The Effect of Protein Relaxation on Charge-Charge Interactions and Dielectric Constants of Proteins

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ABSTRACT The effect of the reorganization of the protein polar groups on charge-charge interaction and the corresponding effective dielectric constant (ϵ_{eff}) is examined by the semimicroscopic version of the Protein Dipole Langevin Dipoles (PDLD/S) method within the framework of the Linear Response Approximation (LRA). This is done by evaluating the interactions between ionized residues in the reaction center of *Rhodobacter sphaeroides*, while taking into account the protein reorganization energy. It is found that an explicit consideration of the protein relaxation leads to a significant increase in ϵ_{eff} and that semimicroscopic models that do not take this relaxation into account force one to use a large value for the so-called "protein dielectric constant," ϵ_p, of the Poisson-Boltzmann model or for the corresponding ϵ_{in} in the PDLD/S model. An additional increase in ϵ_{eff} is expected from the reorganization of ionized residues and from changes in the degree of water penetration. This finding provides further support for the idea that ϵ_{in} (or ϵ_p) represents contributions that are not considered explicitly. The present study also provides a systematic illustration of the nature of ϵ_{eff}, supporting our previously reported view that charge-charge interactions correspond to a large value of this "dielectric constant," even in protein interiors. It is also pointed out that ϵ_{eff} for the interaction between ionizable groups in proteins is very different from the effective dielectric constant, ϵ'_{eff}, that determines the free energy of ion pairs in proteins (ϵ'_{eff} reflects the effect of preoriented protein dipoles). Finally, the problems associated with the search for a general ϵ_{in} are discussed. It is clarified that the ϵ_{in} that reproduces the effect of protein relaxation on charge-charge interaction is not equal to the ϵ_{in} that reproduces the corresponding effect upon formation of individual charges. This reflects fundamental inconsistencies in attempts to cast microscopic concepts in a macroscopic model. Thus one should either use a large ϵ_{in} for charge-charge interactions and a small ϵ_{in} for charge-dipole interactions or consider the protein relaxation microscopically.

INTRODUCTION

Electrostatic energies play a major role in many biological processes (Perutz, 1978; Warshel, 1978, 1981; Warshel and Russell, 1984; Matthew, 1985; Sharp and Honig, 1990; Nakamura, 1996). Thus it is important to develop reliable strategies for correlating the structure of macromolecules with their electrostatic energies. Several approaches have been developed for such calculations in the last two decades (e.g., Warshel and Levitt, 1976; Warwicker and Watson, 1982; Warshel and Russell, 1984; Sharp and Honig, 1990; Buono et al., 1994; Gilson, 1995), but their quantitative level can probably be improved. Among the factors that must be considered in proper calculations of electrostatic energies in proteins are the effects of charging each ionizable group when all other groups are neutral (the self-energy) and the charge-charge interaction. Although significant progress has been made in quantifying the self-energy term (Warshel, 1981; Warshel and Åqvist, 1991; Yang et al., 1993; Buono et al., 1994; Sham et al., 1997), the situation is less advanced with regard to the charge-charge interaction term. Experimental observations have indicated that the term is usually quite small and corresponds to a large effective dielectric constant (e.g., Rees, 1980; Warshel and Russell, 1984; Svensson and Jönsson, 1995). This is intuitively clear in cases of surface groups where the solvent provides large shielding. However, the case in which charges are located in the protein interiors is much less clear. In such cases it was postulated that the protein must reorganize upon formation of the interacting charges (Warshel and Russell, 1984; Warshel et al., 1984), and that leads to a larger than expected ϵ_{eff} in protein interiors. This was found to be consistent with both experimentally observed mutation effects (e.g., Alden et al., 1995; Muegge et al., 1996) and simulation studies (Hwang and Warshel, 1988; Cutler et al., 1989). It was also stated recently that the need of discretized continuum (DC) studies to use a large "protein dielectric," ϵ_{p_i}, reflects the missing contribution of the protein reorganization (Muegge et al., 1996; Sham et al., 1997; Warshel et al., 1997). This issue, however, was not investigated in a systematic way, and the actual effect of the protein reorganization on charge-charge interactions is neither widely recognized nor fully established.

In this work we perform a systematic study of the effect of protein reorganization on the interaction between ionizable groups in the bacterial reaction center (RC) of *Rhodobacter sphaeroides*. It is demonstrated that the protein relaxation has a major effect on the charge-charge interaction and the corresponding ϵ_{eff}. The implication of this finding for the nature and magnitude of the "dielectric

Received for publication 3 September 1997 and in final form 16 December 1997.

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0006-3495/98/04/1744/10 $2.00

constant" ϵ_p used in Poisson-Boltzmann (PB) studies for the corresponding ϵ_{in} in the PDLD/S model is discussed.

