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Macroscopic models for studies of electrostatic interactions in proteins: Limitations and applicability

(protein dielectric/ion pairs in proteins/folding energy)

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ABSTRACT **The validity of macroscopic models for calculations of electrostatic energies in proteins is examined. The Tanford–Kirkwood (TK) model is extended to include the self energy of the ionized groups. It is shown that ionized groups cannot exist inside nonpolar regions of proteins and argued that the experimental finding of ions inside proteins proves that the corresponding local environment is polar. The modified TK model (MTK model), which adjusts charge–charge interactions by the corresponding solvent accessibilities, is found to be inconsistent with the TK model, on which it is based. The MTK model corresponds to a polar interior whereas the TK model assumes a nonpolar interior. It is shown that models that assume a high dielectric constant for proteins give reasonable results for interactions between charged groups at equilibrium. It is then explained why, in contradiction to common belief, protein interiors are *polar* around charged groups. It is argued that in focusing on charge–charge interactions one overlooks the key contribution of the protein dipoles in determining the self energy of charges in the interior of proteins.**

Electrostatic interactions are among the key factors that determine the structure and function of proteins (1). Thus it is important to find ways to calculate electrostatic interactions in proteins using available x-ray structures. The main difficulty in such calculations is the complicated nature of the microscopic dielectric effect. Recent attempts to evaluate electrostatic interactions in proteins are divided into two main approaches: (*i*) calculations that try to model, on a microscopic level, the complete electrostatic energy of the protein charges, protein permanent and induced dipoles, and the surrounding water molecules (see, for example, refs. 2 and 3) and (*ii*) macroscopic approaches. The macroscopic approaches date back to the use of Kirkwood's formula (4) by Tanford and Kirkwood (5). This approach (which was proposed before the availability of x-ray structures of proteins) views the protein as a medium of low dielectric constant and predicts (consistently with its assumptions) that all ionized groups must be near the surface of the protein (6, 7). Experimental findings of some ionized groups inside proteins indicate that the model should be modified. An attempt to overcome this problem, while retaining a macroscopic approach, was made by Gurd and co-workers (8, 9), who modified the Tanford–Kirkwood (TK) model by reducing the electrostatic energy of each group in direct proportion to its solvent accessibility parameter (see ref. 10). This approach, which will be referred to here as the modified TK (MTK) approach, is considered by many as a simple yet reasonable approach for evaluating electrostatic interactions in proteins.

Part of the appeal of the MTK model may be due to the implication that this model is based on the rigorous TK model. If the MTK model is both reliable and based on the TK model, then proteins can be modeled as low-dielectric spheres ("oil drop" models).

We have examined the TK and the MTK approaches and have shown that proteins cannot be treated as low-dielectric spheres and that the local dielectric is *not* related to the corresponding surface accessibility. It is also shown (in contrast to common belief) that protein interiors are polar near charged groups at equilibrium. The relation between this observation and protein flexibility is pointed out.

Energetics of a Charged Group in a Protein and pK_a Calculations

We start by considering a hypothetical nonpolar protein and examining the result of placing charged groups in such a system. As a test case, we evaluate the pK_a of a single ionizable group when all other groups are un-ionized. The standard free energy of ionizing an acid in a protein is given by (3)

$$\Delta G^0_p(AH_p \rightarrow A^-_p + H^+_w) = \Delta\Delta G^{w\rightarrow p}_{sol}(A^-) - \Delta\Delta G^{w\rightarrow p}_{sol}(AH) + \Delta G^0_w(AH_w \rightarrow A^-_w + H^+_w), \quad [1]$$

where $\Delta\Delta G_{sol}$ is the difference in solvation energy of the indicated species in the protein (p) and in water (w). Eq. 1 can be rewritten as $pK^p_a = 2.3RT[\Delta\Delta G^{w\rightarrow p}_{sol}(A^-) - \Delta\Delta G^{w\rightarrow p}_{sol}(AH)] + pK^w_a$. While actual calculations (3) should include evaluation of $\Delta\Delta G^{w\rightarrow p}_{sol}(AH)$, we will concentrate here on solvation of the charged species (A^-), which is much larger than solvation of AH. This approximation gives the pK_a of an ionizable group, relative to its pK_a in water, by

$$pK^p_a - pK^w_a \simeq 2.3RT[\Delta\Delta G^{w\rightarrow p}_{sol}(A^-)] \quad [2]$$

