sample diluted with water to 25 ml. in a volumetric flask. Ornithine was determined by the method of Chinard.[16] Thiosulfate and glycine were found not to interfere with the analysis. Values of 2.9, 3.1 and 2.9 moles ornithine/mole ferrichrome were found in triplicate analyses.

Analysis for L-Ornithine.—The assay was conducted with *E. coli* mutant 160–37 which was kindly supplied by H. J. Vogel.[6] Glycine and D-ornithine were found not to interfere. Triplicate analyses of the HI hydrolysate described above gave 2.8, 3.2 and 2.8 moles L-ornithine/mole ferrichrome.

Syntheses of I.—The preparation of the ω-N-hydroxy derivatives of DL-ornithine and DL-lysine will be the subject of a succeeding communication.[11] The following procedure relates to the preparation of DL-compound I in sufficient quantities for comparison with the natural product.

A solution containing 500 mg. (2.26 mmoles) of DL-5-γ-bromopropylhydantoin,[10] 250 mg. (3.62 mmoles) of $NaNO_2$ and 250 mg. of urea in 5 ml. of dimethylformamide was agitated with a magnetic stirrer for 2 hr. at room temperature as described by Kornblum, *et al.*[17] The solvent was then removed and the residue extracted with acetone. The acetone extracts were filtered to yield a clear yellow solution. The acetone was evaporated and the residue dissolved in 10 ml. of ethanol. After addition of 3 ml. of water and 500 mg. of NH_4Cl, the solution was heated to near boiling and 500 mg. of zinc dust added in portions over a period of several minutes. The solution was then filtered, acidified with dilute HCl and evaporated to dryness. The residue was dissolved in 2–3 ml. of water and applied to a 1 × 5 cm. column of Dowex 50H. The column was washed with water and the tetrazolium positive material eluted with 0.2 *M* NH_4OH. The tetrazolium-positive fractions were pooled, acidified with dilute HCl and evaporated to dryness. The residue was dissolved in 1 ml. of 6 *N* HCl and heated in an autoclave at 135° for 4 hr. After removal of excess HCl, this preparation on electrophoretic analysis (see above) showed the presence of approximately equal portions of ninhydrin positive materials with the mobility characteristics of neutral amino acid(s), compound I and ornithine.

The entire preparation was placed on a 1.5 × 50 cm. column of 200–400 mesh Dowex 50.H × 4 equilibrated with 2.5 *N* HCl. The column was operated at a flow rate of one fraction (4 ml.) per 12 min. with 2.5 *N* HCl as developing solvent. The neutral amino acid(s) as well as an unidentified tetrazolium-reducing peak emerged within the first 50 fractions. Fractions 50–60 gave a positive spot test on paper with tetrazolium. These fractions were pooled, evaporated to dryness and the total residue applied in a narrow band across the entire 15 cm. width of a sheet of Whatman 3 MM filter paper. A spot of authentic ornithine was placed on the origin at one side of the paper. The buffer and operating conditions were the same as those used above. After approximately 1 hr. the paper was air dried and a strip cut off along the edge bearing the authentic ornithine spot. The ninhydrin spray revealed the presence of only two components, the authentic ornithine (15.7 cm.) and compound I (11.1 cm.). A second strip was sprayed with ninhydrin and this showed only a band at 11.1 cm. Finally, a third strip sprayed with tetrazolium reagent gave one band at the 11.1 cm. position.

The 11.1 cm. band from the remainder of the paper was eluted and reduced with HI as was done with the natural product (see above). Paper electrophoretic analysis of the reduced preparation revealed the presence of ornithine as the sole amino acid.

Synthetic and natural I were indistinguishable by ionic mobility on paper and by their behavior toward the ninhydrin and tetrazolium reagents. In the experiment described above, the ionic mobility ratio of synthetic I/ornithine was 11.1/15.7 = 0.708. In a separate but similar analysis the corresponding ratio for natural I/ornithine was 10.1/14.3 = 0.706.

Elementary Analyses. (a) **Ferrichrome.**—Calcd. for $C_{27}H_{42}N_9O_{12}Fe$: C, 43.79; H, 5.72; N, 17.02; Fe, 7.54. Found: C, 44.02; H, 5.90; N, 16.55; Fe, 7.54.

(b) **Ferrichrome A.**—Calcd. for $C_{41}H_{58}N_9O_{20}Fe\cdot 4H_2O$: C, 43.78; H, 5.91; N, 11.21. Found: C, 43.62; H, 5.80; N, 11.18.

A thrice recrystallized sample was dried at 100° under reduced pressure over P_2O_5 for 24 hr. The specimen was then allowed to reach constant weight under atmospheric conditions. The dry sample, 26.5 mg. (25.17 μmoles) reached constant weight at 28.3 mg. The water absorbed, 1.8 mg. (100 μmoles) was 99.3%.

Anal. Calcd. for $C_{41}H_{58}N_9O_{20}Fe$: Fe, 5.3. Found: Fe, 5.3.

(16) F. P. Chinard, *J. Biol. Chem.*, **199**, 91 (1952).

(17) N. Kornblum, H. O. Larson, R. K. Blackwood, D. D. Mooberry, E. P. Oliveto and G. E. Graham, *J. Am. Chem. Soc.*, **78**, 1494 (1956).

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Ionization-linked Changes in Protein Conformation. I. Theory

BY CHARLES TANFORD*

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If a protein molecule can exist in two conformations, α and β, and if the equilibrium constant (β)/(α) depends on *p*H, then a necessary consequence of the laws of thermodynamics is that at least one titratable group has a different *pK* in the two conformations. The objective of this paper is to give exact equations for the relation between the *p*H-dependence of (β)/(α) and the course of titration of the anomalous titratable groups, in a form suitable primarily in situations where electrostatic interaction does not play an important role.

