

Specific Interactions in Proteins Due to Proton Fluctuations

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Synopsis

A theory of site-site interaction due to proton/charge fluctuations in protein molecules has been developed. It is shown that, with the proper geometric configuration of identical ionizable groups on matching sites, a specific attraction may be established. This attractive force has a bell-shaped pH dependence and is maximal close to the pK of the groups involved. Various types of protein interactions are examined in the light of this theory.

Theory and Calculations

Interactions in protein systems may involve a number of different attractive and repulsive forces.¹ The types of bonds involved in protein associations can be classified as hydrogen bonds,¹⁻³ hydrophobic effects,^{1,2,4} and electrostatic interactions.⁵ In as complicated a system as found in proteins these usually act cooperatively, and it is rare that any given interaction can be characterized in terms of a single type of bonding. The purpose of this paper is to discuss one type of electrostatic force, namely, that due to the fluctuation in charge and charge configuration as a result of the fluctuations of protons between a number of ionizable groups with similar free energies of ionization.⁵⁻⁷ Kirkwood and Shumaker^{8,9} have shown that the charge fluctuation phenomenon involving all ionizable groups on a molecule may result in a nonspecific general attraction between two identical molecules. The presence of such a nonspecific attractive force in isoionic proteins has been verified experimentally.¹⁰⁻¹³ This force is strongest at the isoionic point in the absence of supporting electrolyte, and is greatly attenuated by screening electrolyte.^{9,13} The concept of proton fluctuations on protein molecules has been found to be fully compatible with dielectric increment measurements on some proteins.¹⁴⁻¹⁷ Furthermore, Kirkwood¹⁸ has shown that charge fluctuations between identical groups located in a specific site can account for the catalytic activity of some hydrolytic enzymes, while the general concept of charge fluctuations has been invoked to explain enzymatic activity in complex metabolic processes.¹⁹

In their analysis of the nonspecific aspects of this phenomenon, Kirkwood and Shumaker⁹ make the additional suggestion that steric matching of a constellation of basic groups on one molecule with a complementary constellation on another molecule could result in a redistribution of protons leading to a strong specific attraction. This prediction has been examined by Hill²⁰ for some special cases and found to be reasonable.

It will be shown in this paper that with a given geometrical configuration of identical ionizable groups on matching sites in a protein system, a specific attraction between such groups may be established in the pH region of their pK as a result of proton/charge fluctuations, whether the groups be on the same molecule or on different molecules.

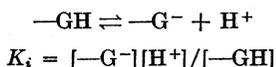
For the sake of simplicity we will examine first the interaction between two identical sites each carrying one ionizable group of type G, with $pK = pK_G$, in a dielectric medium such as is found in the surface of a protein molecule. Taken as a time average at $pH = pK_G$, group G is half ionized. If we now consider a pair of such groups, 1 and 2, taking G as an anionic group (although the identical situation would prevail for a cationic group), the pairings of ionized states between groups 1 and 2 will be as shown in Table I.

TABLE I

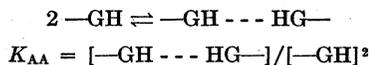
State	Group 1	Group 2
AA	—GH	—GH
AB	—GH	—G ⁻
BA	—G ⁻	—GH
BB	—G ⁻	—G ⁻

Thus, there will be one pair in which both groups are neutral, two pairs in which one group is neutral, the other one charged, and one pair in which both groups will be charged. As a time average the distribution of these pairs will be $1/4$, $1/2$, $1/4$. In calculating the interaction potentials, the neutral (AA) state will be taken as standard. Therefore, the free energy of ionization must be taken into account in states AB, BA, and BB. The corresponding equilibrium equations are as follows.

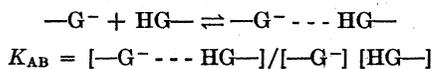
Ionization reaction:



AA reaction:



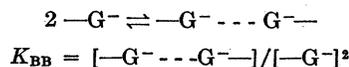
AB reaction:



or, starting with the neutral species:

$$[-G^- \cdots HG^-]/[HG^-]^2 = (K_i/[H^+])K_{AB}$$

BB reaction:



or, starting with the neutral species:

$$[-G^- \cdots G^-]/[HG^-]^2 = (K_i^2/[H^+]^2) K_{BB}$$

Let occupation variables be: $\xi_1 = 0$, when $G^{(1)}$ is in the protonated state; $\xi_2 = 0$, when $G^{(2)}$ is in the protonated state; $\xi_1 = 1$, when $G^{(1)}$ is ionized; $\xi_2 = 1$, when $G^{(2)}$ is ionized. Taking the ionization of groups G into account as described above, and setting the potentials equal to zero at $R = \infty$, where R is the distance between the two groups, the potential of average force between groups 1 and 2, $W_{12}(R)$, is given by:^{9,20}

$$e^{-W_{12}(R)/kT} = \frac{\sum_{\xi_1 \xi_2 = 0}^1 e^{-(1/kT) V(\xi_1 \xi_2, R)} x^{(\xi_1 + \xi_2)}}{\sum_{\xi_1 \xi_2 = 0}^1 x^{(\xi_1 + \xi_2)}} \quad (1)$$

where

$$x = K_i/[H^+]$$

Carrying out the summation and setting the neutral species as standard state, we obtain

$$W_{12}(R) = -2.303kT \log[(1 + 2xe^{-\beta V_{AB}} + x^2e^{-\beta V_{BB}})/(1 + x)^2] \quad (2)$$

with

$$\beta = 1/kT$$

where V_{AB} and V_{BB} are the potentials of interaction for the AB and BB states, respectively.

Taking the derivative of W_{12} with respect to x , it is found that this potential attains a minimal value at a pH which corresponds to a value of x given by:

$$x_{\max} = (e^{-\beta V_{AB}} - 1)/(e^{-\beta V_{AB}} - e^{-\beta V_{BB}}) \quad (3)$$

Examination of eq. (3) shows that the maximum of the potential, $W_{12}(R)$, will be found at values of x close to unity for reasonable intergroup distances, as shown in Figure 1 for several typical cases. At close approach of the groups, the pH of maximal interaction is close to the pK. As the distance between the two groups increases, the maximum is shifted to pH values increasingly distant from the pK, as a result of the different dependence on distance of the potentials V_{AB} and V_{BB} , which must be expressed in specific form for this calculation [see eq. (5)].

