PROTEINS AS BUFFERS

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It was a strange choice to ask me, who has not contributed to the description of the titration behavior of proteins, to discuss that subject. Nevertheless I am pleased to attempt to summarize the state of knowledge for our present purposes. Our best objective in this monograph is surely to make ourselves and each other better informed; we will be examining the meaning of terms for that purpose and not, I hope, to attempt to legislate their use. In that spirit the present assignment certainly promises to be informative to me.

Buffering refers to the minimization of *p*H change on the addition of acid or alkali. We evaluate the extent of buffering by seeing how much acid or alkali is required to change the *p*H; that is, by titrating. The steepness of the titration curve at any point yields the buffer value at that *p*H. About one-third of the amino acid residues in proteins contribute titratable groups; the number of such groups in many common proteins ranges from about 20 to over 200. The common groups include the carboxyl groups, contributed by the sidechains of glutamic and aspartic acid; the amino groups on lysine sidechains; the guanidinium groups contributed by arginine sidechains; the imidazolium groups of histidyl residues; the phenolic groups contributed by tyrosyl residues; and the sulfhydryl groups of cysteine residues. Hemoglobin contains also the propionate sidechains of heme, and an acidic water molecule on each iron atom. In addition, nearly all proteins contain terminal α-amino and α-carboxyl groups on their polypeptide chains.

FIGURE 1 shows a titration curve obtained for β-lactoglobulin by Nozaki.[1] The ordinate label illustrates that the titratable groups may be counted from any desired reference point. The figure shows how the titration curve may be divided into regions; in this case the titration has been terminated before the guanidinium groups were deprotonated, or we should see a fourth region. The groups titrating in the acid range may tentatively be identified as carboxyl groups, those in the middle range as imidazole and terminal amino groups, and those titrating between *p*H 8.5 and 11.5 as sidechain amino, phenolic and sulfhydryl groups. Independent determinations of the number of terminal carboxyl and amino groups permit subtraction of these quantities from the first and second titration regions, respectively, where each is likely to be titrated, to give us a presumptive count of sidechain carboxyl and imidazole groups.

We may then determine how many phenolic groups have been titrated in the upper range, by performing a separate spectro-photometric titration; that is by observing the location and extent of a spectral change at 295 mμ

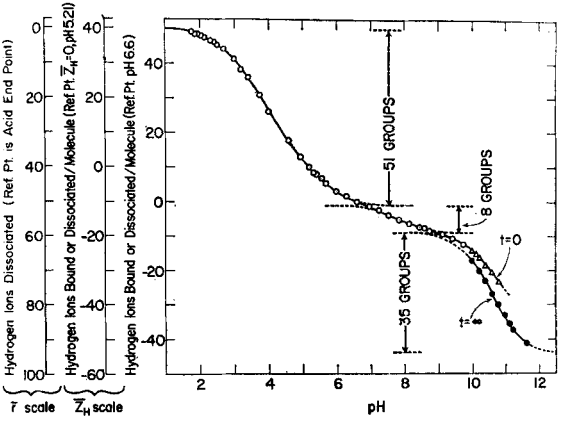

FIGURE 1. Titration curve of β-lactoglobulin at ionic strength 0.15 and 2.50. See text for discussion. From the data of Nozaki, cited by Tanford. 1962. *Advances in Protein Chemistry* **17:** 69 with permission.

characteristic of the deprotonation of each such group. We may also refine our division of our titration curve among the contributing groups by performing the titration at two different temperatures (FIGURE 2). As Jeffries Wyman and his associates showed,[2] this step will have characteristically little effect on the *p*H at which carboxyl groups titrate; but a characteristic shift, corresponding to a characteristic heat of dissociation, will occur at the point where the titration of the carboxyl groups is nearly completed and that of imidazole