If a protein molecule in solution can exist in two different conformations, α and β, and if the equilibrium distribution between these depends on *p*H, then the titration curves characteristic of the two conformations are necessarily different. This proposition is readily proved. If (β)/(α) at pH_1 differs from the same ratio at pH_2, then

$$F^0(\beta, pH_1) - F^0(\alpha, pH_1) \neq F^0(\beta, pH_2) - F^0(\alpha, pH_2)$$

where F^0 is the free energy in a suitable standard state. It follows at once that

$$F^0(\beta, pH_2) - F^0(\beta, pH_1) \neq F^0(\alpha, pH_2) - F^0(\alpha, pH_1)$$

which is equivalent to saying that the titration curve of β between pH_1 and pH_2 differs from the titration curve of α between the same limits.

The purpose of this paper is to derive the equations which connect these two observable events: the effect of *p*H on conformation and the accompanying changes in titration parameters. The titration curve depends on two properties of the protein molecule's side chains,[1-4] their

* Department of Biochemistry, Duke University, Durham, N. C.

(1) K. Linderstrøm-Lang, *Compt. rend. trav. lab. Carlsberg*, **15**, No. 7 (1924).

(2) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).

(3) C. Tanford, "Electrochemistry in Biology and Medicine," T. Shedlovsky, editor, John Wiley and Sons, Inc., New York, N. Y., 1955.

(4) C. Tanford and J. G. Kirkwood, *J. Am. Chem. Soc.*, **79**, 5333 (1957).

intrinsic free energies of ionization (*i.e.*, the free energies of ionization appropriate to a hypothetical discharged protein molecule) and the electrostatic interaction between them. This paper will consider primarily those situations in which the intrinsic free energy of one or more side chains differs in two conformations, but electrostatic interactions between charged side chains remain the same. The results will thus be most important for situations involving "buried" acidic or basic groups or pK anomalies which arise from hydrogen bonding, intramolecular ion-pair formation, etc. They are in principle applicable to situations in which reduced electrostatic interaction is the driving force (*e.g.*, molecular unfolding at extreme pH's), but the results will not be in convenient form for application to that situation.

Conformation Changes Associated with a Single Titratable Group.—We suppose that a protein molecule can exist in two conformations α and β and that the transition between them is associated with the state of a single acidic group AH,[5] such that α is more stable when the group is in its acidic form and β is more stable when it is in its basic form. Where (αAH) and (βAH) represent the concentrations of molecules with undissociated AH groups in the two conformations and (αA) and (βA) the corresponding concentrations of molecules with dissociated A groups, we may write the following equilibrium constants for the transition $\alpha \rightarrow \beta$.

$$k_0 = \frac{(\beta\text{AH})}{(\alpha\text{AH})} < 1 \qquad k_1 = \frac{(\beta\text{A})}{(\alpha\text{A})} > 1 \tag{1}$$

We also have the expressions for the acid dissociation constants (K) of group AH in the two configurations

$$K_\alpha = (\alpha\text{A})(\text{H}^+)/(\alpha\text{AH}) \quad K_\beta = (\beta\text{A})(\text{H}^+)/(\beta\text{AH}) \tag{2}$$

where (H^+) will ordinarily represent hydrogen ion activity.

By considering the two paths along which one can proceed from αAH to βA, one obtains two expressions for (βA)(H^+)/(αAH), from which it follows at once that

$$K_\beta/K_\alpha = k_1/k_0 \tag{3}$$

i.e., if β is stabilized by dissociation of AH, then βAH is necessarily more acidic than αAH.

The assumption that AH is the only group affecting the transition is by equation 3 equivalent to saying that the dissociation constants of other groups are the same in α and β. It also implies, of course, that there is no specific interaction between AH and other titratable groups. Nonspecific electrostatic interaction may be incorporated into the model as shown below.

Two parameters which are subject to experimental measurement are of interest: the extent of dissociation of AH, which we shall call x_A, *i.e.*

$$x_A = \frac{(\beta\text{A}) + (\alpha\text{A})}{(\beta\text{AH}) + (\alpha\text{AH}) + (\beta\text{A}) + (\alpha\text{A})} \tag{4}$$

and the extent of conversion of α to β, which we shall call f_β, *i.e.*, where (α) is the concentration of all forms of the protein molecule in conformation α and (β) the corresponding quantity in conformation β

$$f_\beta = (\beta)/[(\alpha) + (\beta)] \tag{5}$$

It is to be noted, however, that no method is ordinarily available to determine whether all molecules in a given protein solution have the same conformation, *i.e.*, k_0 and k_1 are not directly measurable. In the present situation experimental parameters which depend on conformation will be invariant before titration of AH begins, will change during titration of AH, and will be invariant again when the titration is complete. It will not be possible to distinguish between situations in which the transition proceeds from essentially 100% α to essentially 100% β (equivalent to $k_0 \sim 0$, $k_1 \sim \infty$), and situations in which the transition is from a mixture (say, 80% α, 20% β) to another mixture (say, 20% α, 80% β). It is thus expedient to define an *apparent extent of conversion*, y_β, as

$$y_\beta = \frac{f_\beta - k_0/(1 + k_0)}{k_1/(1 + k_1) - k_0/(1 + k_0)} \tag{6}$$

which will correspond to the observable data regardless of the values of k_0 and k_1.

Using equations 1, 2 and 3, equation 4 becomes

$$x_A = \frac{K^*/(\text{H}^+)}{1 + K^*/(\text{H}^+)} \tag{7}$$

where

$$K^* = \frac{k_0(1 + k_1)}{k_1(1 + k_0)} K_\beta = \frac{1 + k_1}{1 + k_0} K_\alpha \tag{8}$$

Our first important conclusion is then that the link between the titration of AH and the conformation change does not change the *form* of the titration curve of AH, but simply affects the pK.

In the same way we obtain from equation 6 that y_β is also equal to $[K^*/(\text{H}^+)]/[1 + K^*/(\text{H}^+)]$, *i.e.*

$$y_\beta = x_A \tag{9}$$

and the second important conclusion is that with the present assumptions the apparent course of the transition is invariably identical with the course of titration of AH.