Before making calculations of some typical cases, it is of interest to determine whether the system postulated will undergo proton redistribution to give maximal attraction, as suggested by Kirkwood and Shumaker.

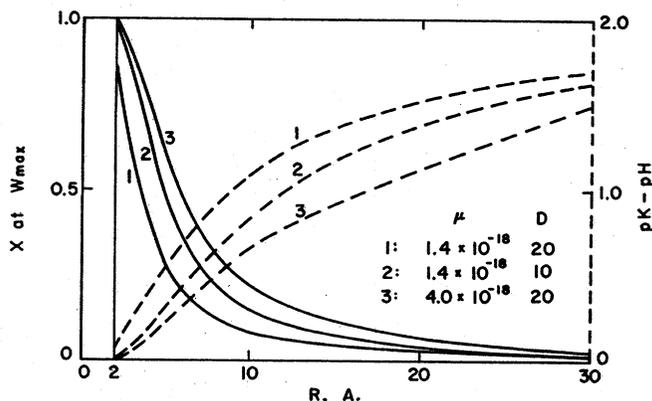


Fig. 1. Dependence of x at W_{max} on the distance of approach between the interacting groups. The corresponding difference between the pK and pH of W_{max} for a pair of anions is shown on the right-hand ordinate. The curves are for three sets of μ and D as indicated on the figure.

For the special case where ionizable groups differ, Hill²⁰ has carried out such an examination in terms of two parameters: a correlation coefficient η and the probability, θ , that a given site is occupied. Where, as here, ionizable groups are identical, these parameters are found to be

$$\eta = \frac{(2xe^{-\beta V_{AB}} - x^2e^{-\beta V_{BB}})/(1 + 2xe^{-\beta V_{AB}} + x^2e^{-\beta V_{BB}})}{\theta = \frac{(1 + xe^{-\beta V_{AB}})/(1 + 2xe^{-\beta V_{AB}} + x^2e^{-\beta V_{BB}})}{\quad} \quad (4)$$

When $\eta = +1$, all pairs are of the AB or BA type, and the matching constellations will have the maximum possible attractive force. θ at this point will reach a value of 0.5, indicating a redistribution of protons to produce the matching constellations.

In carrying out calculations with eq. (2), (3), or (4), specific expressions for the potentials must be used. Taking into account the dipole moment of group B in the protonated state (GH), states AB and BA will be characterized by charge-dipole interactions, while state BB represents a charge-charge repulsion. The corresponding interaction potentials are:

$$V_{AB} = V_{BA} = z\epsilon\mu_G \cos \gamma / DR^2 \quad (5)$$

$$V_{BB} = z^2\epsilon^2 / DR$$

where μ_G is the dipole moment of group G in the protonated state, R is the distance between the two groups, D is the dielectric constant of the medium between them, z is the valence of group G in the ionized form, ϵ is the electronic charge, and γ is the angle formed between the dipole on group 1 and the vector in the direction of charge on group 2. Setting γ at an optimal value for attraction, calculations of η and θ were carried out for various values of x , between 0.1 and 100, taking a hypothetical case of

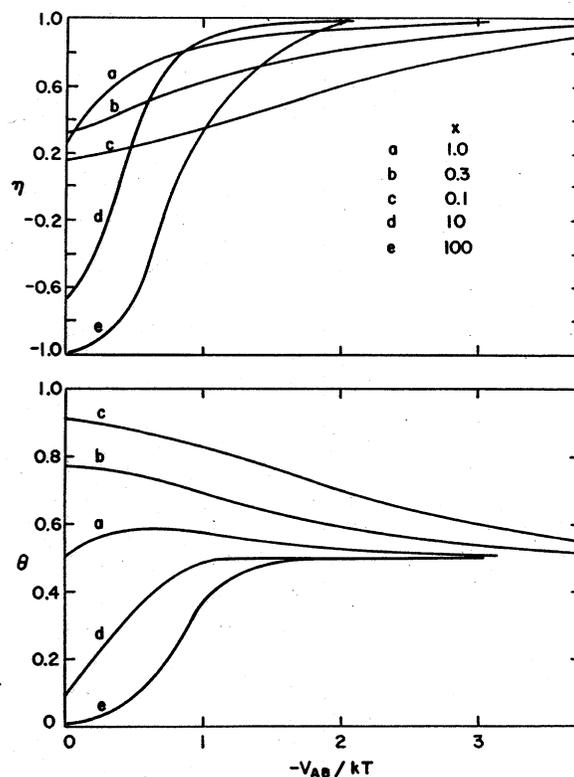


Fig. 2. Dependence of the correlation coefficient η and the probability θ of site occupancy as a function of V_{AB} for various values of x .

$\mu = 4.0$ Debye units, $R = 3$ A., and leaving D unspecified. The results, shown in Figure 2, indicate that in most cases, for reasonable values of V_{AB}/kT , η tends toward +1 and θ toward 0.5, indicating that the "frozen" bound proton distribution suggested by Kirkwood and Shumaker is reasonable, as in the case when the charges on the sites have opposite signs.²⁰ In Table II are listed values of D necessary to give various values of V_{AB}/kT

TABLE II
Values of D Necessary to Give Specific Values of V_{AB} at Various Intergroup Distances