METHODS

Our simulation strategy is based on the semimicroscopic version of the Protein Dipole Langevin Dipole (PDLD/S) method. This method has been described very extensively elsewhere (e.g., Lee et al., 1993; Sham et al., 1997) and is considered only briefly here. The electrostatic energy associated with moving an ionized group with a charge q_1 from water to a specific protein site is evaluated by the PDLD/S thermodynamic cycle (Lee et al., 1993). The change in this free energy upon the change of q_1 from q_1^b to q_1^a is given by

$$\begin{aligned}\Delta U^{w\to p}_{PDLD/S}(q_1^b \to q_1^a) &= \Delta U^{w\to p}_{PDLD/S}(q_1^a) - \Delta U^{w\to p}_{PDLD/S}(q_1^b) \\ &= [-\Delta G^{w}_{qw}(q_1^b \to q_1^a) + \Delta G^{p}_{qw}(q_1^b \to q_1^a)]\left(\frac{1}{\epsilon_{in}} - \frac{1}{\epsilon_w}\right) \\ &\quad + [\Delta V^{p}_{qq}(q_1^b \to q_1^a) + \Delta V^{p}_{q\mu}(q_1^b \to q_1^a)]\frac{1}{\epsilon_{in}} \qquad (1)\end{aligned}$$

where we consider here the PDLD/S free energy as an effective potential (U), since it is taken from a single protein configuration. ΔG^{w}_{qw} and ΔG^{p}_{qw} are, respectively, the contributions of the solvent to the change in solvation free energies of q_1 (when q_1^b becomes q_1^a) in water and in its protein site. These contributions are evaluated by using the microscopic PDLD method, where the solvent molecules are represented by a grid of Langevin-type point dipoles (for details see Warshel and Russell, 1984; Lee et al., 1993). ΔV^{p}_{qq} is the change in the gas phase Coulombic energy for the interaction between q_1 and all other ionized groups, and $\Delta V^{p}_{q\mu}$ is the change in the Coulombic interaction between q_1 and the protein polar groups. ϵ_{in} is a scaling parameter that is closely related to the parameter ϵ_p in PB approaches (e.g., Warwicker and Watson, 1982; Sharp and Honig, 1990), and, as we stated repeatedly, neither ϵ_{in} nor ϵ_p represents the actual "dielectric constant" of the protein, but the contributions that are not treated explicitly in the given model (see King et al., 1991, and the meaning of ϵ_{eff} in the Results). Although Eq. 1 is expressed in terms of the energies of forming a single ionizable group, it can, of course, be used to describe the interaction between two groups. As will be clarified below, the corresponding interaction is reflected by both the ΔG^{p}_{qw} and $\Delta V^{p}_{q\mu}$ terms.

The actual free energy associated with moving a charge from water to its protein site should reflect the proper sampling of the protein configurations during the charging process. This can be done here by using the Linear Response Approximation (LRA) and is given by Lee et al. (1992, 1993). The rigorous implementation of this approximation in the PDLD/S formulation involves rather complex thermodynamic cycles of the type described most clearly by Muegge et al. (1998). At any rate, the final result is quite simple and the relevant free energy change is given by

$$\begin{aligned}\Delta\Delta G^{w\to p}(q_1^b \to q_1^a) &= \frac{1}{2}[\langle \Delta\Delta U^{w\to p}_{PDLD/S}\rangle_{q_1=q_1^a} + \langle \Delta\Delta U^{w\to p}_{PDLD/S}\rangle_{q_1=q_1^b}]. \qquad (2)\end{aligned}$$

Usually q_1^a and q_1^b are the two charge states of the given ionizable group (e.g., the ionized and neutral forms of an ionizable residue), and $\langle\rangle_q$ designates a molecular dynamic (MD) average obtained by using a potential surface (force field) that assigns the indicated charge to the corresponding residue (e.g., q_1^a). This approach takes into account the protein reorganization and the corresponding dielectric effect. It is important to note that the PDLD/S method without the LRA treatment gives results that are similar to those obtained by current PB methods that consider explicitly the protein permanent dipoles. Thus the comparison of the PDLD/S with and without the LRA treatment should be quite instructive.

In this work we are interested in the effect of protein reorganization on charge-charge interaction. The starting point for evaluating this interaction term is the expression for the overall energetics of ionizing the protein ionizable residues, which is given by (Warshel, 1979, 1986; Yang et al., 1993)

$$\begin{aligned}\Delta G^{(m)} &= \sum_i \left\{ -2.3RTq_i^{(m)}[pK^{p}_{int,i} - pH] + \frac{1}{2}\sum_{j\neq i} W_{ij}q_i^{(m)}q_j^{(m)} \right\} \\ &= \sum_i \left\{ -q_i^{(m)}W_i^0 + \frac{1}{2}\sum_{j\neq i} W_{ij}q_i^{(m)}q_j^{(m)} \right\} \qquad (3)\end{aligned}$$

where $q_i^{(m)}$ is the actual charge of the ith group. This can be 0 or -1 for acids and 0 or 1 for bases. W_{ij} is the charge-charge interaction term that will be discussed below. The intrinsic pK_a (pK_{int}) is the pK_a that the given ionized group would have if all other ionizable groups were kept at their neutral state (the evaluation of this term is described in Sham et al., 1997). Equation 3 can also be expressed in terms of the energy of forming the given configuration in a reference state in aqueous solution at infinite separation of the ions and then transforming it to the protein. This gives