Effect of Electrostatic Interaction.—Electrostatic interaction between the charged groups of a protein molecule is usually considered in terms of the approximate theory of Linderstrøm-Lang,[1] in which the electrostatic free energy W at an average net charge $\bar{Z}$ is proportional to $\bar{Z}^2$. The corresponding effect on the titration curve is to replace the dissociation constant K by the product of an intrinsic constant K_{int} and an exponential charge-dependent factor

$$K = K_{\text{int}} e^{2w\bar{Z}} \tag{10}$$

The relation between W and w may be written as $W = 2wRT\bar{Z}^2$.

It is assumed in this section that electrostatic interaction *per se* does not affect the transition $\alpha \rightarrow \beta$. Outside the region of titration of AH, $W_\beta - W_\alpha$ must therefore be independent of charge, which requires that $w_\alpha = w_\beta$. With this restriction, normal electrostatic interaction between titratable groups may be taken into account by replacing the K's of the preceding equations by equation 10. This will make K_α, K_β

(5) We need not distinguish between dissociations of the type $\text{AH}^+ \rightarrow \text{A} + \text{H}^+$ and $\text{AH} \rightarrow \text{A}^- + \text{H}^+$, and charges are therefore omitted in the symbols for AH and A.

and K^* pH-dependent, flattening the titration curve in the usual way.[2] The ratio k_1/k_0 (equation 3) will also be slightly pH-dependent, because, at any pH in the region of the transition, $\bar{Z}$ will have a slightly different value in the two conformations. However, k_1/k_0 will be primarily a measure of $(K_{\text{int}})_\beta/(K_{\text{int}})_\alpha$.

It is to be noted that the principal conclusions of this section, as expressed by equations 7 and 9, are independent of any assumption concerning the actual magnitude of electrostatic interactions.

Titratable Group Buried in a Hydrophobic Region.—A titratable group may be buried in the hydrophobic interior of a globular protein molecule. Ordinarily it must be *uncharged* when so buried because the electrostatic self-energy of a charged site in a medium of low dielectric constant is prohibitively large.[6] We shall therefore assume that if a change in conformation is associated with titration of a buried group, only the uncharged form will be buried. The conformation containing the group in its charged form will be such as to leave the group in contact with free solvent, such that its pK is that normally expected of the group.[7]

If the buried group is an acidic group (AH uncharged) we have K_β equal to the normal K for the group, and $k_1 \sim \infty$. We have (from equation 8)

$$K^* = k_0 K_\beta/(1 + k_0) \qquad (11)$$

Thus k_0 is determinable from the difference between pK^* and the normal pK for the group. Similarly, if the buried group is basic (A uncharged) we have K_α equal to the normal K and $k_0 \sim 0$, giving

$$K^* = (1 + k_1)K_\alpha \qquad (12)$$

so that k_1 becomes determinable.

It is to be noted that we cannot determine whether the buried group is hydrogen-bonded or not. The value of k_0 or k_1 reflects the total relative stability of the two conformations, no matter what the factors which determine the stability.

It should also be noted that the foregoing considerations provide a third possible important mechanism whereby the intrinsic pK of a titratable group, in the normal treatment of titration curves, may be anomalous. Two other mechanisms have been suggested previously: pure hydrogen bonding[8] and strong near-neighbor electrostatic interactions.[9] What the present paper shows is that burying of a group in a hydrophobic region, with rearrangement accompanying ionization, will produce a similar effect. Moreover, this mechanism may be used to explain relatively large shifts in pK, because there is no limit on the magnitudes of k_0 and k_1. There is no evidence at present to suggest that pure hydrogen bonding of a single group or strong vicinal electrostatic effects can change pK values by more than about one pK unit.

Conformation Changes Associated with Two Titratable Groups.—We suppose again that the protein molecule can exist in two conformations α and β but that the transition is associated with two acidic groups AH and BH, such that α is more stable when both of these are in the acid form, and β more stable when both are in the basic form. We shall use αAHBH to designate the species with two undissociated protons, αABH the species with the proton dissociated from AH only, αAHB the species with the proton dissociated from BH only, αAB the species with both protons dissociated, all of these being in conformation α. The corresponding molecules in conformation β are designated βAHBH, etc. The equilibrium for the transition $\alpha \rightarrow \beta$ is now described by the equilibrium constants

$$k_0 = \frac{(\beta\text{AHBH})}{(\alpha\text{AHBH})} \quad k_1 = \frac{(\beta\text{ABH})}{(\alpha\text{ABH})} \quad k_2 = \frac{(\beta\text{AHB})}{(\alpha\text{AHB})} \quad k_3 = \frac{(\beta\text{AB})}{(\alpha\text{AB})} \qquad (13)$$

and the assumption concerning the relation between conformation and titration may be expressed as $k_0 < 1$, $k_3 > 1$, k_1 and k_2 intermediate. Acid dissociation constants are again designated by K. Three subscripts will be used: the first to indicate whether the proton dissociated is the first or second one, the next to indicate conformation, the last to indicate whether the proton is dissociated from AH or BH, *i.e.*

$$K_{1\beta\text{A}} = (\beta\text{ABH})(\text{H}^+)/(\beta\text{AHBH}) \qquad K_{2\beta\text{A}} = (\beta\text{AB})(\text{H}^+)/(\beta\text{AHB})$$
$$K_{1\beta\text{B}} = (\beta\text{AHB})(\text{H}^+)/(\beta\text{AHBH}) \qquad K_{2\beta\text{B}} = (\beta\text{AB})(\text{H}^+)/(\beta\text{ABH}) \qquad (14)$$

and a similar set for conformation α. These constants are not all independent, being related by the equations $K_{1\beta\text{A}}K_{2\beta\text{B}} = K_{1\beta\text{B}}K_{2\beta\text{A}}$, $K_{1\alpha\text{A}}K_{2\alpha\text{B}} = K_{1\alpha\text{B}}K_{2\alpha\text{A}}$. Corresponding to equation 3 we have four relations (three of them independent)

$$\frac{K_{1\beta\text{A}}}{K_{1\alpha\text{A}}} = \frac{k_1}{k_0} \quad \frac{K_{1\beta\text{B}}}{K_{1\alpha\text{B}}} = \frac{k_2}{k_0} \quad \frac{K_{2\beta\text{A}}}{K_{2\alpha\text{A}}} = \frac{k_3}{k_2} \quad \frac{K_{2\beta\text{B}}}{K_{2\alpha\text{B}}} = \frac{k_3}{k_1} \qquad (15)$$