$-V_{AB}/kT$	D		
	$R = 3$ A.	$R = 4$ A.	$R = 5$ A.
1	D = 51	29	18
2	25	14	9
3	17	10	6.1
4	13	7	4.6

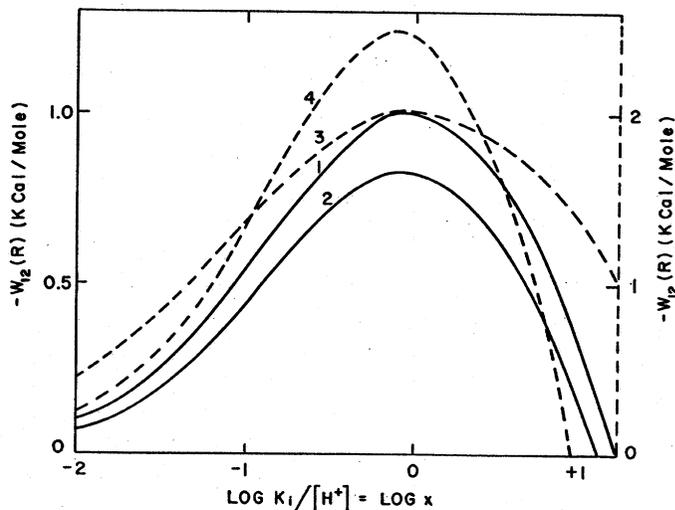


Fig. 3. Dependence of W on pH for groups with identical pK 's ($\log x = \text{pH} - \text{p}K$); (1) one group in each site, $D = 20$, $R = 3$ A., $\mu = 4.0$ Debye units; (2) one group in each site, $D = 20$, $R = 2$ A., $\mu = 1.4$ Debye units, or $D = 20$, $R = 3$ A., $\mu = 3.2$ Debye units; (3) one group in each site, $D = 10$, $R = 2$ A., $\mu = 1.4$ Debye units, or $D = 10$, $R = 3$ A., $\mu = 3.2$ Debye units; (4) two groups in each site, $D = 20$, $R_1 = 3$ A., $\mu = 4.0$ Debye units, $R_2 = 3$ A. Curves 1 and 2 refer to left ordinate; curves 3 and 4 refer to right ordinate.

at $R = 3$ –5 A. The values of D above the dashed line are reasonable for close approach between the groups, and certainly highly probable for interactions between proteins.^{21,22}

Sample calculations have been carried out by use of eq. (2) for the interaction of two anionic groups (e.g., carboxyls) in optimal mutual orientation. For cationic groups the results will be identical, except that all pH dependence curves will be mirror images of those for anionic groups.

The pH dependence of W_{12} is shown in Figure 3. In these calculations, D was assigned values of 20 and 10, which are reasonable in view of Tanford's demonstration that the ionizable groups are located 1 A. below the surface of the molecule.²² R was taken as 2–3 A., and μ was assigned values of 1.4 to 4.0 Debye units. Examination of Figure 3 reveals some characteristic features of this interaction: (1) in the pH region close to pK , the interaction between two identical ionizable groups results in a *net attractive force*; (2) the pH dependence of this attraction assumes an unsymmetrical bell-shaped form, maximal at a pH slightly below the pK of the groups; (3) the attraction is significant over a pH interval of 1.5–2 pH units; (4) the attraction falls off more rapidly on the ionization side of the pK , reflecting the charge–charge repulsion contribution; (5) with reasonable values of the parameters, e.g. $\mu = 4$, $R = 3$ A., $D = 20$ (curve 1), the force of attraction at its maximal point may attain values of the order of 1 kcal./mole. Decreasing D to 10 (a possible value for groups located in a region rich in nonpolar residues) increases the maximal attractive energy.

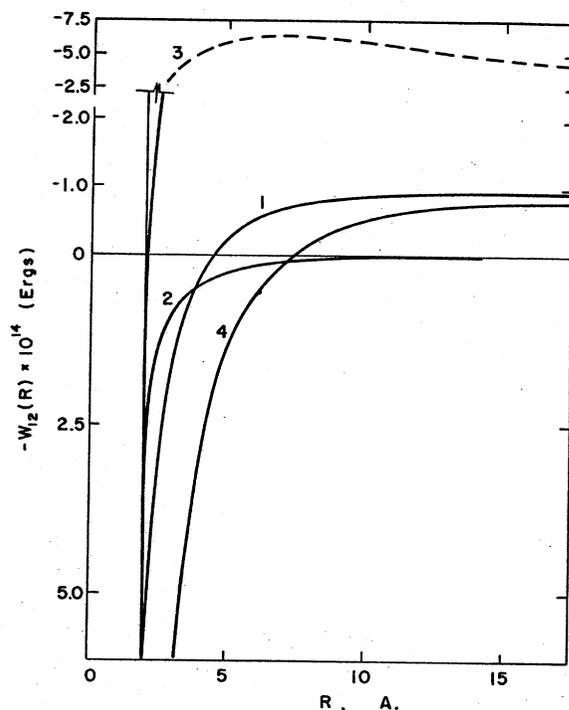


Fig. 4. Dependence of W on distance of separation between the groups, $D = 20$: (1) $\mu = 1.4$ Debye units, $x = 1$; (2) $\mu = 1.4$ Debye units, $x = 0.1$; (3) $\mu = 1.4$ Debye units, $x = 10$; (4) $\mu = 4.0$ Debye units, $x = 1$.

An examination of the range over which this attractive force is operative is presented in Figure 4. Here calculations have been carried out as a function of R at $D = 20$. At $\text{pH} = \text{pK}$ ($x = 1$), the potential of eq. (2) results in an attraction up to an intergroup separation of 4.5 A. ($\mu = 1.4$, curve 1) and 7.5 A. ($\mu = 4.0$, curve 4). At greater distances of separation, W_{12} assumes small positive values, i.e., a weak repulsion, which reflects the fact that in eq. (2) the potential decreasing most slowly with distance is that of charge-charge repulsion. For $x = 10$ (curve 3), i.e., when both groups are almost totally ionized, the zone of attraction essentially disappears; at $x = 0.1$ (curve 2), there is a weak attraction at all distances of separation. Curves 1 and 4 indicate that at $\text{pH} = \text{pK}$, attraction is prevalent up to a separation of the groups of about 5 A. Thus, reasonable distances between groups in matching interaction sites can be accommodated.

The present calculations can be easily extended to the participation of more than one ionizable group in the interacting site. In such a case, the energy of interaction will be the sum of the interactions of the matching groups, as well as cross reactions between the various groups on sites 1 and 2. The exact pattern of interaction will be determined by the geometry

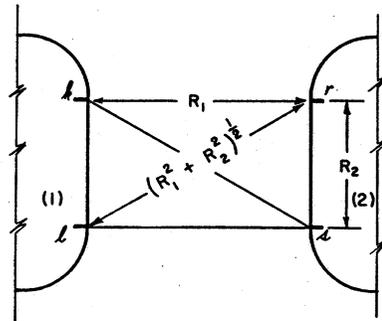


Fig. 5. Schematic representation of the interaction of two sites with two identical ionizable groups each.

of the sites and their distance of separation. The case of two such groups being present in each site is depicted in Figure 5. The interactions are: two direct ones ($k-r$) and ($l-s$), and two cross ones ($k-s$) and ($l-r$). The cross interactions are considerably weaker than the direct ones, since the distance between groups is greater and angle γ is not at optimal orientation (see Fig. 5). A sample calculation has been carried out for $R_1 = 3 \text{ \AA}$, $R_2 = 3 \text{ \AA}$, $\mu = 4$ Debye units, $D = 20$, neglecting intrasite electrostatic interactions, which, however, can be taken into account.^{22,23} The result is shown by curve 4 of Figure 3. At pH_{max} , the energy of attraction between sites 1 and 2 is 2.5 times larger than that between two isolated groups, the cross terms contributing about one quarter of the total. It is obvious that increase in the number of ionizable groups present in a constellation will result in even larger energies of attraction.

