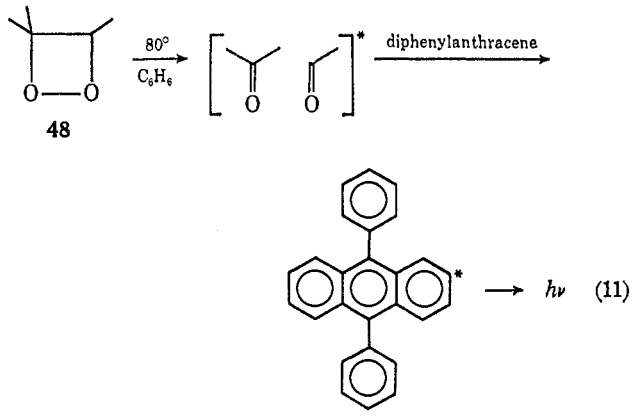
analysis (eq 11) of an oxaoxetane (48) has been reported recently[48] (eq 11). We have found that the



(11)

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decomposition carried out in the presence of *trans*-stilbene leads to about 10% isomerization of the stilbene into the *cis* form.[49] Other "photochemical" reactions have been effected,[49] and now that the principle has been established, efforts will be made to make this a practical, general approach to photochemistry. Two advantages of this method of generating excited states are: (1) the method of excitation ensures that all the energy resides initially in one molecular species (in the donor part of a molecule) and (2) only simple apparatus is needed, beakers, not monochromators.

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Protein–Solvent Interactions and Protein Conformation

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Proteins are natural high polymers with molecular weights in the range between 5000 and several million. They are polycondensates of amino acids, with the general formula $(-NHCHRCO-)_n$, where R can be any of some 22 different amino acid side chains.

At any given set of conditions at which the protein is not undergoing a conformational transition, all the polymeric chains of a given protein are folded in essentially identical fashion, resulting frequently in compact globular structures. Thus, within the limits of thermal fluctuations of the polypeptide chain fold, all the molecules have identical secondary and tertiary structures.[2]

A feature of particular importance is the fact that, in globular proteins which are soluble under physiological conditions, when the molecules are native, *i.e.*, in the conformation in which they occur in their natural surroundings, most polar (and in particular ionizable) residues are located on the surface of the molecule in contact with the aqueous solvent, while the interior (or core) is made up to a large extent of nonpolar amino acid side chains.[3] When the aqueous medium is perturbed by the addition of some organic molecules, such as urea or alcohol, or some salts, such as guanidinium chloride, at a given concentration of the perturbant the protein becomes denatured, *i.e.*, its compact structure opens up and the chain largely unfolds, exposing much of the core to contact with solvent. This pattern of folding and unfolding of the polypeptide chain raises the questions: what are the forces that stabilize the native structure of globular proteins, and in what manner is this structure disrupted by the denaturing agent? It is the purpose of this paper to consider some aspects of the second question.

Protein Structure Stabilizing Forces

It is quite evident that the folding of a long-chain polymer into a compact structure results in loss of freedom of motion of the chain and, thus, in a decrease of the configurational entropy. Thus, for a typical protein of 200 amino acid residues, folding into the native structure may result in the loss of 200–250 kcal/mol of protein of structure stabilization free energy.[4,5] This must, evidently, be overcome by opposite effects of equal magnitude. Let us examine what these may be.

The forces which stabilize the protein structures are predominantly of a noncovalent nature.[6,7] The prin-

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(2) The secondary structure of a protein refers to the manner in which the chain is folded, *i.e.*, to the relative three-dimensional space coordinates of consecutive amino acid residues; the tertiary structure refers to the manner in which folded chain segments are mutually arranged with respect to each other and to the mutual ordering of side chains.

(3) See, for example, J. C. Kendrew, *Sci. Am.*, **205** (6), 96 (1961), or D. C. Phillips, *ibid.*, **215** (5), 78 (1966).

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cipal ones are: (1) hydrogen-bond formation, (2) electrostatic interactions, (3) the hydrophobic effect. In examining their contributions to protein conformational stability, the proper states to compare are the native globular folded structure and the completely unfolded random chain in which all residues are exposed to solvent.