That is, the change in the solvation energy of A^- on moving from water to its protein site determines the corresponding pK_a change. Instead of using discrete microscopic calculations (2), let us try to use a macroscopic approach considering the protein as a sphere of dielectric constant ϵ_i and radius b surrounded by a solution of dielectric constant ϵ_w. The electrostatic interaction between the charge and the solution has been derived by Kirkwood (4, 5). The leading terms in Kirkwood's expression (the terms $n = 0$ and $n = 1$ in his B_{kk}) are given in kcal/mol (for $\epsilon_i << \epsilon_w$)

$$\Delta\Delta G_{sol,K} \simeq -166[(1/\epsilon_i) - (1/\epsilon_w)][(1/b) + (r^2/b^3)], \quad [3]$$

where r is the distance of the charge from the center of the protein. The $(1/b)$ term is the change in Born energy (11) associated with changing the dielectric from ϵ_i to ϵ_w around a charged sphere of radius b. The (r^2/b^3) term is the corresponding Onsager energy (12) of a dipole of length r in a

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Abbreviations: TK model, Tanford–Kirkwood model; MTK model, modified TK model.

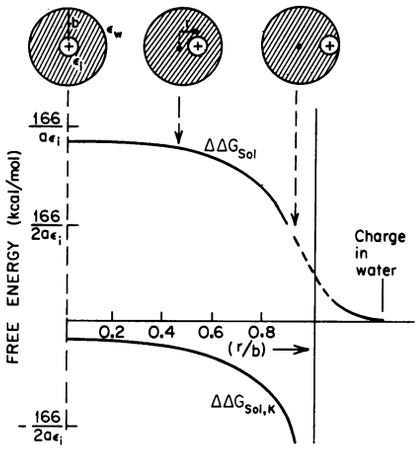

FIG. 1. Electrostatic free energy of a charged group of radius a inside a nonpolar sphere of dielectric ϵ_i and radius b surrounded by a continuum with the dielectric constant of water ($\epsilon >> \epsilon_i$). Calculations are for $b = 10\bar{a}$. $\Delta\Delta G_{sol,K}$ and $\Delta\Delta G_{sol}$ are, respectively, the macroscopic electrostatic free energy obtained by Kirkwood's formula (Eq. 3) and by the extended TK treatment (Eq. 4).

sphere of radius b. However, one must realize a point that is frequently overlooked. Kirkwood's expression *does not* include the reference energy needed for pK_a calculations, as the energy of transferring the charge from vacuum to the given medium is not included. Recognizing this point, Tanford and Kirkwood did not try to calculate the energies of isolated charged groups. To evaluate the proper reference energy, one must add to Kirkwood's formula the energy of transferring a charge of radius $\bar{a}$ from water ($\epsilon = \epsilon_w$) to a continuum with $\epsilon = \epsilon_i$ (for a related treatment, see ref. 13). The energy for this process is given using the Born formula (11) as $\Delta\Delta G' = (166/\bar{a})[(1/\epsilon_i) - (1/\epsilon_w)]$. The solvation energy of a charge in the idealized spherical protein can now be approximated by

$$\Delta\Delta G_{sol} = \Delta\Delta G_{sol,K} + (166/\bar{a})[(1/\epsilon_i) - (1/\epsilon_w)]. \quad [4]$$