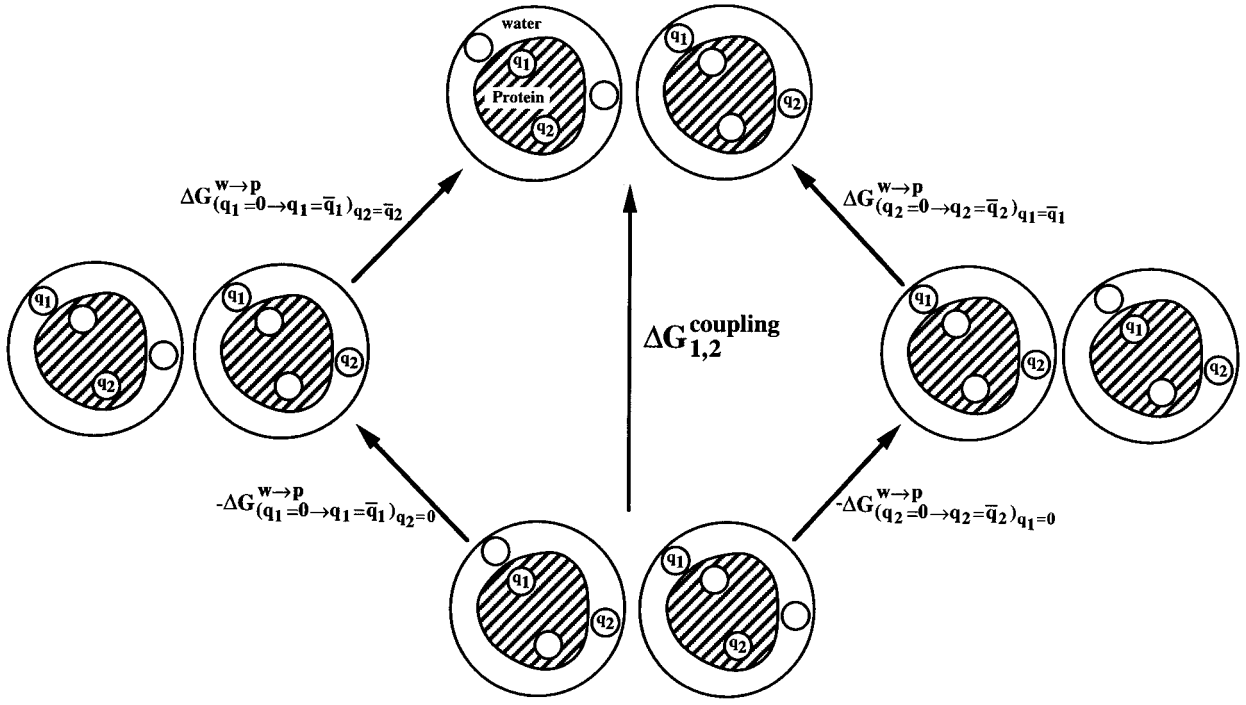
$$\begin{aligned}\Delta G^{(m)} &= (\Delta G^{(m)})^{w} + \sum_i \left\{ -2.3RTq_i^{(m)}[pK^{p}_{int,i} - pK^{w}_{a}] + \frac{1}{2}\sum_{j\neq i} W_{ij}q_i^{(m)}q_j^{(m)} \right\} \\ &= (\Delta G^{(m)})^{w} + \sum_i \left\{ -q_i^{(m)}(\Delta\Delta G^{w\to p}_{sol}(q_i))_0 + \frac{1}{2}\sum_{j\neq i} W_{ij}q_i^{(m)}q_j^{(m)} \right\} \qquad (4)\end{aligned}$$

where $(\Delta\Delta G^{w\to p}_{sol}(q_i))_0$ represents the energy of moving q_i from water to its actual protein site when all other ionizable groups are neutral (this term can be evaluated by Eqs. 1 and 2). The interaction terms in Eq. 4 can be evaluated with the help of the thermodynamic cycle of Fig. 1. This is done by using Eqs. 1 and 2 to evaluate the $\Delta G^{coupling}$ of the steps in Fig. 1 and then comparing the resulting free energy to the corresponding expression obtained from Eq. 4. The resulting coupling term is given by

$$\begin{aligned}\Delta G^{coupling}_{ij} &= W_{ij}\bar{q}_i\bar{q}_j \\ &= \Delta\Delta G^{w\to p}(q_i = 0 \to q_i = \bar{q}_i)_{q_j=\bar{q}} \\ &\quad - \Delta\Delta G^{w\to p}(q_i = 0 \to q_i = \bar{q}_i)_{q_j=0} \qquad (5)\end{aligned}$$

where $\Delta\Delta G^{w\to p}_{sol}$ designates the change of the corresponding $\Delta G^{w\to p}_{sol}$ as a result of the indicated change in charge, and $\bar{q}_i$ is the charge of the ith ionizable group in the ionized form. In this work we treat q_i as the charge q_1 in Eq. 1 and consider q_j as a charged group in the surrounding protein region. The interaction between these two charges reflects both the direct effect of the V_{qq} term and the indirect effect of the ΔG^{p}_{qw} term. The ΔG^{p}_{qw} term gives the contribution to the solvation free energy of q_i from the water molecules in and around the protein. This contribution is influenced, of course, by the surrounding protein and the charge q_j. Equation 5 gives the coupling term for two ionizable groups, A_i and A_j, in terms of the difference between the free energy of charging A_i when A_j is ionized and the free energy of charging A_i when A_j is neutral. An equivalent expression for ΔG_{ij} can also be obtained by exchanging i and j in Eq. 5, and the agreement between the two calculated results can serve as a consistency check. The W_{ij} obtained from Eq. 5 can also be rewritten as

$$W_{ij} = \frac{332}{(r_{ij}\epsilon_{ij})} \qquad (6)$$



$$\Delta G^{coupling}_{1,2} = \Delta G^{w\to p}_{(q_1=0\to q_1=\bar{q}_1)_{q_2=\bar{q}_2}} - \Delta G^{w\to p}_{(q_1=0\to q_1=\bar{q}_1)_{q_2=0}}$$
$$= \Delta G^{w\to p}_{(q_2=0\to q_2=\bar{q}_2)_{q_1=\bar{q}_1}} - \Delta G^{w\to p}_{(q_2=0\to q_2=\bar{q}_2)_{q_1=0}}$$