For hydrogen bonding, the equilibrium to consider is that between internal hydrogen bonds between groups within the protein molecules and external hydrogen bonds between these groups and water molecules. The difference between the energies of these two end states has been shown on model systems to be small,[8-11] and it is generally estimated that the net contribution of hydrogen bonds to the conformational stabilization of proteins is not large.[5,7] On the other hand, the lack of formation of a potential internal hydrogen bond results in destabilization by the energy of that bond.

Electrostatic contributions can be of two kinds: (1) charge–charge interactions between the ionizable groups of the amino acid residues; (2) internal dipolar interactions between peptide groups, in particular in ordered sections of a protein molecule, such as α helices.

Since proteins exist normally in a medium of high ionic strength of the order of $\Gamma/2 = 0.15$ and the charged side chains lie on the surface of the molecule exposed to solvent, any interaction between charges on the surface are mostly screened out, resulting in little stabilization. Conversely, acquisition by the protein of a net nonzero (positive or negative) charge results in repulsion between the groups, *i.e.*, in a net destabilization.[12]

The internal dipolar interactions between peptide groups within a protein molecule are a function of the detailed conformation of any given protein. Brant and Flory[13] have concluded that "a peptide unit buried in a long α-helical sequence experiences a favorable dipole interaction energy of about -1.2 kcal/mole."

The third type of noncovalent contribution to protein structural stability is from the hydrophobic effect. This effect stems from the unfavorable interactions between water molecules and the nonpolar residues of a protein.[5,14-18] When a hydrocarbon molecule is introduced into water, it induces changes in water structure, frequently decreasing its entropy. To minimize this unfavorable entropy change, the nonpolar molecules are forced to coalesce together into droplets or globules, reducing their surface of contact with water. In a protein molecule, the amino acid side chains, which are held together by the polypeptide backbone chain, cover a wide range of polarity from highly polar ones, such as the carboxylic groups of aspartic or glutamic acid, to highly nonpolar ones, such as the benzene ring of phenylalanine. Contact of the nonpolar residues with water lowers the entropy of the system. As a result, the protein chain is forced to fold into a micellar structure with the hydrocarbon moiety on the inside of the globule and the polar groups on the outside. Measurements of the free energy of transfer of amino acids between water and organic solvents[19,20] and theoretical considerations[17] have shown that the removal of the nonpolar residues from contact with water makes a major contribution to the free energy of conformational stabilization.[5] Thus, the hydrophobic effect is the result of the abhorence of water for contact with hydrocarbons. It is *not* due to the mutual attraction between the nonpolar side chains *via* van der Waals forces, as is sometimes implied. In fact, the van der Waals interactions should not change greatly when an amino acid side chain is transferred from water to the interior of a protein molecule, since in both cases the maximal possible number of contacts, consistent with the structure of the medium, is made between the amino acid radical and the surrounding atoms whether water or protein.

Destabilization of Protein Conformation

Addition of various materials to an aqueous protein solution results frequently in denaturation of the protein. The end product can be of two generally different natures. (1) It may be a highly unfolded chain, approaching the random flight conformation of synthetic polymers; such denaturation occurs on addition of urea and guanidine salts. (2) It may assume a structure with long-range order, such as an α helix; such denaturation occurs on addition of many organic solvents, for example, acetone and various alcohols.[21,22]

The ability of various denaturants to alter the structure of proteins has been the subject of broad investigation in recent years.[6] In particular much insight into the action of these agents has been gained from studies on the degree of affinity which these substances have for amino acids and various compounds that may serve as models for structural components of proteins. These studies have taken the form principally of solubility measurements[19,20,23-26] and, thus, of the free en-

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ergies of transfer of the amino acids from water to the denaturing medium. As a result, it has been possible to conclude that the unfolding of proteins by denaturing solvents is closely related to their affinity for the amino acid residues and the peptide groups.[5,24-27] The nature of the unfolded structure appears to be controlled by the interactions between the peptide groups and the solvent. Thus, the free energy of transfer of a peptide group from water to 8 *M* urea is negative,[19] and the protein structure approaches that of a random coil in which the peptide groups are in contact with solvent. In the case of ethylene glycol or ethanol, on the other hand, the free energy of transfer of peptide groups from water to the denaturant is unfavorable;[20] the denatured protein assumes an α-helical structure in which the peptide groups are internally hydrogen bonded and partially shielded from solvent by the surrounding side chains.