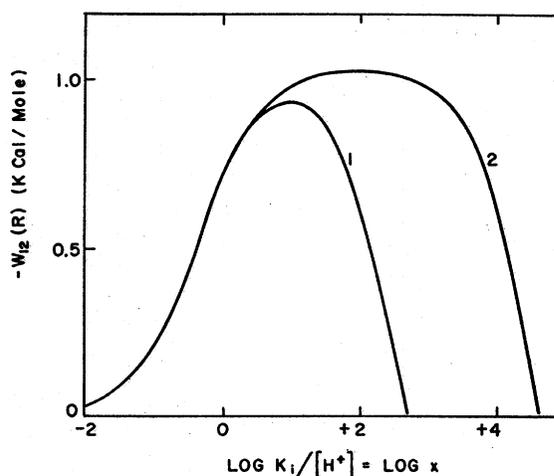


Fig. 6. Dependence of W on pH for two groups with different $\text{pK}'\text{s}$, $D = 20$, $R_1 = 3 \text{ \AA}$, $\mu = 2.7$ Debye units; (1) $\log y = \log x + 2$; (2) $\log y = \log x + 4$.

If groups 1 and 2 do not have identical pK 's, but are of the same charge sign, eq. (2) assumes the form:

$$W_{12}(R) = -2.303kT \log[(1 + xe^{-\beta V_{AB}} + ye^{-\beta V_{BA}} + xye^{-\beta V_{BB}})/(1 + x)(1 + y)] \quad (6)$$

for

$$x = K_A/[H^+]$$

$$y = K_B/[H^+]$$

Calculations ($\mu = 2.7$ Debye units, $R = 3$ A., $D = 20$) are shown in Figure 6 for the interaction of two anionic groups whose pK 's are 2 and 4 pH units apart (curves 1 and 2, respectively). The resulting dependence of W_{12} on pH yields again a net attractive force of close to 1 kcal./mole, and the curve is bell-shaped with a maximum between the two pK 's. In the case of a difference of 4 pH units between the two pK 's (curve 2) attraction persists over 5 pH units. Such a difference in pK is quite possible, for example, in the interaction between cationic imidazole and guanidine groups.

Discussion

The calculations presented above show that a significant attractive force may exist between identical ionizable groups in the pH region of their pK due to fluctuations of protons. In a dielectric medium such as the surface of a protein molecule, an energy of attraction on the order of 1-2 kcal./mole may be available. It is of interest to examine protein systems in which this force may manifest itself. Since the interacting groups may be present in the same or in different protein molecules, the attraction may result in inter- as well as intramolecular bonding. For bonding to take place, the groups must come together in a specific geometrical arrangement determined by local structural features of the protein molecule; the interaction, therefore, will be usually of a specific nature, i.e. it will occur at definite loci on protein molecules. This does not eliminate, however, the possibility of nonspecific attraction if the groups are brought together into favorable alignment by random fluctuations of structure within the molecule as has been suggested by Schellman.²⁴

The most striking feature of the fluctuating charge interactions is that the pH dependence of the attraction passes through a maximum. Thus, it is possible that some protein interactions which have a bell-shaped pH dependence are controlled, at least in part, by the fluctuating charge mechanism described above. Several types of such interactions may be suggested.

Some proteins are known to pass through a pH region in which they form aggregates, for example, α -chymotrypsin,²⁵ β -lactoglobulin,²⁶⁻²⁸ and apomyoglobin.²⁹ In all these cases, a well-defined specific aggregation takes place in a pH region removed from the isoelectric point. The aggre-

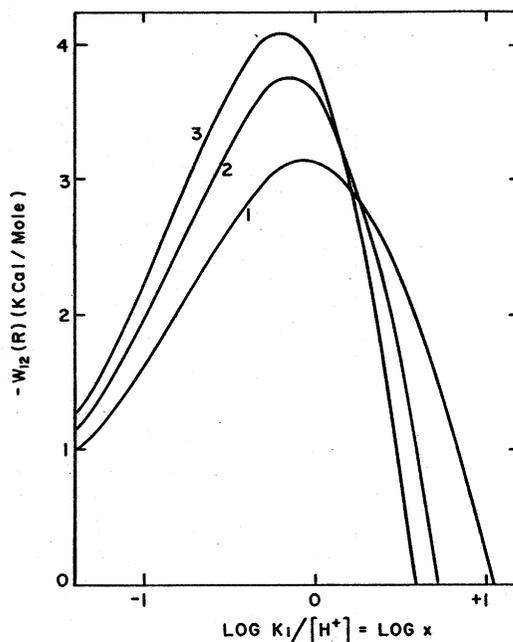


Fig. 7. Dependence of W on x for the interaction of two clusters of four carboxylic groups each, with $R_1 = 3 \text{ \AA}$, $R_2 = 3 \text{ \AA}$, $D = 20$, $\mu = 3.0$ Debye units: (1) direct interactions only; (2) including contribution of cross interactions; (3) including diagonal interactions as well.

gations have a bell-shaped dependence on pH and a spread over two to three pH units. At the pH of maximal interaction, the free energy of the dimerization of chymotrypsin is about 5 kcal./mole,* and that of the tetramerization of β -lactoglobulin A is about 3.9 kcal./mole per bond formed.²⁸ Values of such magnitude can be attained with this mechanism if the attraction is between matching sites containing several ionizable groups each. In the case of β -lactoglobulin A, it is assumed that each site consists of a constellation of four carboxyl groups, since titration³⁰ and peptide analysis³¹ data have suggested that, in the tetramerization reaction, four such side chains per monomer are involved in each interaction site.