FIGURE 1 Thermodynamic cycles used to obtained the coupling term, ΔG_{ij}. Each cycle starts with two identical proteins, each with one ionizable group in its site and the second in the surrounding solvent (water), and ends up with one protein with two ionizable groups in their sites and the second where the two groups are in the solvent.

where r_{ij} is the average distance (in Å) between the ith and jth charge centers and where the energy is given in kcal/mol. This equation does not depend on any "linearity" of the model and is simply a *definition* of the effective dielectric constant for the specific interaction. This reflects our perspective (Warshel and Russell, 1984) that the dielectric constant in proteins depends on its definition and the property studied. Thus we define the effective dielectric constant ϵ^{ij}_{eff} by

$$\epsilon^{ij}_{eff} = \epsilon_{ij} = \frac{332}{r_{ij}W_{ij}} \tag{7}$$

For the purpose of the subsequent discussion it is also useful to define the free energy of an ion pair at a distance r_{ij} relative to the energy of the ions at infinite separation in water. This energy (which is not at all equal to the corresponding W_{ij}) can be obtained from Eq. 4 (see also the original derivation in Warshel, 1981, and the related discussion in Warshel and Russell, 1984):

$$\begin{aligned}(\Delta\Delta G^{+-}_{12})^{w\to p} &= [\Delta G(q_1=1, q_2=-1)^p - \Delta G(q_1=0, q_2=0)^p] \\ &\quad - [\Delta G(q_1=1, q_2=-1)^w_\infty - \Delta G(q_1=0, q_2=0)^w_\infty] \\ &= (-\Delta\Delta G^{w\to p}_{sol}(q^+_1))_0 + (\Delta\Delta G^{w\to p}_{sol}(q^-_2))_0 - W_{12} \\ &\quad + \frac{1}{2}\sum_{k\neq i,j} W_{1k}\langle q_k\rangle - \frac{1}{2}\sum_{k\neq i,j} W_{2k}\langle q_k\rangle \end{aligned} \tag{8}$$

where 1 and 2 are the relevant base and acid, respectively, and $\Delta\Delta G_{solv}$ is defined in Eq. 4. Here the $\langle q_k\rangle$ term represents the average change of the ith ionizable group. Note that this result is not equal to W_{12}, and in particular note the existence of the self-energy terms in Eq. 8.

Because the free energy of forming an ion pair in water from the corresponding neutral acid and base is $(\Delta\Delta G^{+-}_{12})^W_\infty = 2.3RT(pK^W_{a,2} - pK^W_{a,1})$, and the free energy of forming the ion pair in the protein is $(\Delta\Delta G^{+-}_{12})^p = 2.3RT(pK^p_{a,2} - pK^p_{a,1})$ (see Warshel, 1981), it follows that

$$(\Delta\Delta G^{+-}_{12})^{w\to p} = 2.3RT(\Delta pK^{w\to p}_{a,2} - \Delta pK^{w\to p}_{a,1}) \tag{9}$$

where $\Delta pK^{w\to p}_{a,i} = pK^p_{a,i} - pK^w_{a,i}$. This equation can be verified by calculating the right-hand side of Eq. 9 by using Eq. 4 and then comparing the resulting expression to Eq. 8. Now if we try to define an effective dielectric constant by $\Delta\Delta G^{+-}_{12}$, we find that

$$\epsilon'_{eff} = \frac{332}{r_{ij}(\Delta\Delta G^{+-}_{ij})^{w\to p}} \neq \epsilon_{eff} \tag{10}$$

Although this additional definition might confuse some readers, it serves an extremely useful purpose in correlating ion pair stability with the corresponding protein folding energy and is directly related to the Potential of Mean Force (PMF) for charge separation in proteins. This point will be discussed in subsequent sections.

The actual PDLD/S-LRA calculations involve the use of the program package POLARIS 6.28 (Lee et al., 1993). This package includes both the original POLARIS model for performing the PDLD calculations and a built-in ENZYMIX model for performing the MD simulations needed for the LRA treatment. The program divides the protein into several simulating

regions, which are described in detail elsewhere (Lee et al., 1993; Sham et al., 1997). With regard to these regions, we only mention here that in the evaluation of Eq. 5 we place A_i in region I (the region reserved for groups whose charge is being changed) and place A_j in region II with the rest of the protein. It is important to note that the POLARIS program places special emphasis on the proper treatment of long-range effects by using the Local Reaction Field (LRF) model (Lee and Warshel, 1992), and on special spherical boundary conditions with proper polarization constraints of the solvent in the surface region by using the Surface Constraint All Atom Solvent (SCAAS) model (see King and Warshel, 1989; Lee et al., 1993). The calculations of the ΔG_{ij} of Eq. 5 are made by evaluating the relevant $\Delta\Delta G^{w\to p}$ for charging A_i when A_j is charged and when A_j is uncharged. The calculations start with a 2-ps MD relaxation followed by the evaluation of the averages of Eq. 2 from the PDLD/S energies of eight configurations of the charged state and eight configurations of the uncharged state. These configurations are generated in each case at intervals of 0.5 ps for a 4-ps trajectory (the convergence of the calculations is discussed below). All trajectories were generated at a temperature of 300 K and with a 1-fs time step. Longer simulation runs or averaging over more configurations were found to give similar results for the PDLD/S-LRA energies.

