Electrostatic Effects in Proteins

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Summary. Electrostatic effects dominate many aspects of protein behavior. When polypeptide chains fold up, most polar side chains seek the exterior, where they can be solvated. Water bound in the interior has been found between the domains of enzymes of the chymotrypsin family, and between the subunits of hemoglobin and tobacco mosaic virus protein. Assembly of this protein from disk to virus is triggered by electrostatic interactions between neighboring subunits. Lysozyme stabilizes the constellation of charges involved in the transition state of its substrate by both permanent and induced dipoles. All factors that lower the oxygen affinity of hemoglobin act by strengthening the salt bridges that constrain its quaternary deoxy (T) structure. Enzymes of thermophile bacteria owe their extra stability mostly to additional salt bridges. The rate of denaturation of hemoglobins by alkali is determined by the ionization of internal side chains with *pK*'s of about 12.

Twenty years ago investigators tried to apply Debye Hückel theory to polyelectrolytes of undefined structure. Today the challenge is reversed. We now know the structures of the active sites of many enzymes in detail but often lack the theoretical knowledge to understand their mechanism.

In this article I draw your attention to the decisive influence of electrostatic effects on the structure, assembly, and hydration of proteins, and on the catalytic power of enzymes. I stress the importance of salt bridges in allostery and thermophilicity, and finally show how ionization of internal side chains determines the rate of denaturation of proteins by bases and acids.

Protein Structure

Before the structure of any protein had been determined, Kauzmann (*1*) predicted the general principles of protein assembly. He foresaw that all polar groups would be either compensating each other or be solvated by water, and that entropic effects would draw the nonpolar groups together in the interior. As a result, proteins are waxy inside and soapy outside. On heating, the wax melts and they unfold, unless held together by strong electrostatic forces.

Kauzmann's prediction was first verified in myoglobin and hemoglobin. Their tertiary structures are the same in invertebrates and vertebrates despite very different sequences; only two sites are occupied by the same residue in all species. This made me wonder what it was that determined their tertiary structure. I found that most external sites could be occupied by any residue, polar or nonpolar, but 36 internal sites were invariably occupied by nonpolar residues. Along helical segments, there tended to be a regular alternation of polar and nonpolar side chains. In my usual sanguine way, I thought I had discovered the mechanism of protein folding and wrote a paper with a suitably flamboyant title, but Kendrew wisely restrained me, because he foresaw that the problem would prove more complex (*2*).

The first successful attempt at predicting even the approximate structure of a protein from its sequence on the basis of Kauzmann's principles is due to Levitt and Warshel (*3*). They tried to fold up the chain of pancreatic trypsin inhibitor by energy minimization on a computer. They used simplified side chains, and assigned to each of them a hydrophobic energy calculated from the energy of transfer between water and ethanol. This was taken as the difference in energy of the side chain when isolated in water and when surrounded by other residues. Use of this principle together with that of closest packing made the chain fold up to something approaching the right conformation, giving a root-mean-square deviation of 3.7 angstroms from the true model. The result is impressive as a first step, but it also shows that hydrophobic bonding and close-packing alone are not sufficient. To get the structure right, electrostatic effects due to hydrogen bonding would also have had to be taken into account.

Hydration

The apparent density of proteins in water is greater than their dry density in organic solvents; the increase is due to electrostriction of bound water which Adair and Adair estimated from the excluded volume—that is, the volume that is not available to diffusible electrolytes—as 0.3 g per gram of protein (*4*). Refinement of protein structures at high resolution has allowed some of this water to be located. No fixed internal water is found in myoglobin or in the subunits of hemoglobin, but, in the chymotrypsin family of enzymes, 25 water molecules are part of the internal pattern of hydrogen bonding between the two cylinders or domains of pleated sheet that compose the tertiary structure (Fig. 1). Ninety other water molecules are bound to polar groups on the exterior (*5*). In hemoglobin, internally bound water is found at the subunit boundaries and forms an essential part of the pattern of hydrogen bonds that hold the tetramer together (*6*). Other water molecules are bound to polar groups at the protein surface, but the total number of bound water molecules that can be located by x-ray analysis is no more than one-eighth of the number that is unavailable to diffusible electrolytes. Where is the rest? It could be argued that most of them are not held down firmly enough to be located by x-ray analysis at 2-Å resolution. The structure of rubredoxin, refined at 1.2-Å resolution, speaks in favor of that interpretation. This shows about 100 water molecules for a protein molecular weight of 6000, which comes very near to 0.3 g per gram of protein. Electron density peaks representing water molecules bound to the protein by two or more hydrogen bonds are highest. As the number of bonds to the protein decreases and the distance from the protein surface increases, the peaks weaken, suggesting a continuum of water molecules bound less and less firmly the further the distance from the protein surface (*7*).