It is generally accepted at present that the conformational stability of a native protein molecule in aqueous solution is the result primarily of the pressure which water exerts on nonpolar residues, forcing these into the interior of the molecule. Conversely, the disruption of the native structure of denaturing agents is closely related to their interactions with the structural components of the protein.[6,25-29] There is no general agreement, however, on whether it is the effect of these denaturants on the bulk structure of water or their direct interaction with structural elements of the protein which plays the predominant role. In a system at equilibrium the various interactions between components must be related as required by the Gibbs–Duhem equation. Therefore, in the denaturation of proteins, one may expect simultaneous changes in the structure of bulk water, in the conformation of the protein, and in the interaction between solvent components and parts of the protein molecule.

In order to examine the degree of interaction between protein components and solvents which accompany protein denaturation, concomitant studies of conformational transitions and protein–solvent interactions have been carried out in our laboratory.[29-31] The solvent systems used were water plus a helix-inducing organic solvent such as 2-chloroethanol, methoxyethanol, and ethylene glycol. The proteins investigated include β-lactoglobulin, bovine serum albumin, lysozyme, and insulin. These studies consisted of two parallel sets of experiments: (1) measurements of the conformational change induced by the solvent; and (2) measurements of the preferential interactions between the proteins and solvent components. The results obtained with lysozyme will be used as an example in this paper, although similar patterns were obtained with all the proteins.

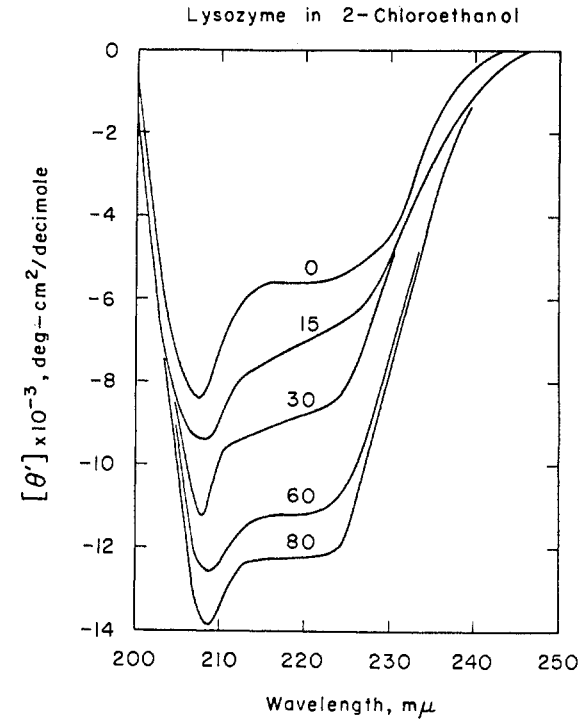

Figure 1. Circular dichroism spectra of lysozyme below 250 mμ in aqueous medium containing 0.01 *M* HCl, 0.02 *M* NaCl, and various amounts of 2-chloroethanol. The numbers on the spectra refer to the contents of 2-chloroethanol expressed as volume per cent.

Conformational Changes

In the first type of experiment, conformational changes were followed by changes in a particular spectral property of the protein. In studies where the induced transition is from the native protein structure to a denatured structure with a high helical content, circular dichroism (CD) is a particularly suitable method. The gradual changes which occur in a protein spectrum as it undergoes a transition from its native structure to a new one consistent with the solvent composition is exemplified in Figure 1, where are shown the CD spectra of lysozyme in aqueous medium and in various mixtures of 2-chloroethanol with water. The observed progressive increase in intensity of the negative band at 207–209 mμ and of the shoulder at 220 mμ is consistent[32-34] with the conclusion of Hamaguchi and Kurono[35] that addition of 2-chloroethanol to aqueous solutions of lysozyme increases the α-helix content of the protein. For the purpose of a denaturation study, however, the transition may be followed by observing the change in spectral intensity as a function of denaturant concentration without knowledge of the exact

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nature of the conformation to which the given spectra correspond. Thus, in lysozyme the conformational transition induced by 2-chloroethanol was followed by plotting the CD band intensity at 207–209 mμ as a function of 2-chloroethanol concentration, as shown in Figure 2. A major change occurs between 10 and 30 vol % of 2-chloroethanol, with a continued gradual increase in band intensity above this solvent composition. For comparison, the viscosity data of Hamaguchi and Kurono[35] are plotted on the same figure, indicating, as well, a major conformational change, with expansion of the protein. Such an expanded structure should be characterized by an increase in side-chain exposure to contact with solvent.