This energy will be referred to as the "self energy" of the given group. The dependence of the self energy $\Delta\Delta G_{sol}$ on the distance of the charge from the center of the sphere is shown in Fig. 1. Since Kirkwood's formula is not applicable for charges of finite radius in the range $r > b - a$ (contact between the charge and the surrounding solution), we approximate $\Delta\Delta G_{sol}$ for this range using a linear interpolation (Fig. 1). The key point that emerges from Fig. 1 is that $\Delta\Delta G_{sol}$ is very sensitive to the position of the charge: $\Delta\Delta G_{sol}$ can change by ≈35 kcal (≈25 pK_a units; 1 cal = 4.18 J) for an ionized acid moving from water to a nonpolar sphere of dielectric 2. Thus, if a protein can be represented as an oil drop, the intrinsic pK_a values of its internal ionizable groups will be 25 pK_a units different than that of the corresponding group in water. This huge environmental effect (which is far more important than the effect of interaction between the charged group and the surface charges) was noticed in the original work of Tanford and Kirkwood (5). However, since experimental findings point out that the changes in pK_a are very small, they concluded that charges in proteins must be at a constant distance from the protein surface.

The MTK model (which tries to use the x-ray results in electrostatic calculations) considers the pK_{int} values as constants* independent of the position of the corresponding groups. This treatment clearly violates the TK assumption that protein interiors are nonpolar.

It might be argued that the fact that the pK_{int} of groups in proteins are not shifted by more than a few pK_a units (relative to the corresponding pK_a values in water) indicates that all ionized groups are exposed to water. However, ionized groups do exist in regions of low accessibility to water (e.g., aspartate-102 in chymotrypsin) and their pK_{int} values are still similar to the corresponding values in water. The reason is that ionized groups in the interiors of proteins can be stabilized by the protein permanent dipoles [e.g., hydrogen bonds (2)]. This corresponds to a polar rather than nonpolar environment even in sites of zero solvent accessibility.

As long as one does not try to evaluate pK_{int} by any molecular model, the assumption that $pK_{int} \simeq pK^w$ is a reasonable approximation for surface groups. The problems start with functionally important buried groups (which are exactly the groups that one would like to understand). Unfortunately the macroscopic model does not tell us how to evaluate the pK_{int} of such groups.

Energetics of Salt Bridges and pK_a Changes

The free energy of an ion pair ($A^- BH^+$) can be expressed relative to the energy of the corresponding ions in the gas phase (2). However, in treating the energetics of proteins it is more convenient to choose as a reference the energy of the ions at infinite separation in water (3). With this reference, the free energy of the ion pair is given (in kcal/mol) as (3) $\Delta G(R) = -332/R + \Delta\Delta G_{sol}(R) + \Delta G^w_{sol}(\infty)$, where $\Delta\Delta G_{sol}$ is the change in solvation free energy of the ions on change of their interionic distance from infinity to R (in Å). The above relation can be expressed as

$$\Delta G(R) = -332/[R\epsilon(R)] + \Delta G^w_{sol}(\infty). \quad [5]$$

The distance-dependent dielectric, $\epsilon(R)$, reflects the compensation of the change in the gas-phase attraction term, $-332/R$, by the change in solvation energy. Here again one can use explicit microscopic models to evaluate $\Delta G(R)$ (3). Before commenting on the microscopic meaning of $\epsilon(R)$, we will examine the validity of the macroscopic models for $\Delta G(R)$. For this purpose, we will consider an ion pair in a sphere of low dielectric constant surrounded by a solvent of high dielectric constant. For simplicity we will examine first the configuration presented in Fig. 2. For this configuration the leading term in Kirkwood's expression (for $\epsilon_i << \epsilon_w$) is

$$\Delta G(R)_K = -332/(R\epsilon_i) - 166(R^2/b^3)[(1/\epsilon_i) - (1/\epsilon_w)]. \quad [6]$$

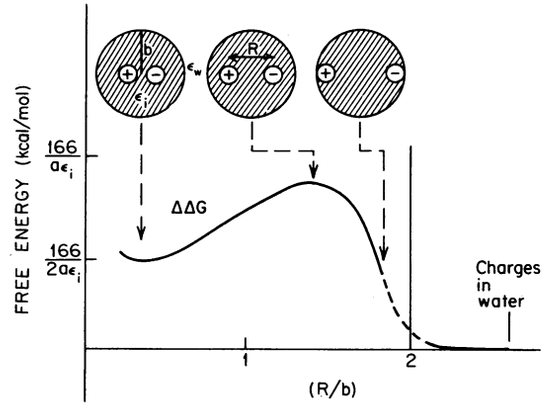

FIG. 2. Electrostatic free energy of an ion pair inside a nonpolar sphere of dielectric ϵ_i surrounded by a continuum with the dielectric constant of water (Eq. 7).