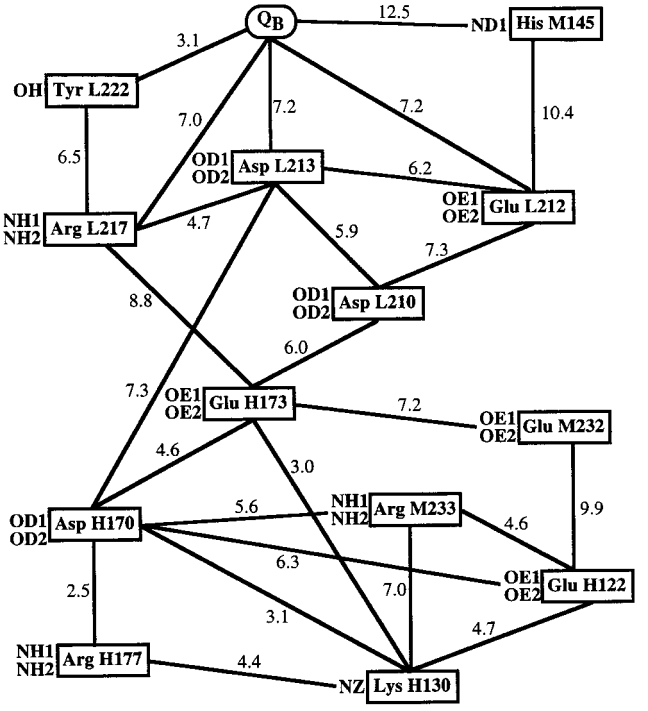


FIGURE 2 A schematic description of the interactions between ionizable groups in the bacterial reaction center (RC) of *Rhodobacter sphaeroides* that were considered in the present work. The relevant distances between interacting groups are given in Ångstroms and are measured between the indicated atoms.

RESULTS AND DISCUSSION

The effect of protein relaxation

To have an extensive benchmark, we studied the interactions between ionizable groups in the bacterial reaction center (RC) of *Rhodobacter sphaeroides* (Ermler et al., 1994). This system was considered recently (Beroza et al., 1995 and Lancaster et al., 1996) with particular emphasis on its role in the proton transport process. The ionizable groups considered are depicted in Fig. 2.

The relevant values of ΔG_{ij} were calculated with and without the LRA treatment. The corresponding results are summarized in Tables 1, 2, and 3, where each calculation corresponds to the average obtained using Eq. 5 and exchanging i and j. The error range of the calculations is rather small, despite the relatively short simulation time. That is, because, as illustrated in Fig. 3 and in our previous studies (e.g. Langen et al., 1992; Sham et al., 1997), the PDLD/S converges much faster than all-atom LRA and FEP simulations. Furthermore, the SCAAS/LRF treatment implemented in ENZYMIX provides significantly faster convergence of electrostatic energies than other current simulation programs. This is due to the SCAAS boundary conditions that allow one to use consistently a relatively small simulation region. The convergence error of the calculations is estimated from Fig. 3 and related considerations to be ~0.5 kcal/mol. Thus we consider the error range obtained by exchanging *i* and *j* as a much more stringent test for the accuracy of our results. The average error range is ~1 kcal/mol, where the largest error is ~2.0 kcal/mol. Averaging the simulations on very different initial conditions should, in principle, help in minimizing the error range, but this is out of the scope of the present work. At any rate, although an error range of ~1 kcal/mol can produce a significant error range in the ϵ_{eff} of weak interactions, this is rather irrelevant, however, because weak interactions are not so important biologically or conceptually. As a result, only the values of ϵ_{eff} for interactions that are greater than 2 pK_a units were considered (Table 3). In these cases we were able to obtain meaningful estimates of the effect of the protein relaxation (see below). As is clear from the tables, the introduction of protein relaxation has a major effect on the calculated interactions. In general, it appears that the explicit treatment of the protein reorganization leads to a decrease in the charge-charge interactions and to a corresponding increase in the effective dielectric constant (see Table 3). It is important to realize in this respect that there is no unique definition for the nonrelaxed result, which was considered here as the result obtained by taking the crystal structure at its face value. This is, of course, problematic, because the orientations of the polar groups in the protein depend on the force field used in the structural refinement process. Furthermore, the x-ray structure evaluated at the ionization states that correspond to the crystallization conditions may not be identical to the equilibrium structures in other pH ranges. And the local environment around groups that are neutral in the given crystallization condition is not the proper structure for studies of the corresponding intrinsic pK_a values. Fortunately, the unrelaxed results depend on the presence of the permanent dipoles only in a second-order way. That is, the "back field," $V_{q\mu}$, from the permanent dipoles (see definition in Warshel and Russell, 1985, and Sham et al., 1997) cancels in Eq. 5 (because the dipoles are kept at the same orientation in both steps of the unrelaxed cycle), and the only effect comes from the polarization of the solvent by these permanent dipoles. Thus it is not

TABLE 1 Calculated unrelaxed interaction energies between relevant ionizable groups of the reaction center of *Rhodobactor sphaeroides