The question still remains why all this

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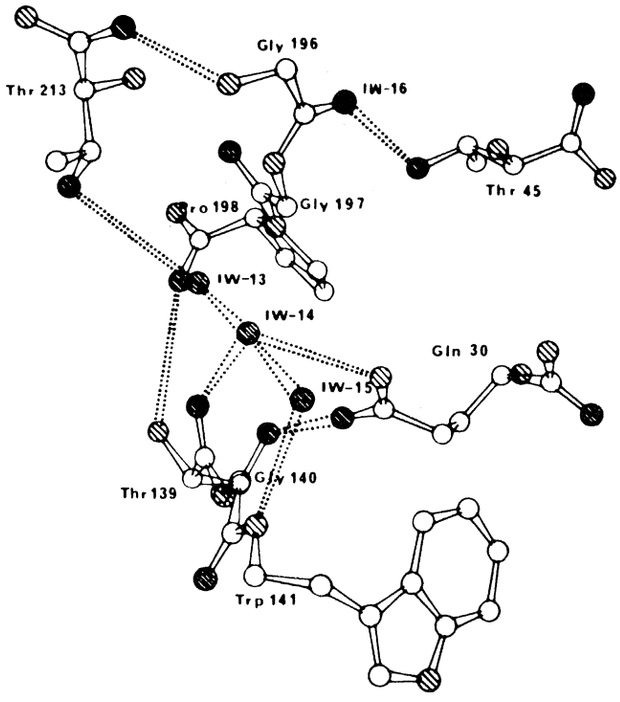

Fig. 1. Three of the bound water molecules (IW-13, -14 and -15) in the interior of elastase. [From (*5*), reproduced by permission of the *Journal of Molecular Biology*]

water should be unavailable to diffusible electrolytes. Kauzmann suggested to me that this may be due to a simple electrostatic effect. Consider a charge, e, immersed in a solvent of high dielectric constant, D_0, which is brought up to a slab of low dielectric constant, D_p. Electrostatic theory shows that an image charge

$$E' = e\,\frac{D_0 - D_p}{D_0 + D_p}$$

appears in the low dielectric slab, and the energy of the charge e at distance R from the slab surface is (relative to R = infinity)

$$E = \frac{e\,e'}{2D_0R} = \frac{D_0 - D_p}{D_0 + D_p}\,\frac{e^2}{2D_0R}$$

If $D_0 \approx 80$ and $D_p \approx 5$, then $E \approx kT$ when $r = 3$ Å, which means that ions cannot penetrate a layer of water about one molecule thick in the neighborhood of a protein or other macromolecule; this gives the observed 0.3 g of water per gram of protein unavailable to diffusible electrolytes (*8*).

Virus Assembly

Assembly of the tobacco mosaic virus from its RNA and more than 2000 protein subunits with a molecular weight of 17,500 presents a problem like nucleation and growth of a crystal. One would have expected the helical protein coat to be assembled by sequential addition of the single subunits, but this is not what happens. They first aggregate into disks made up of two rings of 17 subunits each. On assembly into the virus, these rings undergo a transition from circular to helical symmetry with 16.34 subunits per turn (Fig. 2A). This transition happens on contact with the specific viral RNA but can also be mimicked by low *p*H (*9*). How is it brought about? Determination of the disk structure at 2.8-Å resolution has shown that the subunits are joined by two kinds of salt bridges: one bridge between neighboring subunits in the same ring that is preserved on transition from disk to virus—Arg 122 (arginine residue) from the left radial helix of one subunit to Asp 88 (aspartic acid residue) of the right radial helix of its neighbor; and a network of salt bridges between the two rings of the disk that is lost in the course of that transition (*10*) (Fig. 2B). The transition appears to be brought about by the deionization of two as yet unknown carboxyl groups and the formation of hydrogen bonds between them; these carboxyl groups do not form part of the salt bridges just described (*11*). The detailed mechanism of the transition is still unclear, but electrostatic effects play a decisive part.

