EFFECT OF A MOLECULAR DIPOLE ON THE IONIC STRENGTH DEPENDENCE OF A BIMOLECULAR RATE CONSTANT

IDENTIFICATION OF THE SITE OF REACTION

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ABSTRACT A theory is proposed for determining the location of a reaction site on a protein of known tertiary structure with an asymmetric charge distribution by an analysis of the effect of ionic strength on the rate of reaction of the protein with a small ion, using equations of Brønsted (J. N. Brønsted, 1922, *Z. Phys. Chem.* **102**:169–207), Debye and Hückel (P. Debye and E. Hückel, 1923, *Phys. Z.* **24**:185–206), and Kirkwood (J. G. Kirkwood, 1934, *J. Chem. Phys.* **2**:351–361). The theory is based on the fact that the dipole moment of the transition complex differs from that of the protein, which will be reflected in the ionic strength dependence of the reaction. The location of the small ion with respect to the dipole axis of the protein can be calculated from this difference. For protein-protein reactions, an a priori assumption has to be made about the orientation of one of the reaction partners, since many different orientations of the reactants with respect to each other result in dipole moments of the same magnitude.

INTRODUCTION

It is well known that the presence of an inert, ionized salt diminishes the electrostatic interactions between charged molecules. As a consequence, reactions between oppositely charged molecules proceed slower, and reactions between molecules of the same sign faster, as compared with reactions between uncharged molecules.

In 1922 Brønsted (1) proposed the following equation to describe this effect quantitatively:[1]

$$k_I = k_{I=0} \frac{\gamma_A \gamma_B}{\gamma_{AB^\ddagger}}. \tag{1}$$

[1]Symbols used: D, dielectric constant of water; I, ionic strength, $I = \frac{1}{2}\Sigma_i Z_i^2 m_i$; M, center of mass; N, Avogadro's number; N, center of negative charge; P, center of positive charge; T, temperature; W_I, energy required to charge a molecule at ionic strength I; Y, the smaller of the two parameters n and p; Z, net charge, $Z = p - n$; $a = b + 1.5 \cdot 10^{-8}$ cm; b, radius of protein or ion in centimeters; e, elementary charge in electrostatic units; k_I, bimolecular rate constant at ionic strength I; k, Boltzmann's constant in erg/K; m, molality; n, number of negative charges; p, number of positive charges, $\mathbf{r}$, vector from N to P; $\mathbf{r}_P$, $\mathbf{r}_N$, radius vectors from M to P and M to N, respectively; γ, activity coefficient; κ, reciprocal thickness of ionic atmosphere in cm^{-1}, $\kappa^2 = (8\pi N e^2 I)/(1{,}000DkT)$; μ, dipole moment in esu · cm.

This relation was proven by Bjerrum (2, 3). It applies only to a bimolecular reaction,

$$A + B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AB^{\ddagger} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{products} \tag{2}$$

in which k_2 is much larger than k_1, and k_{-2} can be neglected. No rearrangement is considered to take place within the complex. Thus, the rate limiting step is the formation of $AB^{\ddagger}$. Many electron transfer reactions satisfy these requirements. Substitution of the Debye-Hückel expression (4), Eq. 3,

$$\ln \gamma_i = -\frac{Z_i^2 e^2}{2DkT} \cdot \frac{\kappa}{1 + \kappa a_i} \tag{3}$$

in Eq. 1, and the assumption that all radii are equal lead to[2]

$$\ln k_I = \ln k_{I-0} + \frac{Z_A Z_B e^2}{DkT} \cdot \frac{\kappa}{1 + \kappa a} \tag{4}$$

which simplifies to (5):

$$\ln k_I = \ln k_{I-0} + \frac{Z_A Z_B e^2 \kappa}{DkT}, \tag{5}$$

if $\kappa a \ll 1$. Therefore Eq. 5 is only valid for reactions between two small ions at low ionic strength. The use of Eqs. 4 and 5 for protein-small ion and protein-protein reactions has been criticized, since neither the radii of the reaction partners and transition complex are equal, nor is κa much smaller than 1 (8–13, footnote 3).

Therefore, one should use the complete relation:

$$\ln k_I = \ln k_{I-0} - \frac{e^2 \kappa}{2DkT}\left[\frac{Z_A^2}{1 + \kappa a_A} + \frac{Z_B^2}{1 + \kappa a_B} - \frac{(Z_A + Z_B)^2}{1 + \kappa a_{AB^{\ddagger}}}\right], \tag{6}$$

which will be termed the Brønsted-Debye-Hückel equation.