We shall confine the examples chosen for analysis to situations in which the K's are "normal" in one of the two conformations. In the derivations it will be assumed that the conformation at high pH (*i.e.*, β) has the normal K's. The manner in which the equations must be transposed if the opposite situation prevails will be indicated at the end. The assumptions that the K's in β are normal includes the assumption that AH and BH are independent in β. Thus $K_{1\beta\text{B}} = K_{2\beta\text{B}} = K_{\beta\text{B}}$, $K_{1\beta\text{A}} = K_{2\beta\text{A}} = K_{\beta\text{A}}$. As the corresponding groups in α may not be independent, these equalities do not reduce the number of independent relations in equations 15.

Defining x_A by equation 4, x_B by a similar relation involving BH rather than AH, and y_β as in equations 5 and 6, we obtain

$$x_\text{A} = \left[\frac{k_0(1 + k_1)}{k_1(1 + k_0)}\frac{K_{\beta\text{A}}}{(\text{H}^+)} + \frac{k_0(1 + k_3)}{k_3(1 + k_0)}\frac{K_{\beta\text{A}}K_{\beta\text{B}}}{(\text{H}^+)^2}\right]\Big/ D \qquad (16)$$

(6) Ref. 4, footnote 14.

(7) This does not mean that other situations are impossible. A charged group could be buried as part of an ion pair, for instance. However, we clearly cannot consider all possible examples in a single paper.

(8) M. Laskowski and H. A. Scheraga, *J. Am. Chem. Soc.*, **76**, 6305 (1954). We use the term "pure hydrogen bonding" to describe the situation envisaged by these authors because the calculations they made ascribe the free energy of formation solely to the heat of formation of the hydrogen bond and to the loss in entropy resulting from loss of rotational freedom of the donor group.

(9) C. Tanford, *ibid.*, **79**, 5340 (1957).

$$x_B = \left[\frac{k_0(1+k_2)}{k_2(1+k_0)}\frac{K_{\beta B}}{(H^+)} + \frac{k_0(1+k_3)}{k_3(1+k_0)}\frac{K_{\beta A}K_{\beta B}}{(H^+)^2}\right] \Big/ D \quad (17)$$

$$y_\beta = \frac{k_0(1+k_3)}{k_3(1+k_0)}\left[\frac{k_3(k_1-k_0)}{k_1(k_3-k_0)}\frac{K_{\beta A}}{(H^+)} + \frac{k_3(k_2-k_0)}{k_2(k_3-k_0)}\frac{K_{\beta B}}{(H^+)} + \frac{K_{\beta A}K_{\beta B}}{(H^+)^2}\right] \Big/ D \quad (18)$$

where

$$D = 1 + \frac{k_0(1+k_1)}{k_1(1+k_0)}\frac{K_{\beta A}}{(H^+)} + \frac{k_0(1+k_2)}{k_2(1+k_0)}\frac{K_{1\beta B}}{(H^+)} + \frac{k_0(1+k_3)}{k_3(1+k_0)}\frac{K_{\beta A}K_{\beta B}}{(H^+)^2} \quad (19)$$

This result is clearly not as simple as that given by equations 7 to 9. However, it reduces to simple form whenever the conformational change proceeds from essentially 100%α to essentially 100%β, *i.e.*, when $k_0 \sim 0$, $k_3 \sim \infty$, provided that both k_1 and k_2 are considerably larger than k_0.[10] We obtain, whenever $K_{\beta A} \sim K_{\beta B}$

$$x_A = x_B = y_\beta = \frac{K^*/(H^+)^2}{1 + K^*/(H^+)^2} \quad (20)$$

$$K^* = k_0 K_{\beta A} K_{\beta B} \quad (21)$$

It is to be noted that the course of reaction follows an equation for the simultaneous dissociation of *two* protons.

If, with $k_0 \sim 0$, $k_3 \sim \infty$, $K_{\beta A} >> K_{\beta B}$, then AH and BH are titrated separately

$$x_A = \frac{K_A^*/(H^+)}{1 + K_A^*/(H^+)} \qquad x_B = \frac{K_B^*/(H^+)}{1 + K_B^*/(H^+)} \quad (22)$$

$$K_A^* = \frac{k_0(1+k_1)}{k_1} K_{\beta A} \qquad K_B^* = \frac{k_1}{1+k_1} K_{\beta B} \quad (23)$$

and the interesting result is obtained that the transition $\alpha \rightarrow \beta$ is also split into two parts

$$y_\beta = \frac{k_1}{1+k_1} x_A + \frac{1}{1+k_1} x_B \quad (24)$$

as illustrated for two values of k_1 in Fig. 1.

The criterion for use of equations 20 and 21, derived on the basis $K_{\beta A} \sim K_{\beta B}$ is that $K_{\beta A}/K_{\beta B} < 1/100k_0$. The criterion for use of equations 22 to 24, derived on the basis of $K_{\beta A} >> K_{\beta B}$, is $K_{\beta A}/K_{\beta B} > 100/k_0$. Thus deviations from the two limiting situations here given will occur primarily when $K_{\beta A}/K_{\beta B} \sim 1/k_0$. If, for instance, AH is a carboxyl group and BH a phenolic group we can expect that $K_{\beta A}/K_{\beta B} \sim 10^5$. With $k_0 = 10^{-5}$ we would then be in the intermediate region to which the simple expressions above are inapplicable. Calculations for this case, with $k_1 = 5$ and $k_1 = 0.25$ are shown in Fig. 2. With $k_1 = 5$ the result is close to that obtained when $K_{\beta A} \cong K_{\beta B}$, but the curves for y_β and $\frac{1}{2}(x_A + x_B)$ do not quite coincide; also, both curves are intermediate between one-proton and two-proton dissociation curves. With $k_1 = 0.25$ the inflection point between the titration curves of AH and BH is still barely observed, but the fact that most of the conformational change is associated with the titration of BH (equation 24) leads to a coalescing of the two parts of the curve for y_β. The over-all curve is *flatter* than a one-proton dissociation curve: this is the