Protein–Solvent Interactions

When a protein molecule unfolds, it is the previously buried nonpolar residues which become primarily exposed since the majority of the polar ones are already in contact with solvent. Since nonpolar residues have a greater affinity for nonpolar solvents than for water, when the protein unfolds the amount of the nonpolar solvent component should increase in the immediate domain of the protein.

In order to test this hypothesis, experiments were carried out on the preferential binding to protein[36] of solvent components in water–organic solvent mixtures. In multicomponent systems, the preferential interactions between macromolecules and solvent components may be reflected in the variation of a number of physical properties with solution composition.[29–31,37–43]

Such properties may be the refractive index increment, the amount of light scattered from the solution, the partial specific volume of the macromolecular component, the vapor pressure of the solution, the variation in the equilibrium constant of a reaction with a change in solvent composition, etc. In terms of a physical model the problem may be reduced to the definition of the kinetic unit for which the particular property is being measured. Taking as an example light scattering, which depends on the difference between the refractive indices of the macromolecular kinetic unit and the solvent, the situation may be described in terms of the model of Figure 3. In this technique, the measurements are based on the thermal fluctuations of the macromolecules[44,45] (together with any solvent molecules immobilized by them). In a two-component system (water = component 1; protein = component 2) the refractive index of water bound to the protein will, as a close approximation, not differ from that of free water in the surrounding medium. Thus, any technique based on the difference between the refractive indices of solvent and macromolecule will detect only the macromolecule and yield a result which is essentially valid for the unhydrated macromolecule. When a third component is added (let organic solvent = component 3), the situation changes drastically. If components 1 and 3 are immobilized by component 2 in a

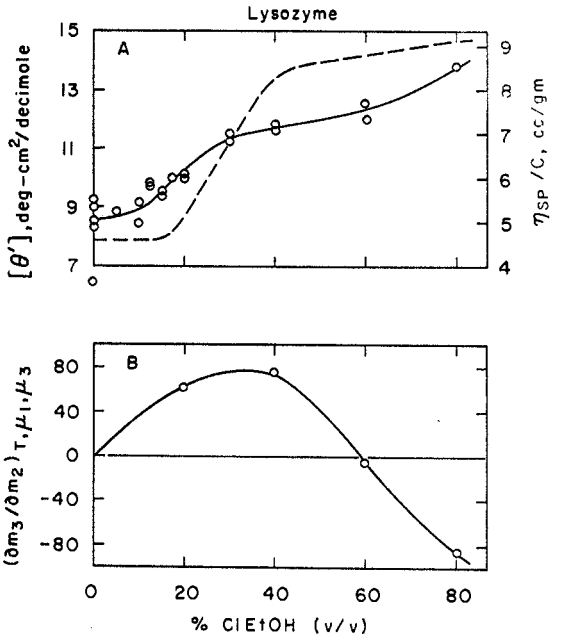

Figure 2. (A) Change in the intensity of the 207–209-mμ circular dichroism band in lysozyme with addition of 2-chloroethanol; the dashed line represents the viscosity data of Hamaguchi and Kurono[35] obtained under similar conditions. (B) Change in preferential binding to lysozyme of 2-chloroethanol as the concentration of the latter increases; component 3 is 2-chloroethanol; component 2 is protein; m_i is concentration of component i on the molal scale, *i.e.*, expressed as moles per 1000 g of water. All measurements were carried out in the presence of 0.01 *M* HCl and 0.02 *M* NaCl.