In 1976, Wherland and Gray (14, 15) proposed an equation according to which the ionic strength dependence of a rate constant is

$$\ln k_I = \ln k_{I-\infty} - 3.576\left[\frac{e^{-\kappa a_A}}{1 + \kappa a_B} + \frac{e^{-\kappa a_B}}{1 + \kappa a_A}\right]\frac{Z_A Z_B}{a_A + a_B}. \tag{7}$$

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[2]The reader may be more familiar with the equations

$$\log k_I = \log k_{I-0} + \frac{2Z_A Z_B A I^{1/2}}{1 + aBI^{1/2}} \tag{4a}$$

and

$$\log k_1 = \log k_{I-0} + 2Z_A Z_B A I^{1/2}. \tag{5a}$$

In these equations $AI^{1/2} = 0{,}434(e^2\kappa/2DkT)$ and $BI^{1/2} = \kappa$. Numerical values for A and B as a function of temperature are given by Manov et al. (6) and Robinson and Stokes (7).

It should be noted that Eq. 7 is not in electrostatic centimeter-gram-second units, since κ was multiplied by 10^{-8} and distances were entered in ångströms. The equation was derived using the theory of Marcus (16) and, although not mentioned, a potential energy equation by Debye (17). It describes ionic strength effects on reactions between small molecules and proteins rather well.[3] This is surprising, since the part of the relation derived from the Debye equation, which is valid only at low ionic strength and for interaction energies smaller than 1 kT, is used to correct a rate constant at infinite ionic strength.

Very recently a modified form of Eq. 7 has been proposed by Stonehuerner et al. (18) to describe the ionic strength dependence between cytochrome c and cytochrome b_5. These studies were carried out at ionic strengths between ~0.05 and 1.7 M. The equation is

$$\ln k_I = \ln k_{I-\infty} + 3.576\, n \frac{e^{-\kappa a}}{a(1 + \kappa a)}, \tag{8}$$

in which n is the number of complimentary charge interactions and a is supposed to represent the radius of an amino- or carboxyl group. In addition to being used outside the valid range of ionic strength, this equation can be criticized for excluding interactions which do not appear to be negligible. It could be argued that Eq. 8 violates Coulomb's law.

In both the Brønsted-Debye-Hückel equation (Eq. 6) and the Marcus-Debye equation (Eq. 7), only the net charge on each molecule is considered. Presented below is an extension of the Brønsted-Debye-Hückel equation that takes into account the dipole moments of the reactants and of the transition complex, using an equation derived by Kirkwood (19). This approach should give a better description of ionic strength effects, and also helps to identify the site of reaction on the surface of the protein. Part of this work has been presented at the Second International Symposium on Mechanisms of Reactions in Solution (20).

THEORY

According to Kirkwood (19), the activity coefficient of a spherical protein in an electrolyte solution is a function of its net charge, dipole, and higher-order moments. For the present, quadrupole and higher-order moments will be neglected. The equation for the activity coefficient is then:

$$\ln \gamma_i = -\frac{Z_i^2 e^2}{2DkT} \cdot \frac{\kappa}{1 + \kappa a_i} - \frac{\kappa^2}{2DkT}\left[\frac{3\mu^2}{4a_i\left(1 + \kappa a_i + \frac{\kappa^2 a_i^2}{3} + \frac{\kappa^2 b_i^3}{6a_i}\right)}\right]. \tag{9}$$

To arrive at this result, Kirkwood assigned a value of unity to the dielectric constant inside the protein. The monopole term is proportional to κ or $I^{1/2}$ and the dipole term to κ^2 or I, indicating that at higher ionic strength this term becomes relatively more important. This is illustrated in Figs. 3 and 4 of reference 10. As expected, the monopole term is identical to the Debye-Hückel expression given in Eq. 3. Since Eq. 9 does not take into account protein-protein interactions, it holds only in a solution in which the protein concentration is small compared with the concentration of small ions.

[3]Feinberg, B. A., and M. D. Ryan. Manuscript submitted for publication.

Let us now consider a reaction between a protein having an asymmetric charge distribution and a small ion. Provided that the spatial structure of the protein is known, we can make reasonable assumptions as to the parameter b_i and as to which residues are charged at a given pH. A calculation of the solvent accessibility of ionizable groups, as carried out by Matthew et al. (21) following the method of Lee and Richards (22), may be helpful in deciding whether a specific group is charged or not.