A model containing four carboxylic residues positioned in a square, 3 \AA . to the side, has been selected for the interacting site of β -lactoglobulin; the two sites have been placed at 3 \AA . separation; μ was set equal to 3.0 Debye units, D to 20. The resulting potentials, involving four direct interactions, four cross interactions, and four along the diagonals, are shown in Figure 7, where curve 1 represents only the contribution of direct interactions, curve 2 takes account also of the cross interactions, and curve 3 of the diagonal ones as well. In the upper curve of Figure 8 the values of curve 1, normalized to the maximum, are compared with the experimental

* This value was calculated from the sedimentation data of a previous paper.¹²

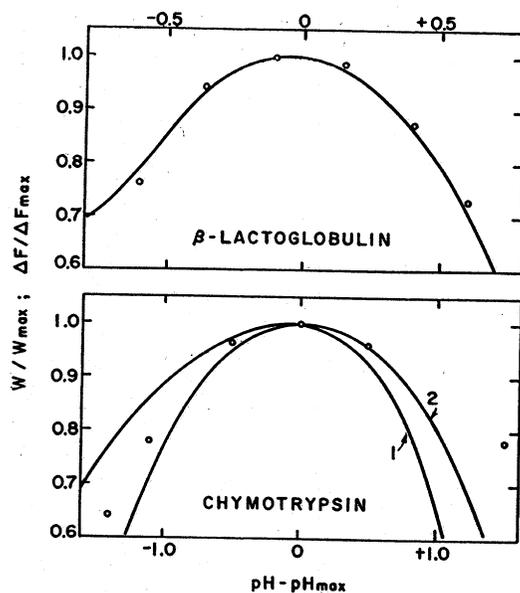


Fig. 8. Comparison of experimental data on β -lactoglobulin and α -chymotrypsin with calculated curves (details in text).

free energies of tetramerization of β -lactoglobulin A, as the function of pH, also normalized to the maximum. It is seen that the assumed cluster of four carboxyls gives a pH dependence curve with a shape similar to the experimentally observed one if the fluctuating charge mechanism is invoked.

A similar comparison is carried out for chymotrypsin in the lower part of Figure 8. Here the theoretical curves were calculated using the data of Figure 6, curve 1, i.e. the two groups have pK values differing by two pH units. In curve 1 of Figure 8 the theoretical curve was normalized to the maximum, in curve 2 normalization was carried out after introducing electrostatic interaction, by setting $x = (K_i / [\text{H}^+] e^{0.868w\bar{z}})$, where \bar{z} is the average charge on the molecule, as obtained from titration data,³² and w is the well-known work function.⁵ Again it is seen that the observed pH dependence curve can be reasonably approximated by use of the fluctuating charge mechanism.

The fluctuating charge attraction between identical groups may also play an important role in the stabilization of the tertiary structure of protein molecules. Such a stabilization would have the very interesting property of being active in only a limited pH zone. This could occur if two groups located on different chains or different parts of the same chain were brought into juxtaposition by the folding of the molecule. Thus, in the pH region of their pK, such a position of the groups would be reinforced by the fluctuating proton attraction, resulting in additional stabilization of a particular conformation of the molecule. As the pH changed from the zone of pK of these particular groups, the fluctuating charge attraction

would weaken and finally disappear. Various conformations of the molecule could then be favored at different pH's depending on the mutual location of particular ionizable side-chains.

It should be emphasized that the fluctuating charge inter- and intramolecular attractions in proteins are not proposed as an answer to all protein interactions nor as implying insignificance to other forms of interaction. Further, it is not asserted that the phenomena described above are uniquely controlled by the action of charge fluctuations nor are charge fluctuations necessarily involved in them. This mechanism, however, is certainly a possible interpretation of such phenomena and should not be arbitrarily omitted from consideration.

In systems as complex as proteins, there cannot help but be a constant interplay between various kinds of attractive and repulsive forces, whether electrostatic, hydrogen bonding, hydrophobic, steric or entropic in nature, and probably very few observed phenomena are mediated by the action of one single type of force. In conclusion, it is proposed that, in addition to other types of forces which are known to act in biological systems, the one due to proton fluctuations be also regarded as a definite and serious possibility.

APPENDIX

It should be pointed out that a similar pH dependence of interactions due to charge fluctuations may be obtained by direct formal application of the equations of Kirkwood and Shumaker.⁹ We take two identical molecules, each possessing a cluster of ionizable groups; their centers of mass are separated by a distance, R_3 , while the active sites on approach are separated by distance, R_1 . Then $R_3 = R_1 + 2b$, where b is the distance from the center of mass of each molecule to the center of the interaction site. From the general theory of Kirkwood and Shumaker⁹ and the enzyme action theory of Kirkwood,¹⁸ the potential of mean force due to site-site interaction is:

$$W_{12}(R) = \frac{\epsilon^2 \langle n \rangle_{Av}^2 e^{-\kappa R_1}}{DR_{1k}} - \frac{\epsilon^4 (\langle n^2 \rangle_{Av}^2 + 2 \langle n \rangle_{Av}^2 \langle n^2 \rangle_{Av}) e^{-2\kappa R_1}}{2kTD^2 \langle R_{1k}^2 \rangle_{Av}} \quad (7)$$

where $\langle n \rangle_{Av}$ is the mean net charge on the site, $\langle n^2 \rangle_{Av}$ is the mean square charge on the site due to proton fluctuations, ϵ is the protonic charge, κ the Debye-Hückel parameter, D the dielectric constant of the medium, and R_{1k} is the average distance between group i on molecule 1 and group k on molecule 2. $\langle n^2 \rangle_{Av}$ can be calculated⁸ to be:

$$\langle n^2 \rangle_{Av} = \nu / (2 + x + x^{-1}) \quad (8)$$

where ν is the total number of ionizable groups involved in the interacting site. In eq. (7), the first term on the right-hand side represents the repulsive potential between the charges on the two sites, while the second term is the attractive potential due to charge-fluctuating charge, and fluctuating