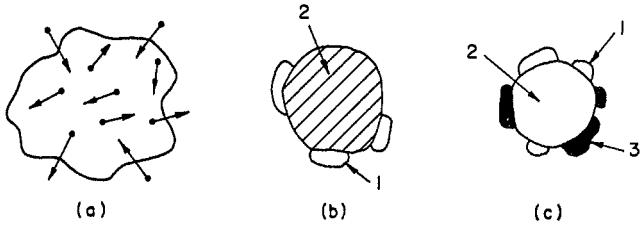

Figure 3. Fluctuations in solution of observed kinetic units. (a) Fluctuations within a volume element. (b) Kinetic unit in a two-component system; solvent (component 1) is bound to protein (component 2). (c) Kinetic unit in a three-component system; two solvents (components 1 and 3) are bound to protein (component 2). Similar schemes apply to a number of thermodynamic methods.

(36) In this paper the term "binding" is used in its most general sense, namely the excess of a solvent component found in the immediate domain of a portion of the macromolecule. There is no implication either on the mechanism of this interaction or on the presence of any specific sites at which solvent molecules become attached in fixed orientation on the macromolecule.

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proportion identical with bulk solvent composition, subtraction of the refractive index of the solvent mixture from that of the solution results in a value very close to that characteristic of the pure macromolecular solute. If, on the other hand, the amounts of solvent components bound to the macromolecule differ from the proportion in which they are present in bulk solvent, then the value of a property, such as the refractive index of the kinetic unit, will be that of the macromolecular solute plus a contribution due to the excess of one solvent component over the amount found in the bulk solvent; this excess is the extent of preferential binding of the solvent component to the macromolecule.

Expressing concentrations in molal units, at equilibrium, the change in solvent composition in the immediate domain of the macromolecule due to binding of component 3 to component 2 is $(\partial m_3/\partial m_2)_{T,p,\mu_3}$, where m_i is the molal concentration of component i, μ is the chemical potential, and T and p are the thermodynamic temperature and pressure. Using refractive index, n, as the measured property, the value of the refractive index increment of the kinetic unit, namely the change in refractive index brought about by the addition of 1 mol of macromolecule, is $(\partial n/\partial m_2)_{T,p,m_3} + (\partial n/\partial m_3)_{T,p,m_2}(\partial m_3/\partial m_2)_{T,p,\mu_3}$, *i.e.*, the refractive index increment of the unsolvated macromolecule plus the refractive increment of the organic solvent component multiplied by its excess in the immediate domain of the macromolecule. It can be easily shown that this is equal to the refractive index increment of the solute over solvent with which it is in equilibrium, *i.e.*, $(\partial n/\partial m_2)_{T,p,\mu_3}$. The extent of preferential interaction of component 3 with protein is then equal to

$$\left(\frac{\partial m_3}{\partial m_2}\right)_{T,p,\mu_3} = \frac{\left(\frac{\partial n}{\partial m_2}\right)_{T,p,\mu_3} - \left(\frac{\partial n}{\partial m_2}\right)_{T,p,m_3}}{\left(\frac{\partial n}{\partial m_3}\right)_{T,p,m_2}} \quad (1)$$

Experimentally this reduces to measuring the refractive index increment of component 3 and two measurements of the refractive index increment of protein. The first is at conditions in which the molalities of solvent components are identical in the solvent and solution. The second measurement is at conditions at which the pressure and chemical potential of component 3 are identical in the protein solution and the mixed solvent. This is very closely approximated by osmotic equilibrium between the two.[39] Similar arguments and procedures apply to a number of other solution properties, such as density or vapor pressure.

The sign of $(\partial m_3/\partial m_2)_{T,p,\mu_3}$ may be either positive or negative. A positive sign means preferential binding of component 3 to a protein; a negative sign means a deficiency in component 3 in the immediate domain of the protein and, thus, preferential interaction with component 1, *i.e.*, water. The two quantities are related by eq 2.

$$\left(\frac{\partial m_1}{\partial m_2}\right)_{T,p,\mu_1} = -\frac{m_1}{m_3}\left(\frac{\partial m_3}{\partial m_2}\right)_{T,p,\mu_3} \quad (2)$$

In our studies, the techniques used for measuring preferential interactions were light scattering, differential refractometry, and sedimentation equilibrium. Results obtained with lysozyme in the water–2-chloroethanol[29] system are shown in Figure 2. It can be seen that the variation of $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ with m_3 is quite complicated. Below 35% of organic solvent, this quantity gradually increases. Then, after passing through a maximum, it decreases and becomes negative above 60%; namely at high contents of the nonpolar solvent component, in the vicinity of the protein, water is found in excess over the amount present in the bulk solvent.