*In some cases, pK_{int} is assumed to be different than the corresponding pK_a but the differences are an order of magnitude smaller than the actual pK_{int} predicted by the TK model.

This is essentially the sum of the charge–charge interaction at R and the Onsager expression (12) for solvation of a dipole. Here again, Kirkwood's expression does not include the self energy of the charges. Extending Eq. **6** to include the crucial energy of taking the ions from water to ϵ_i gives

$$\Delta G(R) = \Delta G(R)_K + (332/\bar{a})[(1/\epsilon_i) - (1/\epsilon_w)]. \quad [7]$$

The dependence of ΔG on R is shown in Fig. 2. Since Kirkwood's formula is not valid for $(r/2) > b - \bar{a}$, where the ion passes the boundary of the sphere, we approximate $\Delta G(R)$ in that range by a linear interpolation (see ref. 13 for a related treatment).

The main point that emerges from Fig. 2 is that ion pairs are *not* stable at the center of a sphere of low dielectric constant. Let us compare this result with the MTK approximation. This approximation was stated to involve a reduction of the energy obtained by scaling Kirkwood's formula (Eq. **6** in our case) by $(1 - SA)$, where SA is the average surface area of the relevant groups accessible to solvent. A consistent implementation of this procedure using the actual positions of the ionized groups would have given, for the case considered in Fig. 2, where $R << b$ (a tight ion pair) the relation $\Delta G(R) \simeq -[332/(R\epsilon_i)](1 - SA) + \Delta G_{int}$, where ΔG_{int} is the free energy associated with the pK_{int} of the ions†. This equation gives incorrect results for two charged groups with $pK_{int} = pK_a^w$ at low local dielectric constant (e.g., the center of the sphere in Fig. 2). For example, with $SA \approx 0$ and $\epsilon_i = 4$ (which is the value used in refs. 8 and 9) one obtains $\Delta G(R) = -332/4R$. This gives the huge interaction of −28 kcal/mol for two ions 3 Å apart. If this were true, then ion pairs would be extracted from water to oil. The reason for this nonphysical result is the omission of the self energy term in Kirkwood's formula. One can argue that the self energy is included in the pK_{int}, which could have been chosen to be much larger than the corresponding pK_a^w. However, the MTK model does not provide a prescription for relating the pK_{int} to the actual polarity of the given site and, if the pK_{int} is an adjustable parameter, then the model has no predictive value†.

Apparently the MTK approach was not implemented by using the physics of Kirkwood's formula. Instead of using the actual positions of the relevant groups they were assumed to be on the surface of a sphere (of the size of the protein) exposed to water. The only relevant parameter left from the actual structure of the protein is the distance between the groups. Since the most important electrostatic interaction occurs when $R_{kj} << b$ one finds, using Kirkwood's expression for two groups on a surface of a sphere (where $r_j = r_k = b$ and $\theta_{kj} \simeq 0$), that the MTK expression is simply

$$\Delta\Delta G_{MTK} \simeq -\{332/[R_{kj}(\epsilon_i + \epsilon_w)/2]\}(1 - SA). \quad [8]$$

This expression, without the surface area correction, is the interaction energy expected from two ions at the surface of an infinite plane separating a medium of low dielectric constant, ϵ_i, from a medium of high dielectric constant. Thus the model simply assumes that the dielectric constant in proteins is high using $\bar{\epsilon} = (\epsilon_i + \epsilon_w)/2 = 41$ for the interior of proteins where SA is zero and an even larger value for regions exposed to water (e.g., for $1 - SA = 0.05$ the model uses $\bar{\epsilon} = 41/0.05 = 820$). Models with high effective dielectric constants are expected to give quite reasonable results for interactions between surface groups because the effective dielectric constant is high in regions that are exposed to water (see below). However, the MTK model also seems, at first glance, to give reasonable results for internal groups. It appears that for such cases the model works, because it is inconsistent with its assumptions. That is, as will be argued below, the effective dielectric constant inside a protein corresponds to a polar rather than a nonpolar environment (this is, of course, inconsistent with the TK model). Thus, for groups inside a protein, one expects high-dielectric-constant models to do much better than low-dielectric-constant models.