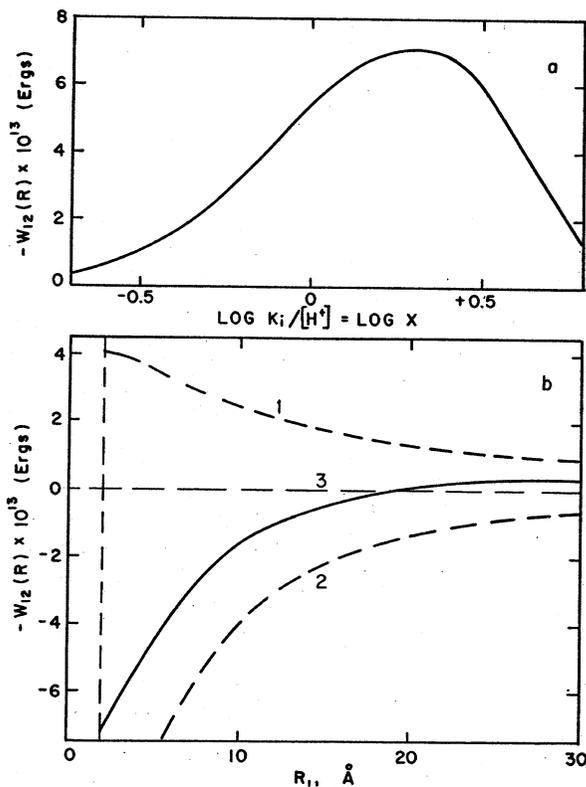


Fig. 9. (a) Dependence of W on pH for the interaction between two sites, each being a cluster of four carboxyls (calculations carried out with the equations of Kirkwood and Shumaker); (b) dependence on distance of separation between the two sites; curve 1: repulsive potential; curve 2: attractive potential; curve 3: total potential.

charge-fluctuating charge interactions. If the site is considered to be a flat disk with a radius r_α , and if the groups are located randomly on its surface, then it is possible to express R_{ik} as:

$$\langle R_{ik}^2 \rangle_{Av} = R_1^2 + (\pi/3)r_\alpha^2 \quad (9)$$

If the groups are considered as randomly distributed along the circumference of the disk, then

$$\langle R_{ik}^2 \rangle_{Av} = R_1^2 + \pi r_\alpha^2 \quad (10)$$

A sample calculation was made for the interaction between two sites, with $\nu = 4$, with the use of the expression for $\langle R_{ik}^2 \rangle_{Av}$ for random circumferential distribution of the groups [eq. (10)]. For $R_1 = 4$ Å., $R_2 = 4$ Å., $D = 20$, and $\kappa = 1.07 \times 10^7$ cm.⁻¹, the pH dependence of $W_{12}(R)$ shown in Figure 9a was obtained. In this calculation, the groups chosen were carboxyls and electrostatic interaction of ionization was neglected. The results show again a net attraction over a range of about one pH unit; the attraction has a bell-shaped dependence on pH and, in the case of

anionic groups, is maximal somewhat above the pK . Introduction of electrostatic interaction between the groups would tend again to extend the attraction over a wider pH range. The contributions of attractive and repulsive forces to the total site-site interaction potential are shown in Figure 9b, as a function of distance between the sites. Once again, at small values of R_1 , attractive forces predominate, while at larger distances a weak repulsion sets in.

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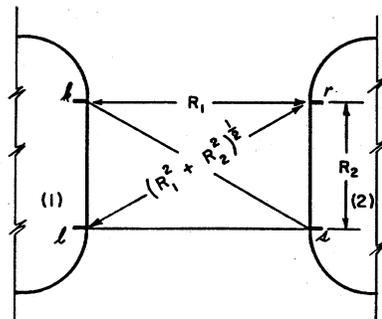


Fig. 5. Schematic representation of the interaction of two sites with two identical ionizable groups each.

of the sites and their distance of separation. The case of two such groups being present in each site is depicted in Figure 5. The interactions are: two direct ones ($k-r$) and ($l-s$), and two cross ones ($k-s$) and ($l-r$). The cross interactions are considerably weaker than the direct ones, since the distance between groups is greater and angle γ is not at optimal orientation (see Fig. 5). A sample calculation has been carried out for $R_1 = 3 \text{ \AA}$, $R_2 = 3 \text{ \AA}$, $\mu = 4$ Debye units, $D = 20$, neglecting intrasite electrostatic interactions, which, however, can be taken into account.^{22,23} The result is shown by curve 4 of Figure 3. At pH_{max} , the energy of attraction between sites 1 and 2 is 2.5 times larger than that between two isolated groups, the cross terms contributing about one quarter of the total. It is obvious that increase in the number of ionizable groups present in a constellation will result in even larger energies of attraction.

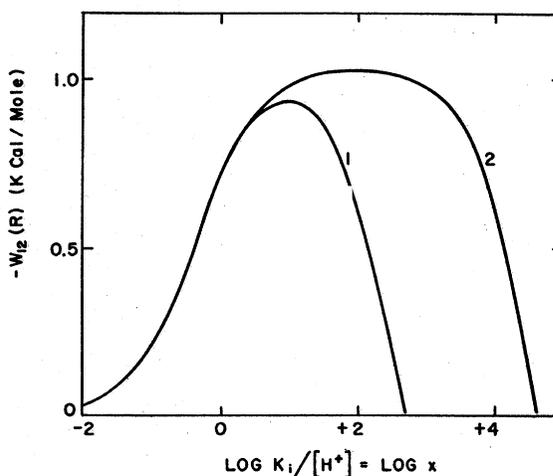


Fig. 6. Dependence of W on pH for two groups with different $\text{pK}'\text{s}$, $D = 20$, $R_1 = 3 \text{ \AA}$, $\mu = 2.7$ Debye units; (1) $\log y = \log x + 2$; (2) $\log y = \log x + 4$.

If groups 1 and 2 do not have identical pK 's, but are of the same charge sign, eq. (2) assumes the form:

$$W_{12}(R) = -2.303kT \log[(1 + xe^{-\beta V_{AB}} + ye^{-\beta V_{BA}} + xye^{-\beta V_{BB}})/(1+x)(1+y)] \quad (6)$$

for

$$x = K_A/[H^+]$$

$$y = K_B/[H^+]$$

Calculations ($\mu = 2.7$ Debye units, $R = 3$ A., $D = 20$) are shown in Figure 6 for the interaction of two anionic groups whose pK 's are 2 and 4 pH units apart (curves 1 and 2, respectively). The resulting dependence of W_{12} on pH yields again a net attractive force of close to 1 kcal./mole, and the curve is bell-shaped with a maximum between the two pK 's. In the case of a difference of 4 pH units between the two pK 's (curve 2) attraction persists over 5 pH units. Such a difference in pK is quite possible, for example, in the interaction between cationic imidazole and guanidine groups.