Preferential and Total Solvation

Comparison of the solvent binding results for lysozyme with those on the conformational transition, also shown on Figure 2, does not reveal any immediate parallel between the solvent composition dependence of $[\theta']$ and $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$. It must be recalled, at this point, that $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ represents the *preferential* interaction between component 3 and protein, *i.e.*, it is essentially a comparison of the solvent compositions in bulk and in the immediate domain of the protein. Thus, if the total amount of water bound to the protein remained unchanged when solvent composition was altered, its amount relative to the bulk solvent would increase proportionately to solvent composition, easily masking an increase in the binding of the nonpolar solvent component to protein. In order to examine this possibility, the observed preferential binding of solvent, $(\partial m_3/\partial m_2)_{obsd}$, may be decomposed into the sum of two contributions; the first, $(\partial m_3/\partial m_2)_{org}$, corresponds to the actual binding of organic solvent; the second, $(\partial m_1/\partial m_2)_{hydr}$, is the contribution from tightly bound water. The variation in the amount of bound water with change in solvent composition may be expressed in general form, without assuming any model, as a power series in organic solvent concentration, namely as $A + Bm_3 + Cm_3^2 + \ldots$. A is the degree of hydration of the protein in the native structure, expressed as moles of water bound to 1 mol of protein; B, C, etc., represent the dependence of hydration (positive or negative) on the solvent composition. The actual binding of the organic component to protein, then, is given by eq 3.

$$\left(\frac{\partial m_3}{\partial m_2}\right)_{org} = \left(\frac{\partial m_3}{\partial m_2}\right)_{obsd} + \frac{m_3}{m_1}(A + Bm_3 + Cm_3^2 + \ldots) \quad (3)$$

Calculation of the total binding of the organic solvent to protein from the experimental value of preferential interaction requires, then, knowledge of the degree of hydration of the protein. This quantity is uncertain since measurements of hydration are functions of the methods used, but a reasonable estimate may be made from various types of measurements. The concept of hydration is quite complicated; it is a summation of contributions from water molecules whose motions are restricted to different extents by protein. The

quantity $(\partial m_1/\partial m_2)_{hydr}$, corresponds to a summation of the free energies of the various protein–water interactions, since $\partial m_i/\partial m_j$, as a first approximation, is a measure of $\partial \mu_i/\partial m_j$, where μ_i is the chemical potential of component i. The quantity needed for the calculation of eq 3 is the effect of protein on the chemical potential of water in a true two-component system, *i.e.*, $\partial \mu_1/\partial m_2$; this last quantity is not readily available. Estimates, however, may be made of protein hydration from a consideration of various experimental values. For the purpose of calculations in the lysozyme system, a reasonable value of hydration was taken as 0.30 g of water/g of protein, giving 240 mol of water bound to 1 mol of lysozyme, *i.e.*, $A = 240$. When protein structure is loosened, water can enter into further interactions with newly exposed peptide groups. Therefore, B in eq 3 was set equal to 20. The results of the calculation are shown in Figure 4. The dotted line represents the contribution of $(\partial m_1/\partial m_2)_{hydr}$. Subtracting this quantity from the experimental value of $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ (solid line) gives the dashed line, *i.e.*, $(\partial m_3/\partial m_2)_{org}$, or the true variation of the binding of the organic solvent to protein as the solvent composition is varied.

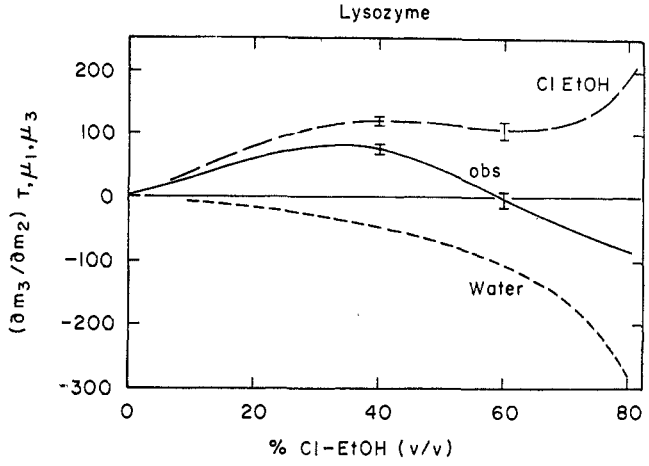

Figure 4. Decomposition of the observed preferential interactions between lysozyme and two solvent components (water and 2-chloroethanol) into contributions from each, in accordance with eq 3, as described in the text. The solid line is the experimental curve; the dotted line is the contribution of protein hydration; the dashed line is the calculated absolute binding of 2-chloroethanol to the protein. The error bars indicate the experimental error in the measurement of binding.