Residue		Asp^- L210	Glu^- L212	Asp^- L213	Arg^+ L217	Tyr^- L222	His^+ M145	Glu^- M232	Arg^+ M233	Glu^- H122	Lys^+ H130	Asp^- H170	Glu^- H173	Arg^+ H177
UQB^-		0.6	2.7	2.4	−2.3	2.8	−0.9	0.4	−0.7	0.4	−1.1	0.8	1.6	−1.1
Asp^-	L210		2.1	4.2	−0.6	0.8	−0.3	1.2	0.3	2.6	−4.1	2.7	3.2	−0.5
Glu^-	L212			3.7	−1.5	0.6	−1.1	1.8	−1.7	2.7	−2.5	2.0	3.4	−2.4
Asp^-	L213				−5.0	1.5	−0.2	2.0	−0.7	2.1	−3.3	3.0	6.2	−2.6
Arg^+	L217					−3.1	0.0	−1.0	0.2	0.2	1.7	−1.9	2.3	1.3
Tyr^-	L222						0.1	0.7	−0.4	0.4	−0.7	0.9	1.5	−1.0
His^+	M145							−0.1	0.0	−0.3	0.2	−0.2	−0.7	0.1
Glu^-	M232								−3.9	2.7	−2.8	3.0	4.2	−9.2
Arg^+	M233									−8.0	3.7	−4.2	−2.7	5.1
Glu^-	H122										−6.7	3.7	4.4	−3.7
Lys^+	H130											−13.0	−9.5	6.7
Asp^-	H170												6.5	−11.1
Glu^-	H173													−7.8

*Calculated PDLD/S results obtained using the crystal structure of the reaction center of *Rhodobactor sphaeroides* (Ermler et al., 1994). The results were obtained without MD relaxation, using $\epsilon_p = 4$. The results are given in pK_a units.

unreasonable to evaluate the nonrelaxed contribution by using the x-ray structure at its face value. The problem is, of course, much more serious when one tries to evaluate the absolute energy of ion pairs (Eq. 8) or the individual pK_a's rather than just the interaction terms. In such cases the permanent dipoles provide the major contribution. In other words, the shielding of charges from each other (which defines the coupling term W_{ij}) involves only a relaxational process that is akin to dielectric response. On the other hand, the stabilization of a particular group (which determines the pK_a change) involves both the relaxation of the permanent dipoles and the effect of these dipoles at their average orientation. This is important because the oriented dipoles may favor or disfavor a given ionized group relative to water.

In addition to the above problem of structural uniqueness, it appears that the effect of relaxation on ϵ_{eff} is not completely predictable. That is, whereas in most cases ϵ_{eff} increases when the protein relaxation is taken into account, there are exceptions (e.g., see LysH130-AspL210 in Table 3). These deviations reflect the dependence of ϵ_{eff} on the difference between two relaxation effects, rather than the dependence of the relaxation effects on the formation of a single charge. Thus although the general trend (see discussion in the next section) is an increase in ϵ_{eff}, some exceptions are possible. This reflects the difficulty in casting microscopic effects in terms of macroscopic concepts. Regardless of this issue, the ϵ_{eff} obtained with a given ϵ_{in} would usually have a larger value with a more microscopic treatment. Furthermore, an additional increase in the effective dielectric constant would be obtained if we consider explicitly the reorganization of the ionized residues around the given interacting pair. In such a case one has to find the proper ionization state of all residues at the given pH and then keep all ionizable groups ionized during the LRA treatment. This is obviously an expensive proposition, which was considered here only in the evaluation of three strong interactions (Table 4). As seen from the table, we

TABLE 2 Calculated relaxed interaction energies between relevant ionizable groups in the relaxed structure of the reaction center of *Rhodobactor sphaeroides

Residue		Asp^- L210	Glu^- L212	Asp^- L213	Arg^+ L217	Tyr^- L222	His^+ M145	Glu^- M232	Arg^+ M233	Glu^- H122	Lys^+ H130	Asp^- H170	Glu^- H173	Arg^+ H177
UQB^-		0.3	1.8	1.9	−2.2	1.7	−1.0	1.5	−0.2	1.0	−1.2	0.6	1.6	1.2
Asp^-	L210		2.0	3.0	−0.5	0.5	−1.0	1.2	−0.3	1.8	−6.4	1.7	2.5	0.9
Glu^-	L212			2.6	−1.4	0.2	−0.9	2.4	−1.0	2.8	−2.0	2.0	3.0	−0.4
Asp^-	L213				−3.7	0.9	0.0	1.5	−0.9	2.1	−2.9	2.4	3.5	−0.6
Arg^+	L217					−3.0	−0.4	−1.0	0.4	0.0	1.2	−1.0	−1.5	2.0
Tyr^-	L222						−0.4	0.4	−0.5	0.8	−1.3	1.0	1.5	0.1
His^+	M145							0.0	0.0	0.2	−0.5	−0.2	−1.0	−0.5
Glu^-	M232								−4.5	2.5	−1.9	4.1	4.8	−7.1
Arg^+	M233									−4.5	1.6	−3.4	−2.7	4.2
Glu^-	H122										−6.1	4.0	2.9	−3.6
Lys^+	H130											−9.5	−6.5	2.9
Asp^-	H170												4.7	−3.5
Glu^-	H173													−3.5

*Calculated PDLD/S-LRA results (in pK_a units) obtained using $\epsilon_{in} = 4$. The LRA calculations were performed using Eq. 2 and the procedure described in the text.