(10) It is difficult to envisage a non-trivial situation in which the condition concerning k_1 and k_2 does not apply. If $k_1 \simeq k_2 \simeq k_0$ at the same time as $k_3 \gg k_0$, this means by equation 15 that $K_{1\alpha A} = K_{\beta A}$, $K_{1\alpha B} = K_{\beta B}$, whereas $K_{2\alpha A} \simeq (k_0/k_3)K_{\beta A} \ll K_{1\alpha A}$, $K_{2\alpha\beta} \simeq (k_0/k_3)K_{\beta B} \ll K_{1\alpha B}$. The condition $k_0 \sim 0$, $k_3 \sim \infty$ requires that k_0/k_3 be certainly no larger than 10^{-4}, and this means then that titration of either of the two groups in conformation α changes the free energy of ionization of the other group by more than 5000 calories (without change in conformation). At the present time we know of no interaction between two titratable groups which would produce so large an effect in the absence of conformational change. On the other hand, it is of course possible that $k_1 \sim k_0$, while $k_2 \gg k_0$, but this is a trivial exception to the rule, for it corresponds to the situation in which only one group (in this case BH) is associated with the conformational change, *i.e.*, it reduces to the situation treated in the previous section.

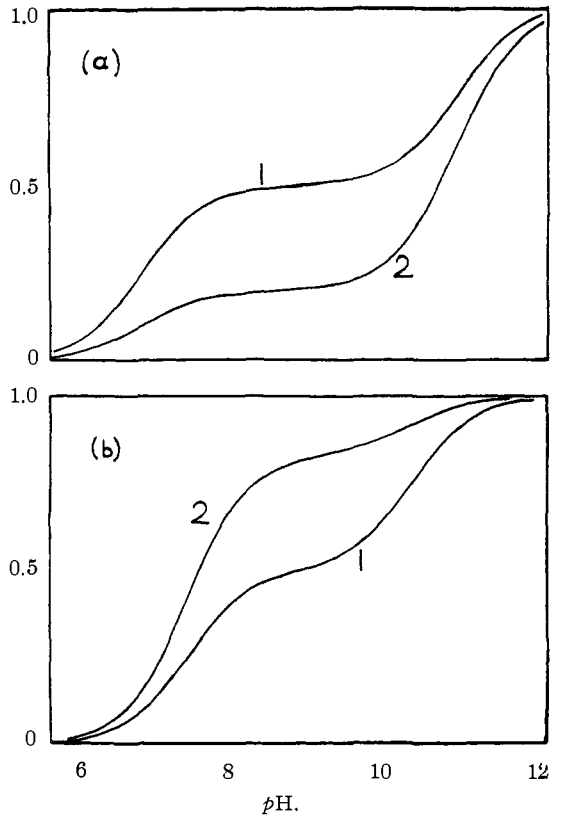

Fig. 1.—The course of titration and conformational change with $K_{\beta A} = 10^{-4}$, $K_{\beta B} = 10^{-10}$, $k_0 = 10^{-3}$, $k_3 > 100$. The value of k_1 is 0.25 in (a) and 5.0 in (b). In each case curve 1 represents $(x_A + x_B)/2$; curve 2 represents y_β.

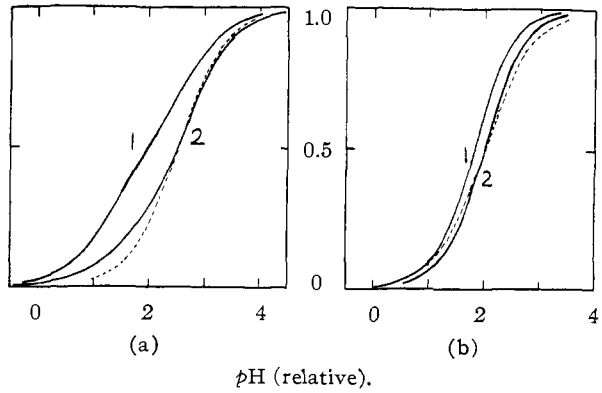

Fig. 2.—The course of titration and conformational change with $K_{\beta A}/K_{\beta B} = 10^5$ and $k_0 = 10^{-5}$. The value of k_1 is 5 in (a) and 0.25 in (b), and k_3 has been assumed >100. In each case curve 1 represents $(x_A + x_B)/2$, curve 2 represents y_β, and the dashed curve represents a one-proton dissociation curve, eq. 7.

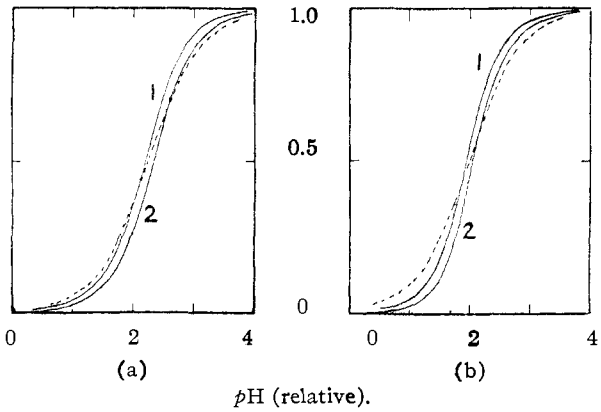

Fig. 3.—The course of titration and conformational change when either k_0 or $1/k_3$ is not very small, *i.e.*, when the conformational change is between a pure conformation (α or β) and a mixture of the two. In (a) we have $k_0 = 0.25$, $k_1 = k_2 = 5$, $k_3 = 10^3$. In (b) we have $k_0 = 10^{-3}$, $k_1 = k_2 = 0.1$, $k_3 = 5$. In both cases $K_{\beta A} = K_{\beta B}$. Curve 1 represents $x_A = x_B$, curve 2 represents y_β, and the dashed curve represents a one-proton dissociation curve.

only instance in which this result has been obtained.