Solvent Binding and Conformational Changes

Comparison of the results of the above calculation with the change in secondary structure of lysozyme induced by 2-chloroethanol (Figure 2) shows that $(\partial m_3/\partial m_2)_{org}$ and $[\theta']$ vary in nearly parallel fashion with changes in solvent composition.

At closer examination, a remaining discrepancy becomes evident: whereas the amount of 2-chloroethanol bound to protein reaches a high value already at 20% of that solvent component, the gross change in structure of the protein occurs mainly between 15 and 40%. Therefore, binding of organic solvent occurs already before the major conformational transition, meaning that the nonpolar solvent component either interacts with the protein in native conformation or induces small conformational changes which do not lead to major changes in CD absorption in the far-ultraviolet spectral region nor to shape changes reflected by an increase in viscosity. Such small conformational changes should involve mainly the tertiary structure of the protein.

In order to check this possibility, the CD of lysozyme was examined as a function of solvent composition in the spectral region between 250 and 330 mμ, which reflects transitions principally of aromatic residues and disulfide bridges. Lysozyme contains four disulfide bridges, three tyrosines, six tryptophans, and three phenylalanines. Their presence in asymmetric environment and their mutual interactions result in the CD spectrum shown in Figure 5. Addition of 2-chloroethanol leads to a series of changes in the spectrum. Below 12.5% 2-chloroethanol, the absorption becomes slightly more positive, possibly reflecting the change in the environment of some chromophores.[35] Between 12.5% and 17.5% 2-chloroethanol, the CD spectrum above 250 mμ changes drastically in character and the signal becomes very weak, as exemplified by the spectrum obtained in 20% 2-chloroethanol, shown on Figure 5. Such a spectral change indicates a decrease in the order between the side chains in question, the residues themselves having acquired an increase in freedom of motion. The spectrum in 15% 2-chloroethanol is intermediate between the two extremes; it points to the partial perturbation of the tertiary structure at a solvent composition at which the globular nature of the protein appears to be still intact (see Figure 2).

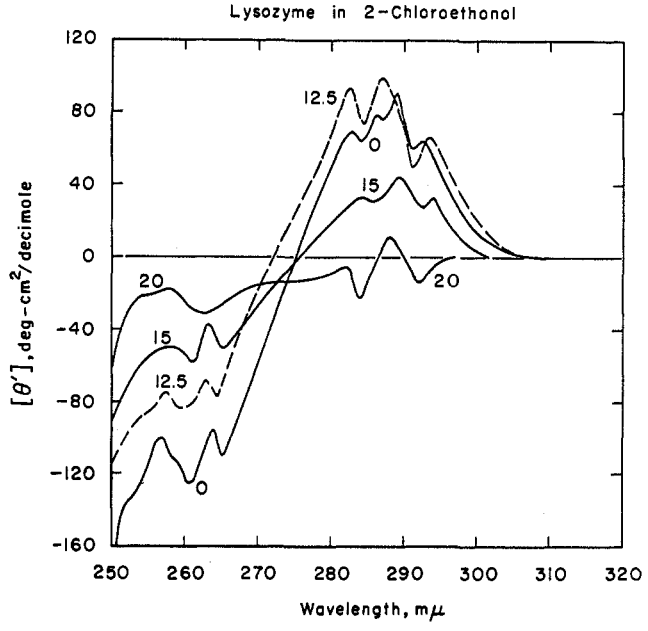

Figure 5. Circular dichroism spectrum of lysozyme between 320 and 250 mμ in the presence of various quantities of 2-chloroethanol. The numbers on the spectra refer to the contents of 2-chloroethanol expressed as volume per cent.