Enzyme Catalysis

Why can chemical reactions which normally require powerful organic solvents or strong acids and bases proceed rapidly in aqueous solution at neutral *p*H in the presence of enzymes? Before the structure of any enzyme was known, Pauling (*12*) predicted that their active sites are complementary to the transition state of the substrate. This prediction was confirmed when the structure of the first enzyme, lysozyme, was solved.

Phillips and his colleagues (*13*, *14*), and Vernon (*15*), suggested the following mechanism.

1) The substrate becomes attached to the enzyme and is held in position by hydrogen bonds and other forces. In the process the ring of residue D becomes distorted and takes up a conformation favorable for the formation of a carbonium ion.

2) A proton is transferred to the glycosidic oxygen atom from Glu 35.

3) Heterolysis of the C_1–O bond gives a carbonium ion which is stabilized by interaction with Asp 52.

4) The disaccharide EF diffuses away and a water molecule attacks the carbonium ion, thus completing the hydrolysis.

These proposals have stimulated much research. Chemists have made synthetic compounds, with carboxyl groups attached to mimic those of the enzyme's active site, but found their hydrolysis in water to be much slower than that of the substrate by the enzyme. Was that due to the absence of distortion of the substrate? Warshel and Levitt (*16*), and recently Warshel (*17*), have made a structural and thermodynamic study of the factors responsible for the catalytic mechanism of lysozyme. They found that distortion of the substrate can be neglected, essentially because the enzyme is too soft to bring it about. The decisive factor consists in the much stronger electrostatic effects that the charged groups can exert on the substrate when clamped to the enzyme, as compared to the effect of the same charged groups in water. This happens because, in bulk water, solvation by water dipoles makes the stability of oppositely charged ions almost independent of distance, while in the active site of the enzyme permanent and induced dipoles can generate a minimum of free energy at a separation of 3 to 5 Å between the charges because the stabilization of charges is larger than in bulk water. In consequence, the calculated free energy of the transition state in the cleft of lysozyme is lower by 6 kcal/mole than that of an equivalent system with fixed anionic charges in water, compared to an experimentally estimated difference of ~7 kcal/mole. Hence the model accounts quantitatively for the observed differences in reaction rates in the enzyme and in water.

Allostery and Salt Bridges

Monod, Changeux, and Jacob proposed the term allostery for enzymes that possess two, or at least two, stereospecifically different, nonoverlapping receptor sites (*18*). One of these, the active site, binds the substrate, while the other, or allosteric site, binds the effector. Such enzymes contain more than one site of each kind, and these act cooperatively, just as the four hemes in hemoglobin bind oxygen cooperatively. Monod *et al.* suggested that such cooperative interactions are mediated by conformational alterations through the protein, that is, through an allosteric transition. Monod, Wyman, and Changeux (*19*) later elaborated this concept and attributed the cooperative effects to an equilibrium between two, or at least two, different structures which are distinguished by the number or energy (or both) of the bonds between the protein subunits. When the bonds are few and weak, the enzyme would be relaxed and fully active, but when they are strong it would be tense and its activity would be damped (*20*).

Because of its cooperative binding of oxygen, Monod *et al.* (*18*) took hemoglobin as a model of an allosteric protein, although at the time little was known about its structure and allosteric regulators. It has since been found that H^+, Cl^-, CO_2, and 2,3-diphosphoglycerate all lower the oxygen affinity and increase the cooperativity of oxygen binding in a manner that is similar to the way allosteric inhibitors lower the substrate affinity and raise the cooperativity of allosteric enzymes, such as aspartate transcarbamylase, so that there really does exist a parallel between them as Monod *et al.* had intuitively guessed. What is remarkable is that so diverse a collection of chemical agents should influence the oxygen equilibrium of hemoglobin in a similar way. How do they act?