We have so far considered only the case $k_0 \sim 0$, $k_3 \sim \infty$. The simple results obtained will not in general apply when either one of these constants is fairly close to unity. Figure 3 shows two examples (both with $K_{\beta A} = K_{\beta B}$), one having $k_0 = 0.25$, $k_3 = 10^3$, the other $k_0 = 10^{-3}$, $k_3 = 5$. In both cases the curve for $x_A = x_B$ does not coincide exactly with the curve for y_β. Moreover the curves are all intermediate between one-proton and two-proton dissociation curves, whereas a two-proton curve is invariably obtained for two identical groups (equation 20) when $k_0 \sim 0$, $k_3 \sim \infty$.

In conclusion we give the equations applicable when the groups AH and BH are "normal" in the acidic form (conformation α) rather than the basic form. In this situation $K_{1\alpha A} = K_{2\alpha A} = K_{\alpha A}$ and $K_{1\alpha B} = K_{2\alpha B} = K_{\alpha B}$ become the reference pK's in terms of which the relations are most conveniently expressed. In place of equations 16 to 19 we get

$$x_A = \left[\frac{1 + k_1}{1 + k_0}\frac{K_{\alpha A}}{(H^+)} + \frac{1 + k_3}{1 + k_0}\frac{K_{\alpha A}K_{\alpha B}}{(H^+)^2}\right]\Big/ D \quad (25)$$

$$x_B = \left[\frac{1 + k_2}{1 + k_0}\frac{K_{\alpha B}}{(H^+)} + \frac{1 + k_3}{1 + k_0}\frac{K_{\alpha A}K_{\alpha B}}{(H^+)^2}\right]\Big/ D \quad (26)$$

$$y_\beta = \frac{1 + k_3}{1 + k_0}\left[\frac{k_1 - k_0}{k_3 - k_0}\frac{K_{\alpha A}}{(H^+)} + \frac{k_2 - k_0}{k_3 - k_0}\frac{K_{\alpha B}}{(H^+)} + \frac{K_{\alpha A}K_{\alpha B}}{(H^+)^2}\right]\Big/ D \quad (27)$$

$$D = 1 + \frac{1 + k_1}{1 + k_0}\frac{K_{\alpha A}}{(H^+)} + \frac{1 + k_2}{1 + k_0}\frac{K_{\alpha B}}{(H^+)} + \frac{1 + k_3}{1 + k_0}\frac{K_{\alpha A}K_{\alpha B}}{(H^+)^2} \quad (28)$$

The condition for applicability of equation 20 now becomes $k_0 \sim 0$, $k_3 \sim \infty$, $K_{\alpha A}/K_{\alpha B} < k_3/100$, and to replace equation 21 we have

$$K^* = k_3 K_{\alpha A} K_{\alpha B} \quad (29)$$

The condition for applicability of equations 22 and 24 becomes $k_0 \sim 0$, $k_3 \sim \infty$ $K_{\alpha A}/K_{\alpha B} > 100k_3$ and to replace equation 23 we have

$$K_A^* = (1 + k_1)K_{\alpha A} \qquad K_B^* = \frac{k_3}{1 + k_1}K_{\alpha B} \quad (30)$$

Similar relations could readily be written down for the situation in which one group has a normal pK in conformation α while the other is normal in conformation β.

As before, electrostatic interaction between charged groups may be taken into account by replacing each of the dissociation constants $K_{1\alpha A}$, etc., by an expression of the form of equation 10.

Two Titratable Groups Buried in a Hydrophobic Region.—We again apply the above equations to titratable groups buried in the interior of a protein molecule.[11] If the two groups are acidic, $K_{\beta A}$ and $K_{\beta B}$ will be normal. Moreover, because of the large self-energy of a charged group far from the solvent,[6] both k_1 and k_2 will usually be $>>1$. (However, k_3 will be even larger.) If $k_0 \sim 0$ and $K_{\beta A} \sim K_{\beta B}$, equations 20 and 21 will apply, for these equations were obtained without specific assumption concerning an upper limit for k_1 or k_2. The value of k_0 can be obtained from the observed value of K^* as given by equation 21. It is to be noted that once again no information can be obtained about k_1, k_2 or the values of the dissociation constants in conformation α. For instance, we cannot distinguish between two carboxyl groups which are hydrogen-bonded to one another in conformation α and two such groups buried in the same region without hydrogen bonding. Such information could be obtained only if we knew the relative values of $K_{1\alpha A}$ and $K_{2\alpha A}$, *i.e.*, of k_0k_3/k_1k_2.

If $k_0 \sim 0$ and $K_{\beta A} >> K_{\beta B}$, such that AH and BH are titrated separately, equations 22 to 24 will be obeyed. However, with k_1 large, $K_A^* = k_0K_{\beta A}$, $K_B^* = K_{\beta B}$, $y_\beta = x_A$, *i.e.*, the reaction would be that characteristic of a single buried group. If a carboxyl group is buried and k_0 is not too small ($k_0 \sim 10^{-3}$), we would be unable to tell whether phenolic groups were buried in the same region.

If the two buried groups are basic, equations 20 and 29 would apply when $k_3 \sim \infty$ $K_{\beta A} \sim K_{\beta B}$, and k_3 could be determined from the data. If $K_{\beta A} >> K_{\beta B}$, then, with k_1 now necessarily very small, the situation would again reduce to that characteristic of a single buried group, in this case the less acidic one (BH).