The dipole moment is generated by the asymmetric charge distribution and the small peptide bond dipoles if these are arranged in an α-helix configuration (23, 24). The effect of near perfect alignment of the small peptide bond dipoles can be approximated by placing a charge of +0.5 e at the N-terminal side and a charge of −0.5 e at the C-terminal side of the α-helix (25). It is assumed that other peptide bond dipoles do not yield a net dipole moment. If the protein has p (positive) and n (negative) charges, the net charge is $(p - n)e = Ze$. The coordinates of the center of positive charge, P, negative charge, N, and mass, M can be calculated. The dipole moment is given by

$$\boldsymbol{\mu} = p\mathbf{r}_P e - n\mathbf{r}_N e, \tag{10}$$

in which $\mathbf{r}_P$ and $\mathbf{r}_N$ are the radius vectors from M to P and from M to N, respectively. For $p > n$ Eq. 10 can be rewritten[4] (26)

$$\boldsymbol{\mu} = n\mathbf{r}e + Z\mathbf{r}_P e, \tag{11}$$

and for $n > p$

$$\boldsymbol{\mu} = p\mathbf{r}e + Z\mathbf{r}_N e, \tag{12}$$

in which $\mathbf{r}$ is the vector from N to P. For the present the second terms of Eqs. 11 and 12 will be neglected, which is equivalent to assuming that M coincides with P (Eq. 11) or N (Eq. 12). Furthermore, we define Y to be the smaller of the two parameters n and p, such that

$$\boldsymbol{\mu} = Y\mathbf{r}e. \tag{13}$$

If $n = p$ then Y is taken to be equal to n. Eq. 9 can now be solved for various ionic strengths.

Similarly, we can calculate the activity coefficient of the small ion. This will be fairly simple since many small ions are monopoles, or their dipole moments are so small as not to contribute significantly to their activity coefficients. The net charge of the transition complex is clearly the sum of $Z_A e$ and $Z_B e$. The dipole moment of the transition complex depends on the orientation of the reactants with respect to each other. For the reaction to proceed it may be necessary that the ion binds at the positive side, or alternatively, at the negative side of the protein. If the ion is negatively charged, the first orientation will yield a transition complex with a dipole moment lower than that of the protein, and the second a transition complex with a higher dipole moment.

We can now calculate the factor $\log \gamma_A\gamma_B/\gamma_{AB^\ddagger}$, as a function of ionic strength for both orientations. It is of course also possible to substitute values for all the variables directly into the Brønsted-Debye-Hückel-Kirkwood equation, which for the case of a reaction between a small ion A and a macromolecule B is (assuming that the transition complex has the same

[4]Mofers, F. J. M. Personal communication.

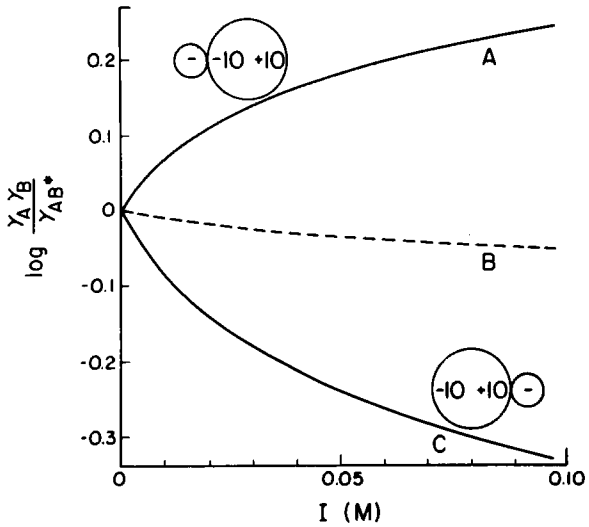

FIGURE 1 Simulated ionic strength dependence of a reaction between a single negatively charged ion with a radius of 4 Å, and a neutral protein with a radius of 18.5 Å and an asymmetric charge distribution leading to a dipole moment of 300 debye (Table I). The orientation of the small molecule with respect to the dipole axis of the protein is as indicated in the figure. Curve *B* is the predicted behavior according to the Brønsted-Debye-Hückel theory (monopoles only), Eq. 6.

radius as the protein):

$$\ln k_I = \ln k_{I=0} - \frac{e^2\kappa}{2DkT}\left[\frac{Z_A^2}{1+\kappa a_A} - \frac{Z_A^2 + 2Z_A Z_B}{1+\kappa a_B} + \frac{3\kappa(Y_B^2 r_B^2 - Y_{AB^\ddagger}^2 r_{AB^\ddagger}^2)}{4a_B\left(1+\kappa a_B + \frac{\kappa^2 a_B^2}{3} + \frac{\kappa^2 b_B^3}{6a_B}\right)}\right], \quad (14)$$

and to plot the second term versus ionic strength.