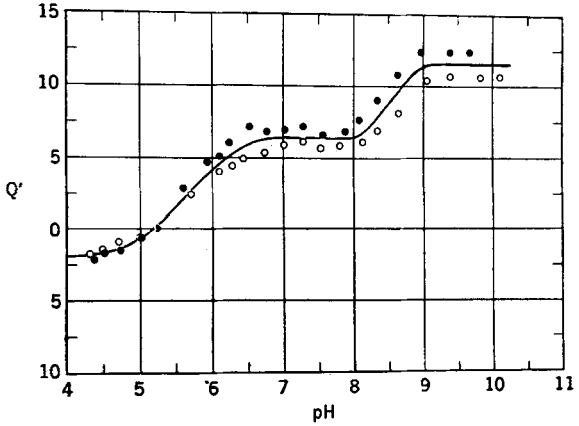

FIGURE 2. Apparent heat of dissociation of oxyhemoglobin as a function of *p*H. From Jeffries Wyman, 1939. *J. Biol. Chem.* **127:** 1 with permission.

groups becomes dominant. The step to a higher heat of dissociation as the amino groups begin to be titrated is not a clearcut one, unfortunately, because a distinction between imidazole, phenolic and sulfhydryl groups is not possible on this basis; nor that between terminal α-amino groups and the sidechain amino groups. An additional method that has been used for discriminating some of the nitrogenous groups from tyrosyl and sulfhydryl groups is to observe the effect of the presence of an excess of formaldehyde on the titration curve. Titrations may also be made in the presence of a chelating cation to assist in identifying dissociating groups.

Although the results obtained by these methods correspond rather well to the predictions from the known amino acid analysis of the protein, there are important deviations from the predictions. One such deviation is seen in FIGURE 1. Above *p*H 9.75, the *p*H values observed immediately on the addition of alkali are substantially higher than those observed when time is permitted for the *p*H to stabilize. If the titration is repeated with protein denatured at *p*H 12.5, the curve in this region becomes stable and reversible.

This phenomenon could arise from the gradual release from a "buried" position in an internal, hydrophobic region, either of uncharged phenolic or sulfhydryl sidechains, or of the unprotonated, apolar form of the sidechain of histidine. It is especially common that phenolic groups cannot be titrated as long as the native conformation of a protein is retained. All of the phenolic groups in ovalbumin, and half those in ribonuclease and chymotrypsinogen, for example, are not titrated at all in the normal position. Alternatively, but less probably, the phenolic groups might be hydrogen-bonded to some other structure. The titration behavior of native β-lactoglobulin in this region has not been entirely rationalized.

For hemoglobin, about half (16–22) of the imidazole groups do not titrate in the normal range, but are only gradually titrated when the *p*H is brought below 4.5. FIGURE 3 shows the results when the cyanide complex of ferrihemoglobin was titrated by Jacinto Steinhardt and his associates.[3] This complex is far more stable to acid denaturation than oxyhemoglobin, and by quick measurements on flowing solutions (lower curve) a considerable discrepancy in the number of groups titrated in the native and denatured (upper curve) forms could be observed. But what this figure shows is that this discrepancy develops in the neutral region, not in the acidic region. Apparently about 22 imidazole groups are restrained in their uncharged state in the native protein and cannot be titrated until denaturation occurs. Hence for the native protein their titration is an accompaniment of denaturation.

The mode of restraint of four of these imidazole groups is presumed to be well understood. Each of these four is bonded to an iron atom of a heme group; and we cannot expect them to be protonated until the hydrogen ion concentration is high enough to displace the iron. The remaining inaccessible imidazole groups must be restrained in some different way.

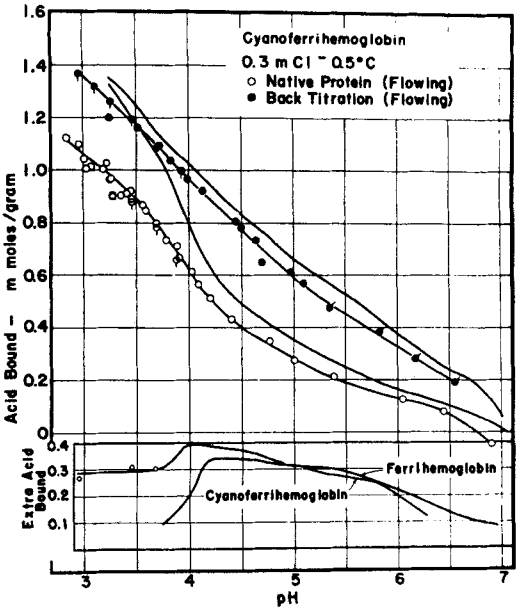

FIGURE 3. Titration data for native and denatured (back titration) carbonyl hemoglobin at 0.5° in the presence of 0.3 *M* chloride and with air excluded. Lower curves, differences in acid bound. From Steinhardt, Ona & Beychok. 1962. *Biochemistry* **1:** 29 with permission.