To show that the results of the MTK model have little to do with the TK model and with solvent accessibility, we compare, in Table 1, the calculated pK_a values obtained by the MTK approach (8, 9) with those obtained using only Coulomb's laws with a large dielectric constant. These evaluations are not in any way an attempt to present a consistent analysis of the pK_a values in myoglobin. They are simply repetition of the calculations of refs. 8 and 9 with the same pK_{int} and a Coulomb's law type model. The table shows that the Coulomb's law type model does as well as the MTK model. The reason for the apparent success of high-dielectric-constant models will be considered below.

The problems associated with the MTK and related models can also be realized by considering the specific case of ion pairs in proteins. Two alternative definitions of pK_{int} can be considered. If pK_{int} is taken as the pK_a of the given ionizable groups in their protein sites, where all other ionizable groups are neutral, then the model cannot account for the pK_a of histidine-159 in the cysteine-25–histidine-159 ion pair of papain (14). The MTK model predicts a pK_a change of about 2 units while the experimentally observed shift is 4 units. The discrepancy of about 3 kcal/mol indicates that in cases of strong electrostatic interactions which are important cases in protein function it might be quite risky to use macroscopic models. If the pK_{int} is taken as the corresponding pK_a in water then one faces problems in explaining the energetics of the aspartate-102–histidine-57 ion pair in chymotrypsin. The MTK model predicts that the pK_a of histidine-57 is shifted by about 3 units from its value in water. Experimentally, the pK_a of histidine-57 is similar to the pK_a of histidine in water. Of course, if pK_{int} is used as an adjustable parameter we can get any result we like. Here, as in many other cases, the key electrostatic effect is in the self energy (pK_{int}), which is not evaluated by the macroscopic models.

The Molecular Meaning of $\epsilon(R)$. As described above, interactions between charged groups in a protein correspond to interactions between charges in a medium with high effec-

Table 1. Comparison of pK_a values of myoglobin groups calculated by the MTK model and by Coulomb's law with $\epsilon = 40$

Ionizable group	pK_{int}*	pK_{MTK}*	$pK_{\epsilon=40}$†	pK_{exp}‡
His-12	6.30	4.80	5.00	5.37
His-48	6.80	6.60	6.68	6.83
His-64	7.80	7.70	7.69	8.05
His-81	6.30	6.10	6.09	6.65
His-113	6.30	5.30	5.32	5.53
His-116	6.30	5.80	5.95	6.44
His-119	6.30	5.50	5.65	6.34

*Taken from refs. 8 and 9 with pK_{MTK} calculated for zero ionic strength.

†Calculated from $pK^{(i)} = pK_{int} + q_iV_i/(2.3RT)$, where $V_i = 332q_j/(40R_{ij})$. For acids, $q_i = \alpha_i$ while for bases $q_i = 1 - \alpha_i$, where $\alpha_i = 1/\{1 + \exp[2.303(pK^{(i)} - pH)]\}$.

‡Taken from refs. 8 and 9 without any attempt to consider more recent work.

†There are only two possible selections of pK_{int} that have a clear physical meaning; either pK_{int} is taken as the pK_a of the corresponding group in water or pK_{int} is the pK_a of the given group in its site in the protein when all other groups are uncharged. In the first case, we obtain the huge attraction discussed above; in the second, we obtain the result drawn in Fig. 2. Neither case corresponds to the experimental energy of ion pairs in proteins.