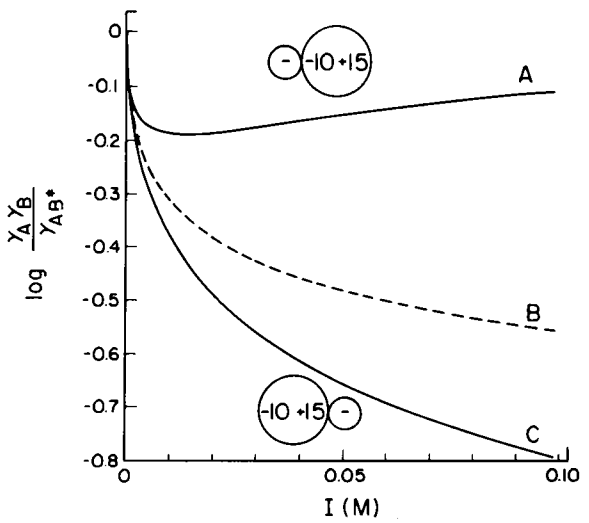

FIGURE 2 Same as legend of Fig. 1, except that the protein has a net charge of +5 e.

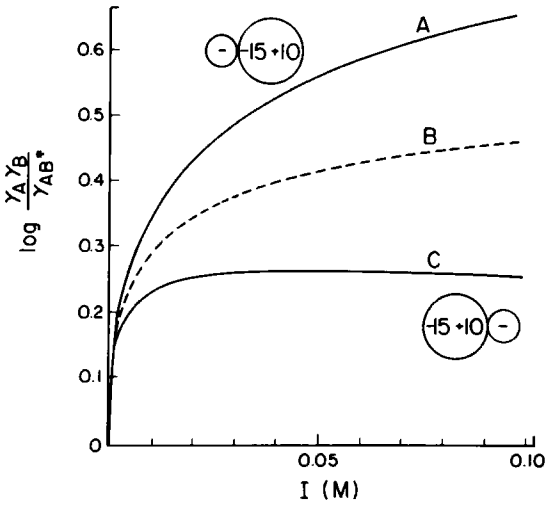

FIGURE 3 See legend of Fig. 1. In this case the protein has a net charge of −5 e.

Using this approach, calculations were performed for a reaction between a small negative molecule and a protein which was either neutral, positive, or negative (Fig. 1–3). For comparison, all figures also show the dependence due to monopoles, as described by Eq. 6. Similar calculations were made for two different protein-protein reactions, one between neutral, and the other between oppositely charged, proteins. Two different values for the radius of the transition complex were used (Figs. 4–7). See Table I for a listing of the physical parameters of reactants and transition complexes which were assumed for these calculations.

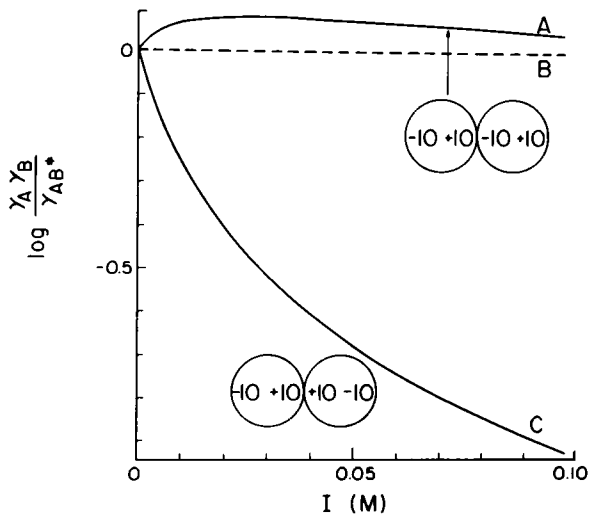

FIGURE 4 Simulated ionic strength dependence of a reaction between two identical neutral proteins, having asymmetric charge distributions leading to dipole moments of 300 debye. The transition complex is supposed to be a sphere with a radius of 25 Å. Orientations of the reaction partners within the transition complex as indicated (Table 1). Curve *B* is the predicted behavior according to the Brønsted-Debye-Hückel theory (monopoles only), Eq. 6.

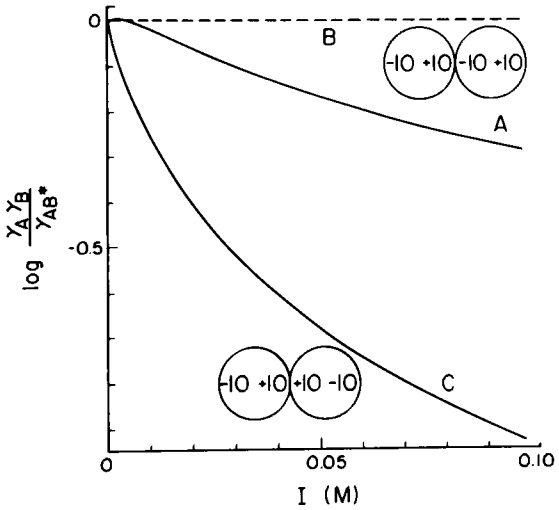

FIGURE 5 Same as legend of Fig. 4, except that the radius of the transition complex is 30 Å. Curves *C* of Fig. 4 and this figure are identical because the transition complexes do not have a dipole moment. Curve *A* is profoundly affected by the change of radius.