TABLE 3 The effect of the protein relaxation on ϵ_{eff} and ϵ_{in}*

Pair		$\Delta G_{ij}^{(ur)}$	$\Delta G_{ij}^{(r)}$	$\epsilon_{eff}^{(ur)}$	$\epsilon_{eff}^{(r)}$	$\epsilon_{in}^{(ur)}$
Lys H130	Asp H170	−13.0	−9.5	7.3	10.1	5.5
Asp H170	Arg H177	−11.1	−3.5	6.9	21.8	12.6
Lys H130	Glu H173	−9.5	−6.5	9.5	14.0	5.9
Arg H177	GluM232	−9.2	−7.1	7.8	10.2	5.2
Glu H122	ArgM233	−8.0	−4.5	6.5	11.6	7.1
Glu H173	Arg H177	−7.8	−3.5	7.7	17.0	8.8
Lys H130	Arg H177	6.7	2.9	8.6	19.7	9.2
Glu H122	Lys H130	−6.7	−6.1	9.4	10.3	4.4
Asp H170	Glu H173	6.5	4.7	10.6	14.5	5.5
Glu H173	Asp L213	6.2	3.5	9.5	16.5	7.0
Arg H177	ArgM233	5.1	4.2	8.8	10.7	4.9
Asp L213	Arg L217	−5.0	−3.7	13.1	17.3	5.3
Glu H122	Glu H173	4.4	2.9	9.4	14.1	6.0
Asp H170	ArgM233	−4.2	−3.4	9.4	11.6	4.9
Glu H173	GluM232	4.2	4.8	11.0	9.6	3.5
Asp L210	Asp L213	4.2	3.0	11.9	16.8	5.7
Lys H130	Asp L210	−4.1	−6.4	13.7	8.9	2.6
Glu M232	ArgM233	−3.9	−4.5	21.0	18.1	3.4
Glu L212	Asp L213	3.7	2.6	12.9	18.8	5.8
Lys H130	ArgM233	3.7	1.6	13.2	31.3	9.4
Glu H122	Asp H170	3.7	4.0	12.0	11.0	3.7
Glu H122	Arg H177	−3.7	−3.6	11.7	11.9	4.1
Glu H173	Glu L212	3.4	3.0	12.1	13.5	4.5
Lys H130	Asp L213	−3.3	−2.9	12.8	14.7	4.6
Glu H173	Asp L210	3.2	2.5	14.4	18.4	5.1
Arg L217	Tyr L222	−3.1	−3.0	15.2	15.5	4.1
Asp H170	Asp L213	3.0	2.4	11.4	14.6	5.1
Asp H170	GluM232	3.0	4.1	12.9	9.5	2.9
Lys H130	GluM232	−2.8	−1.9	13.4	19.8	5.9
UQB	Tyr L222	2.8	1.7	21.2	34.7	6.6
Glu H122	GluM232	2.7	2.5	12.3	13.2	4.3
UQB	Glu L212	2.7	1.8	11.1	16.8	6.0
Glu H122	Glu L212	2.7	2.8	11.4	11.1	3.9
Glu H173	ArgM233	−2.7	−2.7	24.1	23.5	3.9
Asp H170	Asp L210	2.7	1.7	14.8	23.1	6.3
Arg H177	Asp L213	−2.6	−0.6	11.3	48.0	17.0
Glu H122	Asp L210	2.6	1.8	14.4	20.4	5.7
Lys H130	Glu L212	−2.5	−2.0	14.2	17.1	4.8
Arg H177	Glu L212	−2.4	−0.4	11.0	59.4	21.7
UQB	Asp L213	2.4	1.9	15.9	19.9	5.0
UQB	Arg L217	−2.3	−2.2	15.9	17.0	4.3
Glu H173	Arg L217	−2.3	−1.5	14.9	22.8	6.1
Glu H122	Asp L213	2.1	2.1	12.7	12.7	4.0
Asp L210	Glu L212	2.1	2.0	18.2	19.2	4.2
Asp H170	Glu L212	2.0	2.0	13.5	13.7	4.1
Asp L213	GluM232	2.0	1.5	14.0	18.8	5.4

*The table gives the relevant ΔG_{ij} for the unrelaxed (ur) and the relaxed (r) model in pK_a units. The interactions considered are those whose absolute values obtained by the unrelaxed approach are larger than 2 pK_a units. The effective dielectric constants (ϵ_{eff}) in each case are evaluated by using Eq. 6 with the relevant ΔG_{ij} (and the corresponding W_{ij}) and the r_{ij} of the unrelaxed approach. $\epsilon_{in}^{(ur)}$ is the ϵ_{in} that should be used in the unrelaxed model to reproduce the relaxed results that were obtained with $\epsilon_{in} = 4$.

find a significant increase in ϵ_{eff} due to the relaxation of ionized residues. A further increase in ϵ_{eff} will probably be obtained if we perform much longer simulations and consider the change in water penetration during each step in the cycle of Fig. 1. Overall, the present results lend further justification to a model that uses large implicit ϵ_{eff} for charge-charge interactions in proteins (see next section).

It is instructive to ask now what ϵ_{in} is needed to capture the effect of the protein reorganization on charge-charge interactions. To address this issue, we adjusted the ϵ_{in} in the nonrelaxed model until it reproduced the relaxed results obtained with $\epsilon_{in} = 4$. The corresponding results are reported in the last column of Table 3. As seen from the table, the ϵ_{in} that has to be used in the nonrelaxed model is frequently larger than the value of 4 used in the relaxed model. At this point it should be clarified that the view that ϵ_{eff} is large in proteins (Warshel et al., 1984), which is supported by the present finding, does not mean that ϵ_{in} should be large in proteins. That is, as shown above, ϵ_{in} has to be increased to correctly capture the missing effect of

TABLE 4 The effect of relaxation of dipolar and ionized groups*

Pair		$\Delta G_{ij}^{(ur)}$	$\Delta G_{ij}^{(r)}$	$\Delta G_{ij}^{(r,ion)}$	$\epsilon_{ij}^{(ur)}$	$\epsilon_{ij}^{(r)}$	$\epsilon_{ij}^{(r,ion)}$
Lys H130	AspH170	−13.0	−9.5	−4.5	7.3	10.1	15.4
Asp H170	ArgH177	−11.1	−3.5	−3.4	6.9	21.8	16.3
Glu H173	AspL213	6.2	3.5	3.6	9.5	16.5	11.7