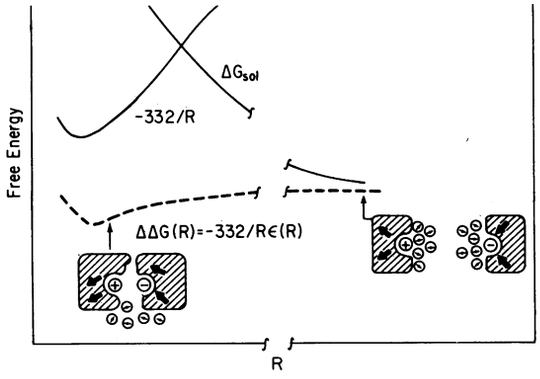

FIG. 3. Energy balance associated with charge separation in proteins. The apparently small value of $\Delta\Delta G(R)$ is due to the compensation of the change in charge–charge interaction ($332/R$) by the change in solvation energy.

tive dielectric constant. In view of the confusion in the literature it is important to clarify the reason for this result.

The dielectric constant, $\epsilon(R)$, for charge–charge interactions in proteins can be defined in different ways, depending on the reference state‡. Here we will consider two alternative definitions. The simplest definition can be obtained by considering Fig. 3, which describes the free energy balance associated with breaking a salt bridge between two subunits. As shown (see also ref. 16), the free energy is composed of two contributions: the interaction between the charges in vacuum and the solvation energy of the two charges by the protein dipoles and the surrounding water molecules. Note that, since the protein is considered as a solvent, its folding energy is included in the solvation energy (in the same way that water–water interactions are included in solvation energy). The free energy change associated with the charge separation process can be written as $\Delta G(R) = \Delta\Delta G(R) + \Delta G^{w}_{sol}(\infty)$, where $\Delta\Delta G(R)$ is the change of $\Delta G(R)$ on change of the charge–charge distance from infinity to R. From the above relation and Eq. **5** we can write

$$\epsilon(R) = \pm(332/R)/[\Delta\Delta G(R)], \qquad [9]$$

where the + and the − correspond to interactions between charges of equal and opposite signs, respectively. As seen from Eq. **9**, $\epsilon(R)$ in the phenomenological Coulomb's law is given by the ratio of the vacuum electrostatic interaction and the *change* in the free energy of the solvated ions. The magnitude of $\epsilon(R)$ reflects the compensation of the change in the vacuum charge–charge interaction by the change in solvation free energy. A large ϵ means that the work of taking the charges from their protein sites to infinity is almost completely compensated by the change in interaction between the ions and their environment (the protein and the water molecules). $\epsilon(R)$ is not a universal function and its use may be quite misleading in analysis of many biological processes (see below). Yet, for interactions between surface charges or charges at a distance of more than 5 Å (at equilibrium), one can fit the effective experimentally observed electrostatic interaction (Fig. 4*a*) to a Coulomb's type law with a distance-dependent dielectric function of the form

$$\epsilon(R) = \{1 + 60[1 - \exp(-0.1R)]\}(1 \pm 0.5), \qquad [10]$$

‡Using a concept of an effective dielectric constant results in different values for different operational definitions of ϵ. The bulk ϵ obtained by averaging the field is different than the ϵ_B needed to reproduce observed solvation energies by Born's formula and from the screening function $\epsilon(r)$ of ref. 15 (which is used to evaluate the local field around an ion). All these ϵ are different than $\epsilon(R)$ in Coulomb's law.