In addition to the possibility that a group will not titrate in the native protein until the native conformation is altered, either because the uncharged form of the group is buried or because one form of the group is bonded to another structure, we have an important factor of *electrostatic effects* in altering titration behavior. This factor can also be modified by conformational changes. Each dissociation reaction occurs in an environment containing many other charged groups. Furthermore the numbers of these groups is changed by the titration itself. In general electrostatic effects tend to stablize the charged form of a dissociating group. Thus the intrinsic p*K* of carboxyl groups is moved downward, and that of amino groups upward. In addition the titration curve tends to be made less steep. These effects can be minimized by raising the ionic strength. Linderstrøm-Lang has treated the matter quantitatively under the simplifying assumption that the charges are spread on the surface of a spherical molecule not penetrated by the solvent. If the solvent is allowed to penetrate the sphere, the dissociating group needs to lie only a short distance inside the molecular surface to have the electrostatic effect greatly accentuated (Tanford).[4] Under the generalized conditions electrostatic effects would occur on all dissociating groups and should not

cause positional anomalies for the titration of only certain specific groups. If, however, we consider that a dissociating group might emerge from or penetrate into a region with a high concentration of charged groups, then the charging electrostatic interactions might cause an unusually large shift in the acid strength of that dissociating group.

For our purposes in this monograph, the most significant deviation from simple titration behavior is that presented by the Bohr effect of hemoglobin. It has been established by several independent methods during the past dozen years, as summarized recently by Reinhold and Ruth Benesch,[5] that hemoglobin undergoes a large, reversible change in conformation when it is oxygenated and deoxygenated. As a result of this change in conformation, its reactivity with many substances, in addition to the hydrogen ion, is strongly modified. Conversely the addition of the hydrogen ion to certain groups, and its removal from them, must be assumed to cause essentially the same reversible conformational change, as can be observed by measuring in turn the affinity for oxygen.

You will recall that Jeffries Wyman showed that the shift in the titration curve on oxygenation of horse hemoglobin could be accounted for by the fall in the pK' of one imidazole group per Hb subunit from 7.93 to 6.68, and by a smaller rise in the pK' of a much more acidic imidazole group. Recently a rather similar downward shift has been calculated for human hemoglobin, from 8.25 to 6.95.[6] These four imidazole groups presumably occur one in each of the four peptide chains. The α and β chains are now of course known not to be identical, and it would be strange if the four imidazole groups had exactly the same intrinsic pK' values. Although an abnormal hemoglobin (hemoglobin H) containing four β chains fails to show any Bohr effect,[7] this result does not necessarily indicate that the oxygen-sensitive imidazole groups are restricted to the α-chains. Rather, it must be the relation established between the chains that is decisive to the Bohr effect.

We must suppose that these oxygenation-sensitive imidazole groups find themselves in a new environment when the reversible conformational change occurs. It is interesting that ionizable dye residues introduced into the hemoglobin molecule at some distance from the heme groups also show abnormally high pK' values in the deoxygenated state, as Irving Klotz and Luisa Tosi showed.[8] The new comparison with x-ray diffraction by Perutz and associates of crystalline reduced and crystalline oxygenated horse hemoglobin, each with two mercury atoms attached to the reactive sulfhydryl groups as markers (FIGURE 4), shows that on reduction the distance between two mercury atoms is increased from 30.0 to 37.3 angstroms.[9] This increase in separation occurs almost entirely in the axis describing the separation of the two β chains, which appear to be completely out of contact in the reduced form. That being the case, one cannot suppose that the change in this distance is caused by an interaction between the two β chains.[10]