Hemoglobin exists in two alternative structures: the oxy or R structure which has about the same oxygen affinity as isolated subunits, so that one regards it as relaxed, and the deoxy or T structure whose oxygen affinity is several hundred times lower, so that it is tense in the Monod sense. When this structure had been solved, I was pleased to find the extra bonds between the subunits which Monod *et al.* had predicted (*18*), but surprised that they all consisted of salt bridges, located either on the surface or in clefts accessible to water. It has since been found that all the agents which lower the oxygen affinity do so by strengthening existing salt bridges or by forming additional ones (Fig. 3). Why is that bridged structure more stable than one in which each of the isolated charges is solvated by water? No one has attacked this problem, but the explanation must be similar to that given by Warshel (*17*) for the stability of the transition

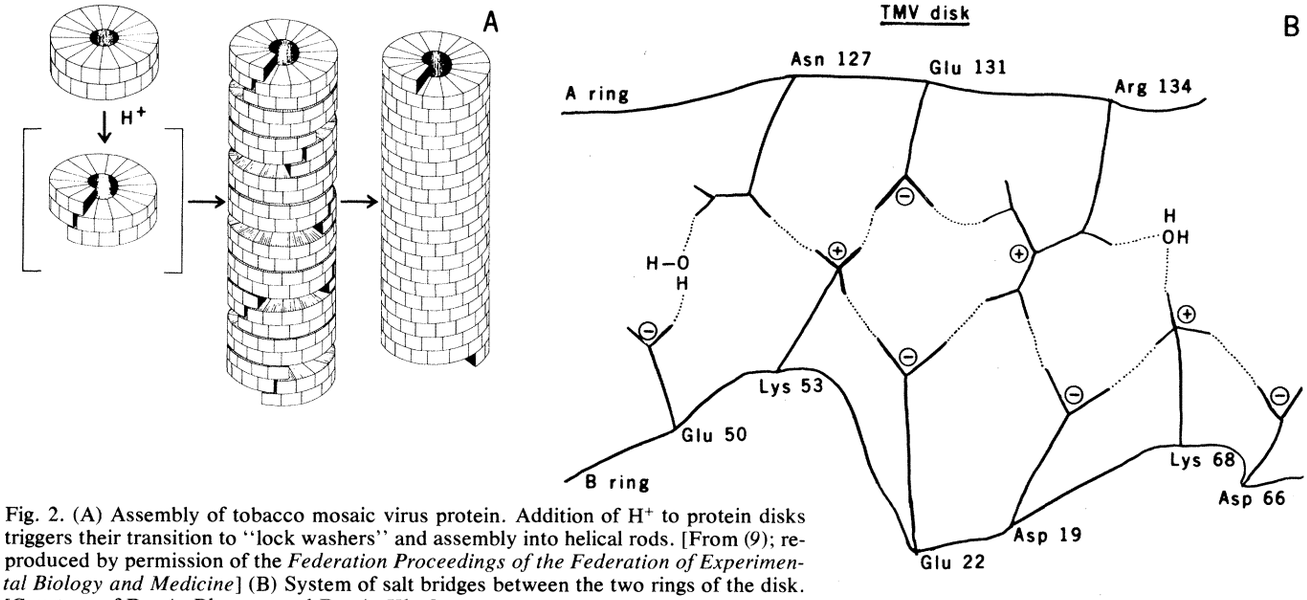

Fig. 2. (A) Assembly of tobacco mosaic virus protein. Addition of H^+ to protein disks triggers their transition to "lock washers" and assembly into helical rods. [From (*9*); reproduced by permission of the *Federation Proceedings of the Federation of Experimental Biology and Medicine*] (B) System of salt bridges between the two rings of the disk. [Courtesy of Dr. A. Bloomer and Dr. A. Klug]