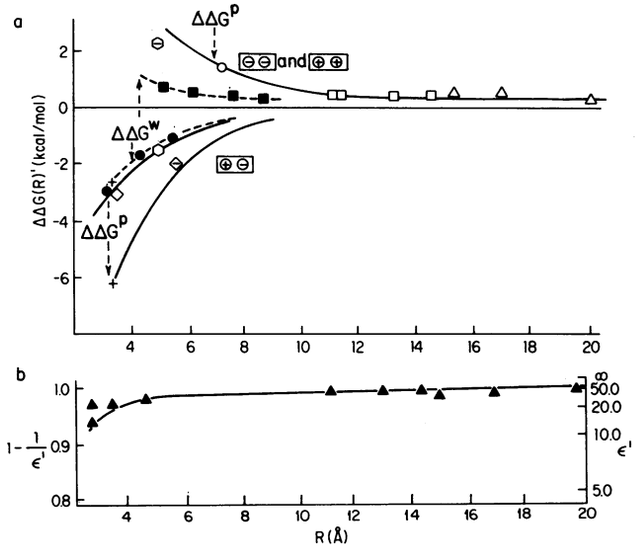

FIG. 4. Experimental estimates of $\Delta\Delta G(R)$ for interactions in proteins and in solutions, using the following observed changes in pK_a values and redox potentials: △ and □, heme groups and surface charges in cytochrome *c*, data of refs. 17 and 18, respectively; ○, glutamate-35 and aspartate-52 in lysozyme (19); ⊖, histidine-36 and glutamate-38 in myoglobin (20); ○, histidine-146 and aspartate-94 in hemoglobin (21); ⊖, histidine-89 and lysine-139 in hemoglobin (21); +, histidine-159 and cysteine-25 in papain (14) (this interaction has two estimates depending on the reference state of histidine-159; $\Delta\Delta G$ is approximately −3 for $pK_{int} \simeq pK^{w}_{a}$ and −6 for $pK_{int} \simeq 4.5$); ◇, aspartate-194 and isoleucine-16 in chymotrypsin (22); ●, zwitterions; ■, and dicarboxylic acids in water. The reference state is taken as an isolated acid in water. (*b*) Empirical estimate of the dielectric function $\epsilon'(R)$ using Eq. **9** and the results of *a*. ϵ' is defined where the reference state is the acid in its site in the protein where all other groups are neutral.

where this equation is valid for $R > 3$ Å. The factor (1 + 0.5) corresponds to an estimate of the error associated with the fact that $\epsilon(R)$ is different for different systems. The dielectric function is given in Fig. 4*b* as $1 - 1/\epsilon(R)$ rather than ϵ itself. The function $1 - 1/\epsilon$ approaches unity for polar solvents when the charge–charge energy is completely compensated by the change in solvation energy. This function seems to have more physical meaning than $\epsilon(R)$. It should be emphasized that the empirical $\epsilon(R)$ of Eq. **10** is not recommended for calculations of the energy of internal groups in proteins. Such calculations should be done by using microscopic models (3).

The risk of using the $\epsilon(R)$ of Eq. **10** can be appreciated by considering the fact that, for ion pairs in an oil drop surrounded by water, the correct $\epsilon(R)$ is *negative*. The reason is quite simple; $\Delta\Delta G(R)$ is larger than zero (see Fig. 2) and Eq. **9** gives a negative ϵ. Such a negative ϵ may be relevant to light-induced formation of charges in low dielectric regions. Similar problems are expected in analyzing the energy of ionic transition states (3).

An alternative definition of $\epsilon(R)$ may be obtained by choosing as a reference state the ions in their protein sites when all other ionizable groups are neutral (23) (this reference state is used in the analysis presented in Fig. 4). This definition gives, of course, a different ϵ, which is referred to here as $\epsilon'(R)$. However, the same compensation effects that cause $\epsilon(R)$ to be large also cause $\epsilon'(R)$ to be large. It should be noted that, although $\epsilon(R)$ is more relevant to the overall energetics of the protein charges, $\epsilon'(R)$ is more relevant to catalysis and to the relative energy of ionic transition states (2). This point is particularly important in view of the results presented in Fig. 4 for papain. The electrostatic interaction

of the histidine-159–cysteine-25 pair is larger when defined relative to the neutral state than when taken relative to the two ions in water.

Polarity and the Folding Energy of a Protein. Thus, $\epsilon(R)$ can have all types of values, yet experimentally we find at equilibrium only small values of $\Delta\Delta G(R)$ and large $\epsilon(R)$. The reason for this apparent polarity of the protein sites around ions is instructive. At equilibrium the value of $\Delta\Delta G(R)$ is limited by the folding energy of the protein (1, 3). If the two ions are unstable then the protein will unfold and allow its dipoles and/or water molecules to stabilize the ions. The unfolding can be a complete or a local unfolding, depending on the situation. Recent molecular dynamics calculations (24) have shown that reorientation of the protein dipoles toward its charges does not cost significant energy.