Discussion

The calculations presented above show that a significant attractive force may exist between identical ionizable groups in the pH region of their pK due to fluctuations of protons. In a dielectric medium such as the surface of a protein molecule, an energy of attraction on the order of 1-2 kcal./mole may be available. It is of interest to examine protein systems in which this force may manifest itself. Since the interacting groups may be present in the same or in different protein molecules, the attraction may result in inter- as well as intramolecular bonding. For bonding to take place, the groups must come together in a specific geometrical arrangement determined by local structural features of the protein molecule; the interaction, therefore, will be usually of a specific nature, i.e. it will occur at definite loci on protein molecules. This does not eliminate, however, the possibility of nonspecific attraction if the groups are brought together into favorable alignment by random fluctuations of structure within the molecule as has been suggested by Schellman.²⁴

The most striking feature of the fluctuating charge interactions is that the pH dependence of the attraction passes through a maximum. Thus, it is possible that some protein interactions which have a bell-shaped pH dependence are controlled, at least in part, by the fluctuating charge mechanism described above. Several types of such interactions may be suggested.

Some proteins are known to pass through a pH region in which they form aggregates, for example, α -chymotrypsin,²⁵ β -lactoglobulin,²⁶⁻²⁸ and apomyoglobin.²⁹ In all these cases, a well-defined specific aggregation takes place in a pH region removed from the isoelectric point. The aggre-

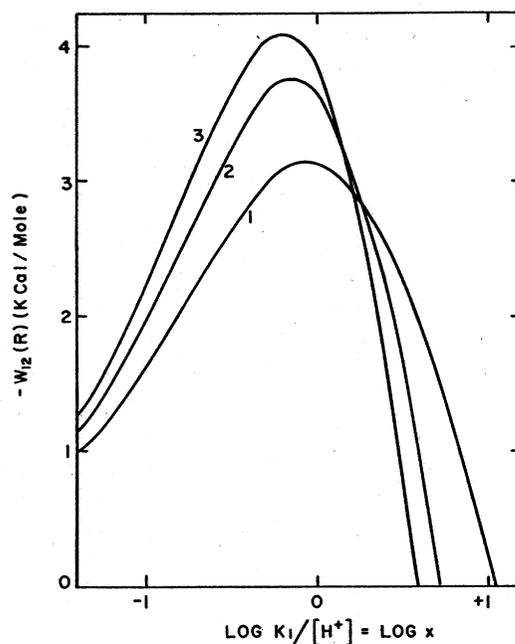


Fig. 7. Dependence of W on x for the interaction of two clusters of four carboxylic groups each, with $R_1 = 3 \text{ \AA}$, $R_2 = 3 \text{ \AA}$, $D = 20$, $\mu = 3.0$ Debye units: (1) direct interactions only; (2) including contribution of cross interactions; (3) including diagonal interactions as well.

gations have a bell-shaped dependence on pH and a spread over two to three pH units. At the pH of maximal interaction, the free energy of the dimerization of chymotrypsin is about 5 kcal./mole,* and that of the tetramerization of β -lactoglobulin A is about 3.9 kcal./mole per bond formed.²⁸ Values of such magnitude can be attained with this mechanism if the attraction is between matching sites containing several ionizable groups each. In the case of β -lactoglobulin A, it is assumed that each site consists of a constellation of four carboxyl groups, since titration³⁰ and peptide analysis³¹ data have suggested that, in the tetramerization reaction, four such side chains per monomer are involved in each interaction site.

A model containing four carboxylic residues positioned in a square, 3 \AA . to the side, has been selected for the interacting site of β -lactoglobulin; the two sites have been placed at 3 \AA . separation; μ was set equal to 3.0 Debye units, D to 20. The resulting potentials, involving four direct interactions, four cross interactions, and four along the diagonals, are shown in Figure 7, where curve 1 represents only the contribution of direct interactions, curve 2 takes account also of the cross interactions, and curve 3 of the diagonal ones as well. In the upper curve of Figure 8 the values of curve 1, normalized to the maximum, are compared with the experimental

* This value was calculated from the sedimentation data of a previous paper.¹²

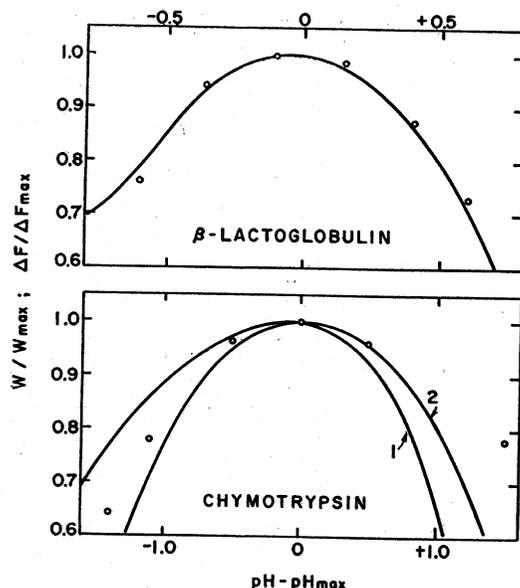


Fig. 8. Comparison of experimental data on β -lactoglobulin and α -chymotrypsin with calculated curves (details in text).

free energies of tetramerization of β -lactoglobulin A, as the function of pH, also normalized to the maximum. It is seen that the assumed cluster of four carboxyls gives a pH dependence curve with a shape similar to the experimentally observed one if the fluctuating charge mechanism is invoked.

A similar comparison is carried out for chymotrypsin in the lower part of Figure 8. Here the theoretical curves were calculated using the data of Figure 6, curve 1, i.e. the two groups have pK values differing by two pH units. In curve 1 of Figure 8 the theoretical curve was normalized to the maximum, in curve 2 normalization was carried out after introducing electrostatic interaction, by setting $x = (K_i / [\text{H}^+] e^{0.868w\bar{z}})$, where \bar{z} is the average charge on the molecule, as obtained from titration data,³² and w is the well-known work function.⁵ Again it is seen that the observed pH dependence curve can be reasonably approximated by use of the fluctuating charge mechanism.