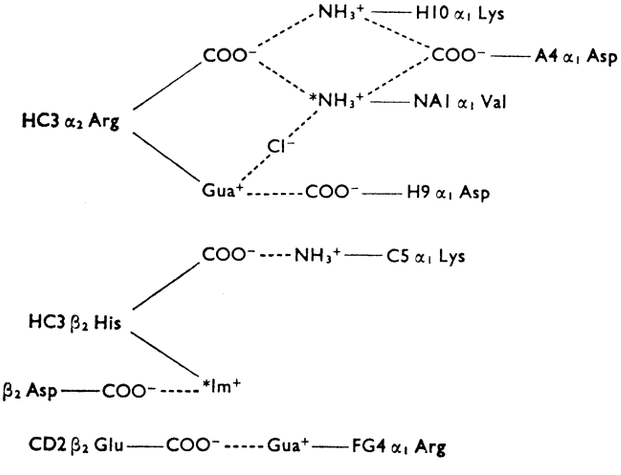

Fig. 3. Salt bridges in human deoxyhemoglobin. Residues are marked in the helix notation (*21*). Protons strengthen salt bridges by ionizing the weak conjugate bases: the imidazoles of histidine and the α amino groups. Chloride ions form additional salt bridges such as that between Val Na1 α_1 and Arg HC3 α_2 shown here. Carbon dioxide combines with α-NH_2 to form carbamino compounds whose carboxylate groups form salt bridges with neighboring cationic groups (not shown). 2,3-Diphosphoglycerate forms salt bridges linking cationic groups of neighboring β chains (not shown). For details see (*21*). Gua indicates the guanidinium group of arginine. [From Baldwin in (*21*); reproduced by permission of *British Medical Bulletin*]

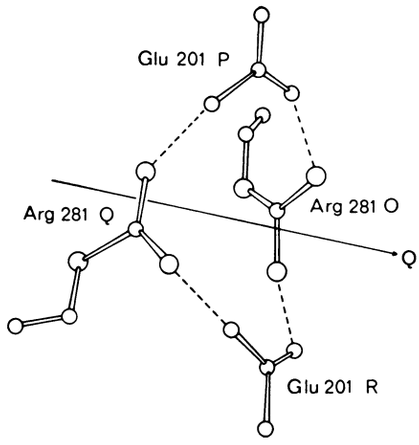

Fig. 4. Salt bridges between the four subunits of *B. stearothermophilus* glyceraldehyde phosphate dehydrogenase. [From (*25*), reproduced by permission of W. de Gruyter & Co.]

state in lysozyme. In clefts of the protein or even close to its surface, charge pairs are solvated by fixed dipoles of the protein or by fixed water molecules which minimize their free energy at close distance, while that energy would be independent of distance if they were free in solution.

The cooperativity of the reaction of hemoglobin with oxygen arises, not from any direct interaction between the four hemes which lie far apart in isolated pockets on the protein surface, but by transmission of stereochemical effects between the hemes and the salt bridges, so that combination of the iron atoms with oxygen loosens the salt bridges, and joining of their ion pairs loosens the iron-oxygen bonds. Hemoglobin transduces stereochemical effects at the heme into electrostatic ones near the protein surface, and they in turn regulate the allosteric equilibrium between the T and R structures (*21*, *22*).

Thermal Stability

Most enzymes are inactivated quickly above 55°C, but those from thermostable bacteria retain their activities over long periods at higher temperatures. I wondered why, and decided to look at the simplest possible enzyme of known structure, the electron transfer protein ferredoxin. This consists of a single chain containing fewer than 60 residues plus two Fe_4-S_4 clusters (*20*). The enzyme from *Clostridium pasteurianum* is completely inactivated after 2 hours at 70°C, that from *C. acidiurici* retains 20 percent, that from *C. tartarivorum* half, and that from *C. thermosaccharolyticum* 90 percent of their respective activities (*23*). Homologies of the amino acid sequences of these two proteins show that their structures must be closely similar. Raidt and I (*24*) built an atomic model of the protein, using coordinates supplied by Jensen (*7*), and we replaced its amino acid side chains in turn to correspond to the sequences in the different organisms. We found that the only possible sources of greater heat stability in the enzyme from the thermophile organisms were extra salt bridges on the protein surface, especially bridges linking residues near the amino terminus to others near the carboxyl terminus (*24*).