The above argument may leave the impression that proteins can always be considered as polar systems and that Fig. 2 is just an irrelevant hypothetical case. However, in considering key nonequilibrium photobiological processes (such as the action of the light-driven proton pump of bacteriorhodopsin), it is likely that charged groups are introduced in low-dielectric regions (23). In such cases, $\epsilon(R)$ is a time-dependent function (23) that can be quite small at the beginning of the protein relaxation process.

Concluding Remarks

We have examined the basis of the TK and MTK approaches and here we report the following points. (*i*) The TK approach predicts that no charged group can exist in the interior of a protein. This prediction is consistent with treating the protein as a low-dielectric-constant medium but inconsistent with experimental facts. (*ii*) The MTK approach is inconsistent with the TK model, on which it is supposed to be based. This model, which assumes that protein interiors are nonpolar, results in an expression that corresponds to a polar interior. (*iii*) Models that assume that the interior of proteins are polar (e.g., Eq. **10**) work as well as the MTK model without the need to involve the inconsistent implementation of the TK model or solvent accessibility. This point is demonstrated by comparing the MTK results with those obtained with a simple Coulomb's law model with a large dielectric constant. (*iv*) Reliable calculations of the energetics of ion pairs in protein interiors cannot be done by the MTK model or any other macroscopic model. As reported here the MTK model gives incorrect results for key cases of ion pairs in protein interiors. Thus, to treat correctly the energetics of ion pairs in a protein one must use a microscopic approach that takes into account the local environment. The fact that the local dielectric effect is not always correlated with the effect of ionizable groups is illustrated in ref. 25. (*v*) The local environment around charges in a protein is polar at equilibrium. This view is confirmed by x-ray structures (e.g., the active site of trypsin and papain; see also ref. 2).

It is important to realize that the TK model gives the effect of the water molecules around the nonpolar protein correctly. However, as shown in Figs. 1 and 2, this dielectric stabilization does not help at all in stabilizing internal charges (because the water molecules are too far from the charge). The MTK model tries to stabilize the charges *not* by using actual structural information about the charges but by placing them at the surface of the protein. The protein, on the other hand, stabilizes the internal charges by its dipoles.

Perhaps the most serious deficiency of the MTK and related models is the implication that electrostatic energies in proteins are determined and controlled by charge–charge interactions. Although charge–charge interactions are important, neglecting the key role of the protein permanent dipoles in determining self energies of charged groups is risky. For example, there is currently significant interest in using the MTK model in determining the binding of ions by proteins. The problem with such calculations can be demonstrated by using them in determining ion binding to crown ethers, which resemble active sites of proteins (2); the calculations do not result in binding. The same is true for the energetics of ions in biological ion channels.

In concluding this discussion, it might be useful to comment on a proposal (26) that ion pairs can exist in low-dielectric regions of proteins. This proposal used considerations similar to that introduced in ref. 3 to argue that the polar ($A^- BH^+$) and nonpolar (A HB) forms of a salt bridge are of similar energy in a low-dielectric region. However, the relevant question is related to the energy of moving the ion pair from water to the low-dielectric medium. As shown in Fig. 2, the $A^- BH^+$ form is much more stable in water than in nonpolar solvents. Thus, formation of a salt bridge in a nonpolar region will lead to unfolding of the protein or more likely to a local relaxation of the protein dipoles that will create a polar environment around the ions[§].

[§]One may argue that stabilizing ions by the protein dipoles (e.g., hydrogen bonds) corresponds to specific dipoles in a nonpolar environment and not to a polar site. However, in the same way one can argue that the solvation of ions by methanol corresponds to specific interactions in a nonpolar medium. In fact, the dielectric constant around charges in a protein is similar to that in polar solvents by all definitions, although it involves a slower dielectric relaxation time (23).

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