The progressive disruption of orders of protein structure may be understood in terms of the following con-

cepts. The structure of a globular protein, while unique and rigidly folded as a first approximation, is not absolutely impenetrable to solvent. The polypeptide chain of a protein molecule is constantly in thermal motion and, as a result, it "breathes," *i.e.*, openings are constantly being formed and closed, through which some solvent molecules may come into contact with interior regions.[46,47] Also, the difference in free energy between a native globular structure and the unfolded chain is of the order of 10–20 kcal/mol.[5] This means that, at any moment, there is a very small, but nevertheless finite, number of molecules in conformational states other than the native fold, including highly unfolded ones. In such opened molecules, solvent may come into contact with protein regions which normally are in the interior. This enables nonpolar solvent molecules to enter into hydrophobic interactions with nonpolar interior residues. When the activity of the nonpolar solvent becomes sufficiently high, the interactions between the nonpolar solvent molecules and some nonpolar protein side chains should acquire sufficient energy to replace the internal interresidue hydrophobic interactions with external ones between residues and solvent molecules, essentially solubilizing local order in the protein and relaxing the mutual immobilization of side chains and in particular of those located within the protein surface. In this manner the hydrophobic pressure of water on the nonpolar residues can now be satisfied by bringing them into contact with the nonpolar solvent component.

Such a relaxation of the conformation of side chains located on the protein surface should randomize their positions in space and lead to a decrease in their optical rotatory power and, thus, in the intensities of their CD bands. The interactions between the denaturant solvent and chromophoric groups can also be expected to affect the transition moments of the latter, again affecting their optical rotatory properties. This pattern is consistent with the observations on lysozyme. The structure of lysozyme may be described roughly as consisting of two compact moieties with a cleft between them. The aromatic residues are distributed through the protein on both sides of the cleft in different states of accessibility to solvent. The mutual orientation of tryptophans in particular permits extensive dipolar interactions between residues. Interactions of the nonpolar solvent with nonpolar residues in the protein surface and in the cleft can perturb their order, affecting the mutual orientation of the aromatic side chains. The change in the CD spectrum above 250 mμ when the amount of 2-chloroethanol is increased from 12.5 to 17.5% strongly suggests that such a mechanism may indeed be operative in this protein.

A further increase in the nonpolar solvent component brings about additional interactions with nonpolar regions of the protein. At a given solvent composition, the internal hydrophobic interactions become so relaxed and are replaced by external ones to such an extent that the loss in entropy due to the folding into globular structure is no longer compensated and the protein unfolds. This occurs in lysozyme between 15 and 40% 2-chloroethanol.

Quite interestingly, a similar situation may exist in the case of random structure-forming denaturants. In measurements of the binding of urea to bovine serum albumin, Gordon and Warren have shown that urea molecules are increasingly bound to protein as urea concentration is increased even before reaching the level at which conformational changes occur.[48] Then, at a given urea concentration, the optical rotation of the protein starts increasing rapidly with further urea binding. Thus, in this case as well, a conformational change from the native structure to a highly unfolded one is preceded by the binding of the denaturant.

Concluding Remarks

The qualitative picture presented above of the events which occur during the denaturation of a protein, in the present instance lysozyme, although similar patterns have been observed with β-lactoglobulin and bovine serum albumin, in mixed solvents supports the concept that local interactions between protein and denaturant play an important role in inducing the structural change. In the case of "helix-forming" solvents, the major feature appears to be the replacement of internal hydrophobic interactions between amino acid residues by external ones between the residues and molecules of the nonpolar solvent. This permits the chain to unfold without imparting an unfavorable entropy to water and to assume a final structure with greater freedom of motion consistent with the minimum free-energy characteristics of the various constituent groups of the protein. The question of the effects of the denaturant on the general structure of water in the bulk solvent is not directly answered by the present studies. Clearly, changes in water structure must occur when organic solvent is added; these may lead to changes in the strength of hydrophobic interactions. In fact, the chemical potential of all components must change as the solvent composition is altered and the protein structure is transformed. Therefore, while local protein–solvent interactions play an important role in the induction of the conformational change, a contribution must also be made by concomitant changes in water structure. Whether these last contributions are protein structure stabilizing or destabilizing will depend on the nature of the changes induced in the structure of water by each particular perturbant.

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