In recent years, my late colleague J. I. Harris initiated the isolation of many enzymes from thermophile organisms. (His tragic death is a great loss to our laboratory and to protein chemistry generally.) Harris and Walker (*25*) determined the sequence of glyceraldehyde phosphate dehydrogenase from *Bacillus stearothermophilus* while Wonacott, Thierry, and Biesecker (*26*) determined its structure by x-ray analysis and compared it with that of the lobster enzyme solved by Rossmann and his colleagues (*27*). The results showed that the greater heat stability of the thermophile enzyme is due to salt bridges between the four subunits which the mesophile enzyme lacks. Serine 281 and Gln 201 (glutamine) in lobster are replaced by Arg (arginine) and Glu (glutamic acid) in *B. stearothermophilus*, where they form salt bridges between symmetry-related subunits in a cavity that is shielded from solvent (Fig. 4). Another buried ion pair that is unique to the thermostable enzyme is formed between an Arg residue in one subunit and an Asp residue (aspartic acid) in a symmetry-related one.

We wondered at first whether a few salt bridges would be sufficient to account for the thermostability of these enzymes, but remembering that a tenfold reduction in rate at 70°C needs only 1.56 kcal of extra energy of stabilization, the differences in the rates of denaturation of mesophile and thermophile enzymes suggest that in ferredoxin the extra stabilization energy amounts to no more than 2 kcal/mole, and in glyceraldehyde phosphate dehydrogenase to no more than 5 to 10 kcal/mole. Judging by hemoglobin, an external salt bridge can contribute up to 1 kcal/mole. Fersht (*28*) showed that, in chymotrypsin, an internal salt bridge contributes 3 kcal/mole. Hence the energy of a few salt bridges is

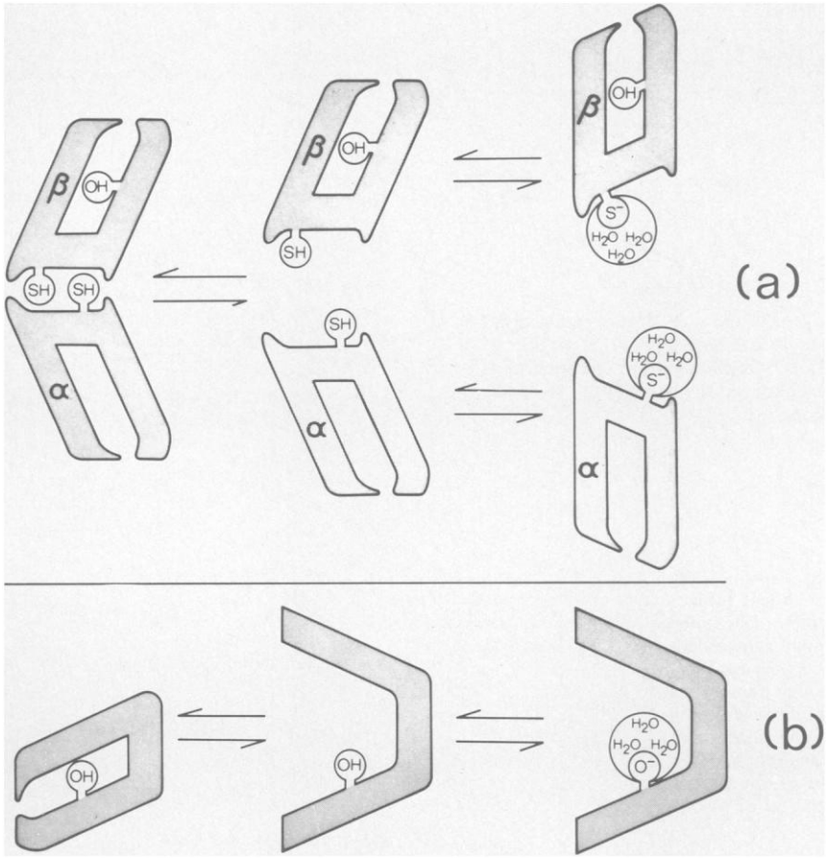

Fig. 5. Effect of solvation of buried ionizable groups in adult human hemoglobin on the equilibrium between: (a) dimers and monomers, (b) the native and denatured monomer. [From (*32*); reproduced by permission of *Nature*]

sufficient to protect thermostable enzymes from denaturation by heat.

Why are mesophile enzymes unstable when it would be so cheap, in structural terms, to stabilize them? Many have been found to turn over rapidly because they become denatured and are then broken down by proteases while fresh ones are being synthesized (*29*). This apparently wasteful process may be an essential part of metabolic regulation.