RESULTS

With one exception (Fig. 5), the curves based on monopoles fall in between the curves based on monopoles and dipoles. Considerable variation in the ionic strength dependence is possible, as determined by the orientation of the reactants within the transition complex. A prior conclusion (10, 27) that the effects of dipoles cancel each other applies only to special cases (see below) and in general appears unjustified. It is even conceivable that the rate constant for

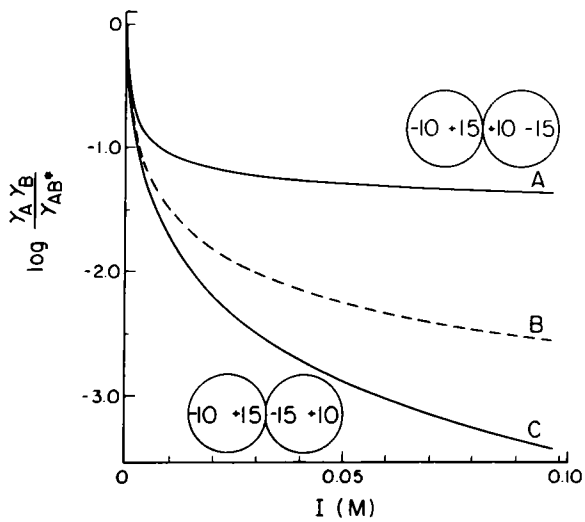

FIGURE 6 Simulated ionic strength dependence of a reaction between two oppositely charged (+5 e and −5 e) proteins, having asymmetric charge distributions leading to dipole moments of 300 debye. The transition complex is supposed to be a sphere with a radius of 25 Å. See legend of Fig. 4.

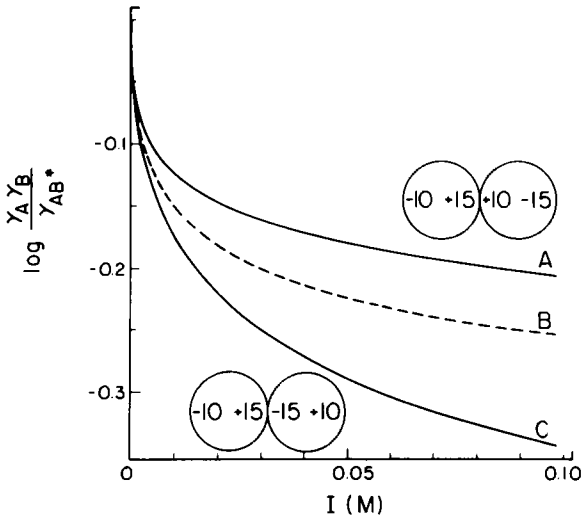

FIGURE 7 See legend of Fig. 6. In this case the radius of the transition complex is taken to be 30 Å.

a reaction between a positive macromolecule and a negative ion increases slightly with ionic strength after a small decrease (Fig. 2, curve *A*). This results when the ion must react at the negative side of the macromolecule. The electrostatic potential at that location does not necessarily have to be negative, merely less positive than at the other side of the molecule. In the case of small ion-protein reactions an experimental curve should fall in between the two theoretical curves, based on monopoles and dipoles, because the orientations used in the calculations lead to extreme dipole moments. From such an experimental curve we can calculate the dipole moment of the transition complex, and since we know the dipole moment