Extension to n Titratable Groups. Conformation Changes Due to Electrostatic Repulsion.—When this treatment is extended to consider conformational changes associated with any number (n) of titratable groups, it is necessary to abandon the "microscopic" dissociation constants used in the earlier portion of this paper and to substitute, as one does in the treatment of titration curves, dissociation constants which are appropriate averages of many "microscopic" constants. One method is to group together all molecules with the same number of protons, dissociated from the n groups considered. Thus (βPH_n) and (αPH_n) may be used to designate concentrations of molecules in the two conformations which have all n acidic groups intact, and ($\beta PH_{n-\nu}$) and ($\alpha PH_{n-\nu}$)

(11) If the protein molecule contains several interior regions which can be independently unfolded, then both groups must be in the same region. Otherwise, we should be dealing with two independent conformation changes.

may designate concentrations of molecules with ν of the n protons dissociated. The state of titration of groups not connected with the transition from α to β is, as before, not explicitly considered.

We may now define the dissociation constants

$$K_{\alpha\nu} = \frac{(\alpha PH_{n-\nu})(H^+)}{(\alpha PH_{n-\nu+1})} \qquad K_{\beta\nu} = \frac{(\beta PH_{n-\nu})(H^+)}{(\beta PH_{n-\nu+1})} \quad (31)$$

with $1 \leq \nu \leq n$ and the equilibrium constants for the transition

$$k_\nu = \frac{(\beta PH_{n-\nu})}{(\alpha PH_{n-\nu})} \quad (32)$$

with $0 \leq \nu \leq n$. For the relation between these we now get

$$k_\nu/k_{\nu-1} = K_{\beta\nu}/K_{\alpha\nu} \quad (33)$$

and, again taking β as the conformation stable at high pH, this means that at least some of the $K_{\beta\nu}$ (in the absence of unusual coöperative effects *all* of the $K_{\beta\nu}$) must be larger than the corresponding $K_{\alpha\nu}$.

We obtain, for the titration curve of the n groups

$$\bar{\nu} = \frac{\sum_{\nu=0}^{n} \nu(1 + k_\nu)K_{\alpha1}K_{\alpha2}\ldots K_{\alpha\nu}/(H^+)^\nu}{\sum_{\nu=0}^{n} (1 + k_\nu)K_{\alpha1}K_{\alpha2}\ldots K_{\alpha\nu}/(H^+)^\nu} \quad (34a)$$

$$= \frac{\sum_{\nu=0}^{n} \nu(1 + 1/k_\nu)K_{\beta1}K_{\beta2}\ldots K_{\beta\nu}/(H^+)^\nu}{\sum_{\nu=0}^{n} (1 + 1/k_\nu)K_{\beta1}K_{\beta2}\ldots K_{\beta\nu}/(H^+)^\nu} \quad (34b)$$

while for the extent of conversion to β

$$f_\beta = \frac{\sum_{\nu=0}^{n} k_\nu K_{\alpha1}K_{\alpha2}\ldots K_{\alpha\nu}/(H^+)^\nu}{\sum_{\nu=0}^{n} (1 + k_\nu)K_{\alpha1}K_{\alpha2}\ldots K_{\alpha\nu}/(H^+)^\nu} \quad (35a)$$

$$= \frac{\sum_{\nu=0}^{n} K_{\beta1}K_{\beta2}\ldots K_{\beta\nu}/(H^+)^\nu}{\sum_{\nu=0}^{n} (1 + 1/k_\nu)K_{\beta1}K_{\beta2}\ldots K_{\beta\nu}/(H^+)^\nu} \quad (35b)$$

We shall consider in detail only the simple situation in which all groups are identical in both conformations and independent of each other except for non-specific electrostatic interaction. In that case the various $K_{\alpha\nu}$ and $K_{\beta\nu}$ are related to the corresponding constants K_α and K_β, for any single group, such that[12]

$$K_{\beta\nu}/K_\beta = K_{\alpha\nu}/K_\alpha = (n - \nu + 1)/\nu \quad (36)$$

Combining this relation with equation 33, we have, independent of ν

$$K_{\beta\nu}/K_{\alpha\nu} = k_\nu/k_{\nu-1} = K_\beta/K_\alpha = \rho \quad (37)$$

where ρ is a constant defined by the equation. Electrostatic interaction may be incorporated into this equation by expressing K_β and K_α as products of an intrinsic K and an electrostatic factor, as in equation 10. This will make ρ pH-dependent, even if $w_\alpha = w_\beta$, since the two conformations will differ in the relation between charge and pH. (It is to be noted that defining β as the conformation stable at high pH requires that $\rho > 1$; also that $k_\nu = k_0\rho^\nu$.)

We may make use of the identity

$$\sum_{\nu=0}^{n} x^\nu n!/(n - \nu)!\nu! \equiv (1 + x)^n \quad (38)$$

and x times its derivative with respect to ν

$$\sum_{\nu=0}^{n} \nu x^\nu n!/(n - \nu)!\nu! \equiv nx(1 + x)^{n-1} \quad (39)$$

and define an auxiliary function

$$\sigma = \frac{1 + \rho K_\alpha(H^+)}{1 + K_\alpha/(H^+)} = \frac{1 + K_\beta/(H^+)}{1 + K_\beta/\rho(H^+)} \quad (40)$$

which varies from 1 to ρ between the beginning and the end of the transition. With these relations we get

$$\bar{\nu} = \bar{\nu}_\alpha \frac{1 + k_0\rho\sigma^{n-1}}{1 + k_0\sigma^n} = \bar{\nu}_\beta \frac{1 + 1/k_0\rho\sigma^{n-1}}{1 + 1/k_0\sigma^n} \quad (41)$$

where $\bar{\nu}_\alpha = [nK_\alpha/(H^+)]/[1 + K_\alpha/(H^+)]$ gives the titration curve of conformation α alone and $\bar{\nu}_\beta$ is the corresponding expression for conformation β. For f_β we obtain

$$f_\beta = \frac{k_0\sigma^n}{1 + k_0\sigma^n} \quad (42)$$

which gives for the experimental parameter y_β (equation 6)

$$y_\beta = \frac{(1 + k_0)\sigma^n/(1 + k_0\sigma^n) - 1}{(1 + k_0)\rho^n/(1 + k_0\rho^n) - 1} \quad (43)$$

A single calculation using equations 40 to 43 is shown in Fig. 4. We have used $n = 6$, $k_0 = 10^{-3}$,

