *et al.*, 1967; Bornstein, 1967a,b).

In order to extend our knowledge to a collagen of another species and with a different function, we have applied CNBr cleavage to the α1 chain from chick bone collagen. The solubility and chromatographic properties of this collagen have been studied (Miller

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