TABLE I
PHYSICAL PROPERTIES OF REACTANTS AND TRANSITION COMPLEXES

Figure	*A*					*B*					Orientation§	*AB*‡				
	Z	*Y*	*b**	Dipole moment‖	$\lvert r\rvert$*	*Z*	*Y*	*b*	Dipole moment	$\lvert r\rvert$		*Z*	*Y*	*b*	Dipole moment	$\lvert r\rvert$
1	−1	0	4	0	0	0	10	18.5	300	6.25	(−10 +10) (−1)	−1	10	18.5	188	3.92
											(+10 −10) (−1)	−1	10	18.5	385	8.01
2	−1	0	4	0	0	+5	10	18.5	300	6.25	(−10 +15) (−1)	−4	11	18.5	207	3.92
											(+15 −10) (−1)	−4	11	18.5	422	8.01
3	−1	0	4	0	0	−5	10	18.5	300	6.25	(−15 +10) (−1)	−6	10	18.5	223	4.65
											(+10 −15) (−1)	−6	10	18.5	358	7.46
4	0	10	18.5	300	6.25	0	10	18.5	300	6.25	(−10 +10) (+10 −10)	0	20	25	0	0
											(−10 +10) (−10 +10)	0	20	25	600	6.25
5	0	10	18.5	300	6.25	0	10	18.5	300	6.25	(−10 +10) (+10 −10)	0	20	30	0	0
											(−10 +10) (−10 +10)	0	20	30	600	6.25
6	+5	10	18.5	300	6.25	−5	10	18.5	300	6.25	(−10 +15) (−15 +10)	0	25	25	137	1.14
											(−19 +15) (+10 −15)	0	25	25	885	7.38
7	+5	10	18.5	300	6.25	−5	10	18.5	300	6.25	(−10 +15) (−15 +10)	0	25	30	137	1.14
											(−10 +15) (+10 −15)	0	25	30	885	7.38

*All distances are in ångströms.

‖Dipole moments are expressed in debye (1 debye = 1.10^{-18} esu · cm = $3.3 \cdot 10^{-30}$ C · m).

§An orientation (−10 +10) (−1) indicates that the ion binds exactly at that point of the protein surface where the "positive" part of the dipole axis emerges.

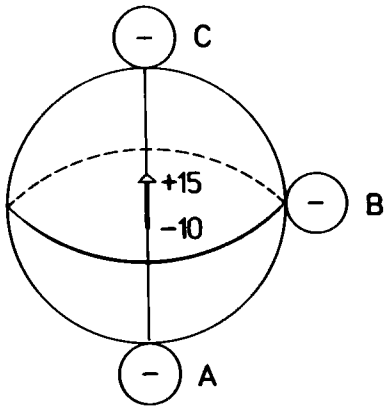

FIGURE 8

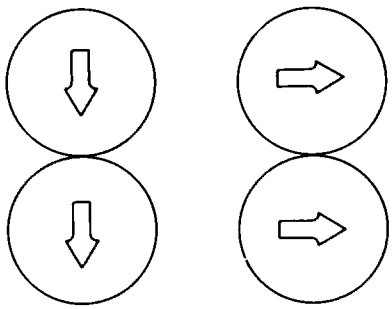

FIGURE 9

FIGURE 8 Orientations of the small ion in the transition complex with respect to the dipole axis of the protein. Orientations *A*, *B*, and *C* lead to ionic strength dependences *A*, *B*, and C, respectively, as shown in Fig. 2.

FIGURE 9 Two different orientatins of two identical protein molecules with respect to each other, each orientation yielding the same dipole moment. This result will be obtained for every orientation in which the dipoles are parallel. The arrows indicate the direction of the dipole moments.

of the protein, postulate one or several transition complexes that have the correct dipole moment.

For instance, let us assume that curve *B* in Fig. 2 is an experimental curve. Such an ionic strength dependence is expected when the transition complex has a dipole moment of 300 debye, because under such conditions the contributions of the dipole moments of the protein and the transition complex to the respective activity coefficients cancel. We then obtain a dependence as if only monopoles are important. Which transition complex has a dipole moment of 300 debye? The answer is: all complexes in which the ion binds near the "equator" of the protein. This equator is defined by the direction of the dipole axis of the protein. Were *A* or *C* the experimental curve, only one orientation would be possible (Fig. 8). This is how ionic strength studies of reactions between small ions and proteins can be used to determine the site of reaction.

For protein-protein reactions such identifications are not possible for two reasons. First, it is not clear what value one should use for the radius of the transition complex and, as seen in Figs. 4–7, the results depend on this value. The shape of the transition complex might be approximated by an ellipsoid, but because of the complexity of the equations and/or the fact that they are valid only at very low ionic strength (see reference 28, Eqs. 17 and 20, and reference 29), this would not be practical.[5] It is therefore suggested that a hypothetical spherical transition complex be used, which had a radius $b_{AB^\ddagger}$ equal to $(b_A^2 + b_B^2)^{1/2}$, so that its surface area is the same as the sum of the surface areas of the reactants. Accordingly, Figs. 4 and 6 should present better simulations of ionic strength effects than Figs. 5 and 7. Second,