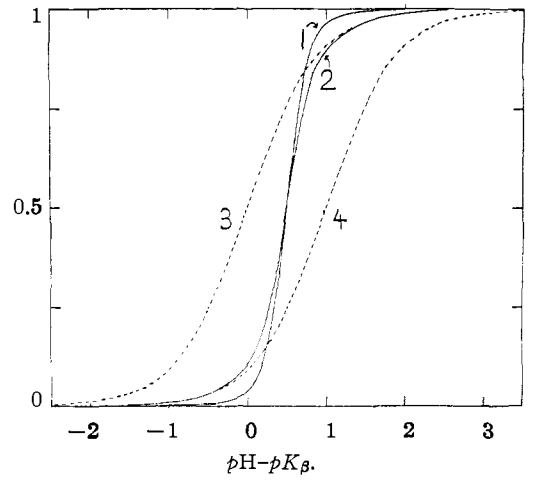

Fig. 4.—Calculations for $n = 6$, $k_0 = 0.001$, $k_6 = 1000$, with electrostatic effects assumed negligible. Curve 1 represents y_β, curve 2 represents the degree of dissociation, $x = \bar{\nu}/6$, curves 3 and 4 are the titration curves in conformations β and α, respectively, *i.e.*, they represent $x_\beta = \bar{\nu}_\beta/6$ and $x_\alpha = \bar{\nu}_\alpha/6$.

$k_6 = 10^3$. We have neglected electrostatic interaction so that ρ = constant = 10. We see that both the transition curve and the titration curve are much steeper than the curves which would correspond to dissociation of a single proton: the curve for y_β approximately follows a relation of the form

$$y_\beta = \frac{K^*/(H^+)^m}{1 + K^*/(H^+)^m} \quad (44)$$

(12) I. M. Klotz, in H. Neurath and K. Bailey, "The Proteins," Vol. I, Academic Press, Inc., New York, N. Y., 1953, Chapter 8.

with $m \sim 3$. The curve for $\bar{x} = \bar{\nu}/n$ approximately follows a similar curve with $m \sim 2$. While these results apply only to the particular situation treated, we may expect that their qualitative aspects are typical, *i.e.*, we shall often expect close correspondence between the titration curve and the transition curve and shall usually find both to be steep, such that m of equation 44 lies somewhere between 1 and n. For n titratable groups buried in the interior of a protein molecule we can expect ρ to be large, which leads to equation 44 with $m \rightarrow n$ for both y_β and $\bar{\nu}$.

The equations of this section may be applied to the expansion of a compact conformation to a more loosely coiled one under the influence of electrostatic repulsion. In this case the change in conformation is associated with *all* titratable groups. However, the effect of electrostatic interaction on K_α and K_β, and, hence, on ρ and σ, now plays the major role in the process, in contrast to the examples discussed explicitly in this paper. We shall postpone consideration of this situation to a later paper.

Discussion

An example of an ionization-linked change in conformation is the N → R transition in β-lactoglobulin, which is discussed in the following paper.[13] It will be seen that this transition is associated with a single titratable group. An example of a transition associated with several titratable groups is the acid denaturation of hemoglobin.[14] This transition obeys equation 44 with $m \simeq 5$ and it is accompanied by the titration of a large number of anomalous groups. However, both intrinsic and electrostatic factors are involved, so that the present treatment is not directly applicable.

It should be observed that the general approach we have formulated is not restricted to changes in conformation. For example, α could represent a free protein molecule and β a protein molecule to which a small molecule has been bound. The parameter y_β would represent the extent of binding, and the conclusion would be that if the extent of binding depends on pH, then there must be titratable groups which change their pK values when binding occurs. This particular situation has been discussed in considerable detail in connection with the binding of O_2 by hemoglobin.[15,16] Another possibility is that β represents a polymer of α, rather than a new conformation. In this case, if the degree of polymerization depends on pH, then the titration curve of the polymer must differ from that of the monomer. A well-known example is the polymerization of fibrin monomer.[17,18]

In conclusion, it must be noted that the general approach of this paper is not original. It should properly be regarded as an extension of the principles already formulated by Wyman[15] in connection with the hemoglobin–oxygen reaction.

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Ionization-linked Changes in Protein Conformation. II. The N → R Transition in β-Lactoglobulin

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Near pH 7.5 native β-lactoglobulin (N) undergoes a transition to a new conformation (R), as previously described. The reaction satisfies the criteria for a conformational change involving a single titratable group. This group is a carboxyl group which appears to be buried in the hydrophobic interior of the protein molecule in conformation N. Our earlier study of this reaction showed two rather than one anomalous carboxyl group per molecule, so that there must be two locations in the protein molecule where the N → R transition occurs independently. There is presumably one location in each of β-lactoglobulin's two polypeptide chains. The thermodynamic parameters of the transition, at 25°, at each of the two locations before titration of the buried groups, are $k_0 = (\mathrm{R})/(\mathrm{N}) = 0.0025$, $\Delta F^0 = 3500$ cal./mole, $\Delta H^0 = 7000$ cal./mole, $\Delta S^0 = 12$ cal./deg. mole. These estimates depend on assignment of normal values to the thermodynamic parameters for dissociation of the carboxyl group in conformation R, so that ΔF^0 has an uncertainty of perhaps 1000 cal./mole. The uncertainty in ΔH^0 is somewhat greater than this, while ΔS^0 has an uncertainty of the order of ±7 cal./deg. mole.

A previous paper from this Laboratory[1] has described a reversible change in conformation which β-lactoglobulin undergoes near pH 7.5. It was shown that this transition is accompanied by a change in specific rotation (but not in the dispersion parameter b_0[2]), by a small change in sedimentation coefficient,[3] (but not in molecular weight[4]) and by the titration of two anomalous carboxyl groups. The curves describing the changes in optical rotation and in sedimentation coefficient are essentially superimposable, and both coincide within experimental error with the titration curve of the two anomalous groups.[5]

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(5) Figure 1 of ref. 1 shows the titration curve to be somewhat steeper.