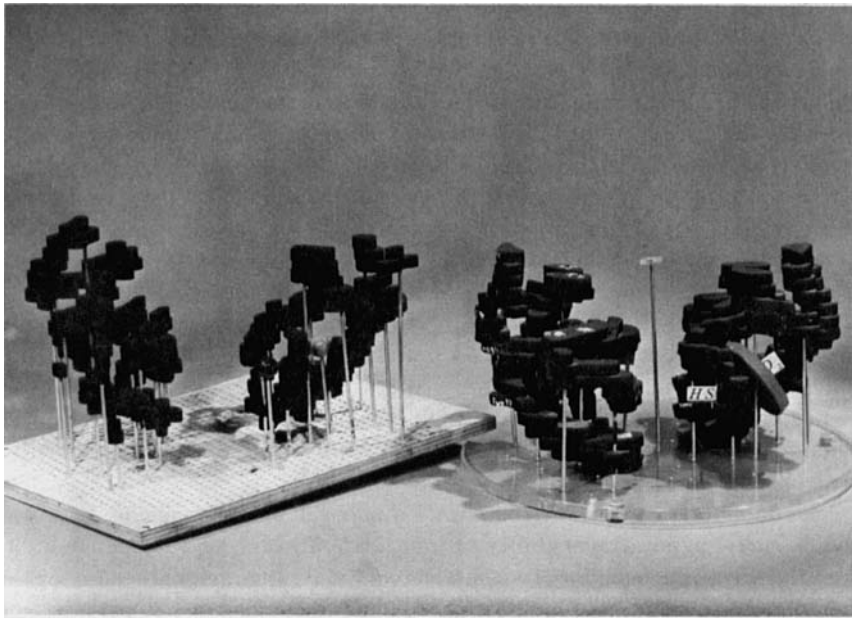

FIGURE 4. View of the two pairs of β-chains, showing the widening of the gap between them in the human reduced form *(left)*, as compared with horse oxyhemoglobin *(right)*. From Muirhead & Perutz. 1964. Nature **199:** 633 with permission.

In that connection it is interesting that results of Eraldo Antonini and his associates at The University of Rome and the Regina Elena Cancer Research Institute in Rome, appear to require that the half molecule, $\alpha\beta$, be intact for a Bohr effect; there is, however, no discontinuity in the change in magnitude of the Bohr effect as the ionic strength attains high enough levels to cause dissociation of hemoglobin to the half molecule.[11] Furthermore, the abnormal hemoglobin H containing four β chains, which shows the shape of a rectangular hyperbola for its oxygen dissociation curve, and no Bohr effect, suffers no change in the separation of the mercury atom markers on deoxygenation.[12]

One of the most interesting observations of the reversible structural change of hemoglobin is that made by Quentin Gibson,[13] who showed that carbon monoxide-hemoglobin, dissociated by flash protolysis, is able for a short interval of time to react with oxygen or carbon monoxide at a much higher rate than ordinary deoxyhemoglobin. After the CO has been removed a measurable interval is required for the usual change in conformation to occur to the more stable form of deoxyhemoglobin. Again, in the case of

hemoglobin H, no tendency could be observed for reactivity with oxygen to decrease for a time after flash protolysis of its carbon monoxide derivative.[14]

The character of the change in the environment of the sensitive dissociating groups of hemoglobin, and the way in which this environmental change modifies the hydrogen-ion dissociation so sharply, remains undetermined. I believe that none of the three factors discussed above, namely a change in the electrostatic environment, a change in the hydrophobic character of the environment, or a change in specific bonding of these imidazole groups, can be entirely excluded at this time. Nevertheless, in the present intense phase of study, the effect seems likely soon to be largely explained.

The Bohr effect is in any case the first observed and best documented case in which structural change of a protein has been linked with biological function. The importance of such structural changes appears likely, as hemoglobin illustrates, to extend well beyond the subjects of contractile behavior and biological transport, but probably also to the physiological control of enzymatic reactivity.

Serum albumin also shows on titration many of the phenomena discussed above, including an irreversibility of the titration in the acid region. In addition the titration behavior is complicated by its characteristic binding of the anion of the acid used in titrating it. This phenomenon has perhaps not been studied as much as it deserves to be, for its relation to the subject of CO_2 transport and the acid-base balance.

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