[5]Recently Eq. 17 of reference 28 was incorrectly interpreted to represent the logarithm of the activity coefficient as a linear function of the dipole moment (30). In this equations, as well as in Eqs. 12 and 20 of reference 28 log γ is proportional to the square of the dipole moment, divided by a distance which in Eq. 17 is the interfocal distance. Furthermore, this equation cannot be used at high ionic strength, as was done by the authors of reference 30.

and more importantly, there are many transition complexes which yield the same dipole moment. For instance, one would calculate a dipole moment of 600 debye, whether the reaction represented in Figs. 4 and 5 proceeded through a parallel apposition, or an in-tandem orientation (Fig. 9). Therefore, for protein-protein reactions ionic strength studies can be used to verify a certain orientation for which evidence has been obtained by another technique. However, it cannot prove a certain orientation without at least one a priori assumption, namely the identity of the reaction site of one of the proteins.

Are the differences between the theoretical curves in Figs. 1-7 significant? We can assume experimental rate constants to have errors of 10% or less and that would lead to vertical error bars of maximum length 0.087[6] in a graph of log k_I vs. I. Since in all cases the curves based on monopoles and dipoles are separated by more than 0.087, it is possible to distinguish between the two transition states.

DISCUSSION

The Kirkwood Model

In Eqs. 3 and 8 there occurs a parameter a_i which is defined as the closest distance of approach between the centers of the ions. For a protein molecule of which the activity coefficient is determined by the concentration of a simple electrolyte, one can approximate a_i as the crystallographic radius, b_i, plus the mean radius of the electrolyte. For instance, in a solution containing cytochrome c and sodium chloride, a_i is equal to 16.5 + 1.5 Å (cytochrome c chloride distance) or 16.5 + 2.0 Å (cytochrome c-sodium distance). Whether one uses 18.0 or 18.5 Å for a_i is of no significance with respect to the results of the calculation of ln γ. One finds that theory and experiment agree well when calculated a_i values for cytochromes c are used (31–33). For simple electrolytes containing small positive ions it has been found that the radius, which fits the results of an experimental activity coeffficient determination best—the Debye-Hückel radius—, is significantly larger than the value based on crystallographic data (compare references 34 and 35). This phenomenon has been the subject of discussions by Robinson and Stokes (36, reference 7, pp. 235–238) who showed that it is caused by a shell of water molecules around the positive ion, which increases the "exclusion distance," a_i-b_i. Using "hydrated" radii, these authors (37, reference 7, pp. 235–238) found that theoretical and experimental activity coefficients agreed in all essentials up to ionic strengths of 0.1 and 0.2 M.

According to Guggenheim (37) Eq. 3 does not satisfy the thermodynamic relation

$$\left(\frac{\partial \log \gamma_i}{\partial m_j}\right)_i = \left(\frac{\partial \log \gamma_j}{\partial m_i}\right)_j, \tag{15}$$

in a solution containing more than one electrolyte, unless all ionic species have the same radius a_i. Guggenheim's objection must apply also to Eq. 9. This had led several authors to abandon the parameter a_i in Eq. 3 and to add a term which is linear in ionic strength and contains an empirical factor (8) However, Eq. 15 does not hold in the case of a reaction between components of the solution. Therefore, the use of the parameter a_i in the present analysis may

[6] If $(R \pm r) = \log (X \pm x)$, in which $X = k_I$, and $x/X = 0.1$, then $r = (\mathrm{d} \log X/\mathrm{d}X)\, x = 0.0434$.

be justified. Rigorous theories have now become available to describe solutions containing several nonreacting components (38).

In Eq. 9 the dielectric constant of the protein was taken to be unity. In calculations regarding titration data of proteins a value of 2 or 4 is usually assumed (39–43). When the model of Ramachandran and Srinivasan (44) is applied to our protein of Fig. 1, an effective dielectric constant of 1.4 is obtained. The actual value is unknown and will undoubtedly vary within the protein. The presence of a low dielectric cavity, formed by the protein, leads to a repulsion between an ion in solution and its image charge within this cavity (28). The repulsion between the dipole charges and their image charges in the solution appears to be smaller and can be neglected. Unfortunately, Kirkwood's revised equation for the activity coefficient, which takes the former effect into account (28), holds only for very low ionic strength and cannot be used here.

That ln γ_{AB^*} depends on the orientation of the reactants with respect to each other is not surprising. Tanford (39, 45) has shown in another, but fundamentally similar approach, that the energy W_I required to charge a protein or, in our case, a transition complex, depends on the configuration of the charges on the protein surface. W_I is related to ln γ by $\ln \gamma = (W_I - W_{I-0})/kT$. Interestingly, the complex formed by the binding of long chain fatty acids to serum albumin has been shown experimentally to possess a smaller dipole moment than serum albumin itself (46).