Denaturation

Having discussed what holds proteins together, let us see what breaks them apart. Two effects may contribute to the denaturation of a protein by acid or alkali. First, the free energy of a spherical polyelectrolyte is proportional to the square of its net surface charge, so that its stability decreases on either side of its isoelectric point. Second, titration with acid or base may ionize groups that lie buried in the nonpolar interior. On becoming ionized, these groups attract hydration shells which are misfits in the native structure and therefore shift the equilibrium toward the unfolded form. The question is which of these effects dominates.

X-ray analyses and sequence studies show that the molecular architecture of mammalian hemoglobins must be closely similar, yet they vary greatly in their susceptibility to denaturation by alkali (*30*). For instance, in 0.1*N* NaOH at 20°C adult and fetal human carbonmonoxyhemoglobin are denatured with half-times of 20 seconds and 12 minutes, respectively, while bovine hemoglobin is stable. It takes 0.5*N* NaOH to denature it with a half-time of 3 hours (*31*). Analysis of the differences in amino acid sequence between the three hemoglobins shows that most of the replacements are at positions that are either external or that lie in the water-filled internal cavity. There is no difference between the number of groups that would be discharged at high *p*H so that the net surface charge of all three hemoglobins must remain the same and cannot affect the rate of denaturation. Three of the other differences involve exchanges of nonpolar residues that could not affect the rate of alkali denaturation. Significant replacements occur at the three remaining positions: two of these lie at the boundary between the subunits of the $\alpha\beta$ dimer and are occupied by cysteines, while the third position lies buried in the β chain and is occupied by tyrosine, in adult human hemoglobin. In fetal human hemoglobin one of the cysteines is replaced by threonine and the tyrosine is replaced by tryptophan. In bovine hemoglobin one of the cysteines is replaced by serine and the other by valine, and the tyrosine by phenylalanine. The buried cysteines and the tyrosine would be uncharged below *p*H 12 but are likely to become ionized above that *p*H, while serines, threonines, and tryptophans would remain unionized even in 0.1*N* NaOH. In short, adult human hemoglobin has three buried side chains ionizable above *p*H 12, while fetal human hemoglobin has only one and bovine none. The differences in the rate of alkali denaturation arise because isolated, nonsolvated charges in the interior of proteins are unstable and tend to surround themselves with water. Dissociation of the $\alpha\beta$ dimer allows the two cysteines at the interface between the two subunits to become ionized and hydrated; the bulk of the hydration shell opposes reassociation, so that the equilibrium is shifted toward the unstable monomeric form. Similarly, opening of the tertiary structure of the β chain allows the buried tyrosine to become ionized and hydrated, and access of water into the interior displaces the equilibrium toward the unfolded form (Fig. 5) (*32*). A similar displacement of the equilibrium is observed in an abnormal human hemoglobin in which the same internal tyrosine is replaced by aspartate. This hemoglobin is denatured and precipitated in the red cell in vivo (hemoglobin Wien: Tyr 130β → Asp) (*33*). Alkali denaturation of human oxyhemoglobin has an entropy of activation of −39 electrostatic units while bovine has one of only −6 electrostatic units, consistent with denaturation immobilizing more water molecules in the human than in the bovine form. The rate of denaturation by acid is probably determined by the ionization of internal histidines but no one has tested this yet.

I have tried to show that electrostatic effects dominate the activities and properties of many proteins. The general distribution of charges on their surface is of little importance compared to the microscopic interaction between ionizable groups selected for some specific purpose such as subunit assembly, substrate binding and activation, cooperativity or thermal stability. Even in denaturation by acid and alkali, the rate-determining step consists of the ionization of specific internal residues. Earlier in this century, when the structure of proteins was still unknown, theoreticians, Edsall prominent among them, attempted to explain their properties in terms of idealized electrostatic models (*34*). Now that the structures of so many enzymes have been determined, we need new approaches to interpret their function in terms of electrostatic interactions between precisely placed atoms in real models. The development of such theories has only just begun.

In conclusion, I wish to acknowledge my great debt to John T. Edsall. His books (*34*) inspired me, and he stood out among biochemists in the 1940's and 1950's as the champion of x-ray crystallography. During the long lean years before the structures of myoglobin and hemoglobin were solved, Kendrew and I received more encouragement from him than from any other scientist outside the circle of our teachers in England.

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