The fluctuating charge attraction between identical groups may also play an important role in the stabilization of the tertiary structure of protein molecules. Such a stabilization would have the very interesting property of being active in only a limited pH zone. This could occur if two groups located on different chains or different parts of the same chain were brought into juxtaposition by the folding of the molecule. Thus, in the pH region of their pK, such a position of the groups would be reinforced by the fluctuating proton attraction, resulting in additional stabilization of a particular conformation of the molecule. As the pH changed from the zone of pK of these particular groups, the fluctuating charge attraction

would weaken and finally disappear. Various conformations of the molecule could then be favored at different pH's depending on the mutual location of particular ionizable side-chains.

It should be emphasized that the fluctuating charge inter- and intramolecular attractions in proteins are not proposed as an answer to all protein interactions nor as implying insignificance to other forms of interaction. Further, it is not asserted that the phenomena described above are uniquely controlled by the action of charge fluctuations nor are charge fluctuations necessarily involved in them. This mechanism, however, is certainly a possible interpretation of such phenomena and should not be arbitrarily omitted from consideration.

In systems as complex as proteins, there cannot help but be a constant interplay between various kinds of attractive and repulsive forces, whether electrostatic, hydrogen bonding, hydrophobic, steric or entropic in nature, and probably very few observed phenomena are mediated by the action of one single type of force. In conclusion, it is proposed that, in addition to other types of forces which are known to act in biological systems, the one due to proton fluctuations be also regarded as a definite and serious possibility.

APPENDIX

It should be pointed out that a similar pH dependence of interactions due to charge fluctuations may be obtained by direct formal application of the equations of Kirkwood and Shumaker.⁹ We take two identical molecules, each possessing a cluster of ionizable groups; their centers of mass are separated by a distance, R_3 , while the active sites on approach are separated by distance, R_1 . Then $R_3 = R_1 + 2b$, where b is the distance from the center of mass of each molecule to the center of the interaction site. From the general theory of Kirkwood and Shumaker⁹ and the enzyme action theory of Kirkwood,¹⁸ the potential of mean force due to site-site interaction is:

$$W_{12}(R) = \frac{\epsilon^2 \langle n \rangle_{Av}^2 e^{-\kappa R_1}}{DR_{1k}} - \frac{\epsilon^4 (\langle n^2 \rangle_{Av}^2 + 2 \langle n \rangle_{Av}^2 \langle n^2 \rangle_{Av}) e^{-2\kappa R_1}}{2kTD^2 \langle R_{1k}^2 \rangle_{Av}} \quad (7)$$

where $\langle n \rangle_{Av}$ is the mean net charge on the site, $\langle n^2 \rangle_{Av}$ is the mean square charge on the site due to proton fluctuations, ϵ is the protonic charge, κ the Debye-Hückel parameter, D the dielectric constant of the medium, and R_{1k} is the average distance between group i on molecule 1 and group k on molecule 2. $\langle n^2 \rangle_{Av}$ can be calculated⁸ to be:

$$\langle n^2 \rangle_{Av} = \nu / (2 + x + x^{-1}) \quad (8)$$

where ν is the total number of ionizable groups involved in the interacting site. In eq. (7), the first term on the right-hand side represents the repulsive potential between the charges on the two sites, while the second term is the attractive potential due to charge-fluctuating charge, and fluctuating

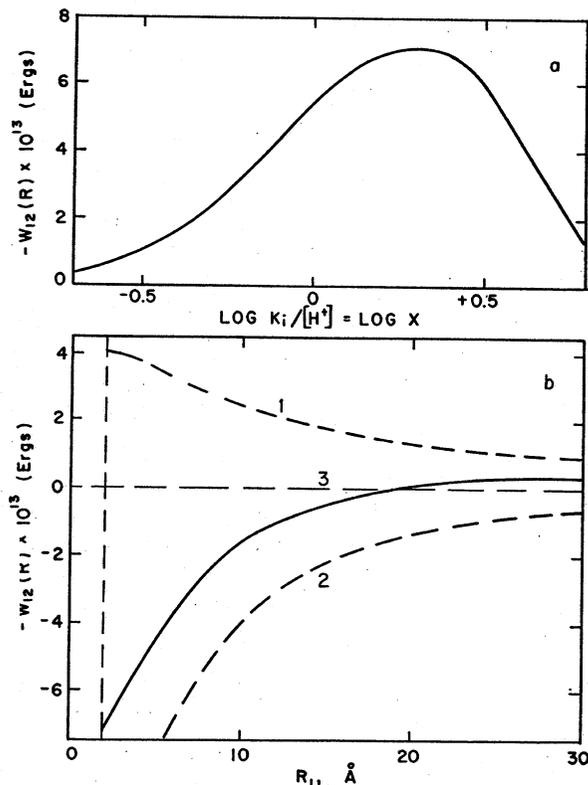


Fig. 9. (a) Dependence of W on pH for the interaction between two sites, each being a cluster of four carboxyls (calculations carried out with the equations of Kirkwood and Shumaker); (b) dependence on distance of separation between the two sites; curve 1: repulsive potential; curve 2: attractive potential; curve 3: total potential.

charge-fluctuating charge interactions. If the site is considered to be a flat disk with a radius r_α , and if the groups are located randomly on its surface, then it is possible to express R_{ik} as:

$$\langle R_{ik}^2 \rangle_{Av} = R_1^2 + (\pi/3)r_\alpha^2 \quad (9)$$

If the groups are considered as randomly distributed along the circumference of the disk, then

$$\langle R_{ik}^2 \rangle_{Av} = R_1^2 + \pi r_\alpha^2 \quad (10)$$

A sample calculation was made for the interaction between two sites, with $\nu = 4$, with the use of the expression for $\langle R_{ik}^2 \rangle_{Av}$ for random circumferential distribution of the groups [eq. (10)]. For $R_1 = 4 \text{ \AA}$, $R_2 = 4 \text{ \AA}$, $D = 20$, and $\kappa = 1.07 \times 10^7 \text{ cm.}^{-1}$, the pH dependence of $W_{12}(R)$ shown in Figure 9a was obtained. In this calculation, the groups chosen were carboxyls and electrostatic interaction of ionization was neglected. The results show again a net attraction over a range of about one pH unit; the attraction has a bell-shaped dependence on pH and, in the case of

anionic groups, is maximal somewhat above the pK . Introduction of electrostatic interaction between the groups would tend again to extend the attraction over a wider pH range. The contributions of attractive and repulsive forces to the total site-site interaction potential are shown in Figure 9b, as a function of distance between the sites. Once again, at small values of R_1 , attractive forces predominate, while at larger distances a weak repulsion sets in.

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