It was assumed that the reactants have their equilibrium ion-atmosphere. This is not necessarily the case for some diffusion-limited reactions, in which the inert ions cannot orient themselves around one of the reaction partners because it diffuses so fast. The effect of ionic strength on rates of these reactions has been discussed by Logan (47–49).

Fluctuating Dipole Moment

The number of sites on a protein surface which will accept a proton ($-NH_2$, $-COO^-$, etc.) usually exceeds the number of protons bound, such that many configurations or protein-proton complexes are possible. These configurations change in time, giving rise to a nonvanishing mean square dipole moment $\Delta\mu_f^2$ (50):

$$\Delta\mu_f^2 = e^2 \sum_{j=1}^{\nu} \frac{b_j^2}{2 + K_j/(H^+) + (H^+)/K_j}, \tag{16}$$

in which the summation is over all sites, and K_j is the dissociation constant of site j. The center of the molecule is taken to be the center of mass, and the distance of site j to that center is represented by b_j. This equation is valid if the equivalent proton accepting sites are distributed homogeneously over the protein surface (50). It can be shown that at pH 7 $\Delta\mu_f^2$ will be large if the protein contains many sites with a pK near 7. For instance, eight histidines of harbor seal myoglobin have a mean pK of 6.25 (51). This leads theoretically to a $\Delta\mu_f$ of 88 debye, which might be an appreciable part of the total dipole moment. The actual variation in the dipole moment depends on the specific locations of the histidine residues on the surface of the molecule. For example, horse cytochrome *c* contains two ionizable histidines, which, according to Eq. 6, would cause a $\Delta\mu_f$ of 41 debye. However, the calculated $\Delta\mu_f$ is only 21 debye, a value which takes into account not only the location of the histidines, but also that one of them has a low pK of 3.2 (52). The dipole moment of horse ferricytochrome *c* is 325 debye, taking

into account the center of mass and the contribution of α-helices (53). The general conclusion is that calculated dipole moments of proteins do not vary much at pH 7 if these proteins contain only one or two histidines. Interestingly, fluctuating dipoles were shown theoretically to assist in forming transition complexes between proteins (54, 55, but see 56 and 57). Cytochromes *c*, because of their small fluctuating dipole moment, apparently do not make use of this mechanism.

Limitations

Ionic strength equations have been used for purposes and under conditions for which they were not intended. The kind of information obtainable from ionic strength studies is discussed above. The conditions under which these studies should be carried out, insofar as they are not self-evident from the preceding discussion, are considered below.

The protein concentration should be as low as possible for two reasons: first, Eqs. 3 and 9 do not take into account protein-protein interactions, and second, they neglect the counter-ions of the protein. In fact, the principle of electroneutrality is violated by applying these equations (8). Therefore, the concentration of the protein should not contribute significantly to the ionic strength. It is suggested that the ionic strength generated by the protein should not be more than one tenth of the total ionic strength. This would correspond to a minimum concentration of 5 mM of a monovalent salt for a solution in which the concentration of a protein with a net charge of $+10$ e is 10 μM. Such a concentration also helps to reduce the energy of interaction between protein and reactant, because Eq. 3 is only valid for interaction energies less than 1 kT (4, 58, reference 39, p. 463). This is particularly important for reactions of small ions with highly charged proteins such as the ferredoxins.

The Debye-Hückel expression, Eq. 3, for the activity coefficient of a simple ion, for instance sodium in a solution of sodium chloride, breaks down between ionic strengths of 100 and 150 mM (i.e., Fig. 2.15 in reference 59). Although the system considered here is different, it is not expected to obey the theory beyond these ionic strengths. Therefore one should use for calculation only data obtained between ~5 and 100 mM ionic strength. Many studies reported in literature have been carried out at too high ionic strengths.

Ideally, the system under study should consist of four components: water, the reaction partners, each with their counter-ions, and inert salt ions. Very often there will be a fifth component present in the form of a buffer. Various complications are possible. Ions from the buffer or the inert salt may bind to the protein, changing net charge and dipole moment. These ions may be competition for a common binding site, or bind at different places. Also, the reactants may bind to each other, as in the case of ferricyanide and cytochrome *c* (60). It should be stressed that, although in some cases the binding constants are known, the corresponding binding sites are yet to be identified. Furthermore, the inert salt may not be fully dissociated, as is well known in the case of sulphates (61). To minimize interference of this kind, monovalent reagents, inert salts, and buffers are preferred. These complications, especially the binding of ions, have prevented the application of the present approach to kinetic ionic strength studies presented in the literature. Only when careful studies are made, under appropriate conditions, will it be possible to draw conclusions regarding the site of reaction.

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