*Calculations and notation are the same as in Table 3, except that $\Delta G_{ij}^{(r,ion)}$ designates the interaction term obtained when the ionized groups around the given pair are kept at their most likely ionization state (rather than in the neutral state).

protein relaxation on charge-charge interactions, but in doing so one would get incorrect results for the self-energies and the corresponding pK_a^{int} of ionizable groups in protein interiors (e.g., Sham et al., 1997). This problem is associated, of course, with the fact that ϵ_{in} is not a true dielectric constant, but just a parameter that reflects the implicit contributions, and as such it cannot consistently reproduce charge-charge interactions and self-energies. The fact that ϵ_{in} can become rather small when more terms are treated explicitly has nothing to do with the "proper" dielectric constant of proteins. That is, many workers would be happy to argue that because more consistent treatments would reduce ϵ_{in}, it follows that the "correct" ϵ_{in} will eventually approach what they assume to be the "low" dielectric constant of proteins. Unfortunately, such an argument completely overlooks the nature of ϵ_{in}, because the most consistent treatment will lead to $\epsilon_{in} = 1$, which is obviously not related to the dielectric properties of proteins. This issue will be addressed further in subsequent sections.

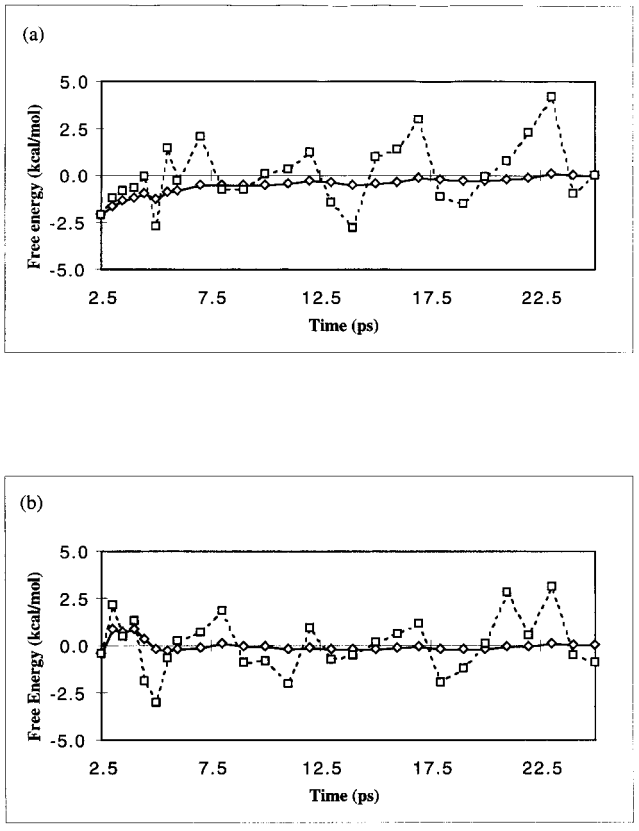


FIGURE 3 Demonstrating the convergence of the calculated PDLD/S free energy. The figures describe the fluctuation (– – –) and the accumulated average values (——) of ΔG_{ij} for (*a*) Asp H170-Lys H130 and (*b*) Asp H170-Arg H177 over a 25-ps trajectory. As shown in the figure, we obtain a reasonable convergence after ~4 ps. (The first PDLD/S calculation was performed for the configuration generated after 2.5 ps.)

The meaning of ϵ_{eff}

Many of our works (e.g., Warshel and Russell, 1984; Muegge et al., 1996) and that of others (e.g., Rees, 1980; Mehler, 1996) used a large ϵ_{eff} to estimate interactions between ionized residues. The large values of ϵ_{eff} do not reflect arbitrary assumptions, but are rather the results of a long series (see, for example, pp. 347–364 in Warshel and Russell, 1984) of computational and conceptual studies and their experimental verifications, including rather rigorous and physically consistent PDLD and FEP calculations (e.g., Russell and Warshel, 1985; Cutler et al., 1989). Despite these works, it seems that the underlying microscopic physics of ϵ_{eff} is not fully appreciated (see discussions in Warshel et al., 1997). Some might assume that ϵ_{eff} simply reflects the effective interactions obtained by a macroscopic model with a small ϵ_{in} in the protein region and a high dielectric constant for the solvent region. Or, in other words, that ϵ_{eff} only represents the effect of the solvent around the protein. Such an assumption might reflect in fact a confusion of the rigorous results of an assumed model and the actual physics of a real protein. It seems to us that microscopic considerations of charge separation are the best and perhaps the only way to understand the origin of ϵ_{eff}. Such considerations do show that ϵ_{eff} reflects the compensation between the vacuum charge-charge interaction and the reorganization of the surrounding environment (e.g., Warshel and Russell, 1984), which includes reorientation of the protein and solvent dipoles and in some cases solvent penetration to the site of the relevant charges. To clarify this issue, it is useful to define ϵ_{eff} in terms of a charge separation process. Such a definition is not unique and depends on the reference state used. In this work we define ϵ_{eff} by Eqs. 4 and 6, where the reference state is the self-energy of the two ionizable groups in their protein site (Fig. 1). Another definition can be obtained by considering the energy of the two interacting charges in the protein relative to their energies at infinite separation in water. This definition, which is very useful when one is interested in the relationship between ϵ_{eff} and the folding energy of the protein (Warshel and Russell, 1984), is not identical at all to the definition of

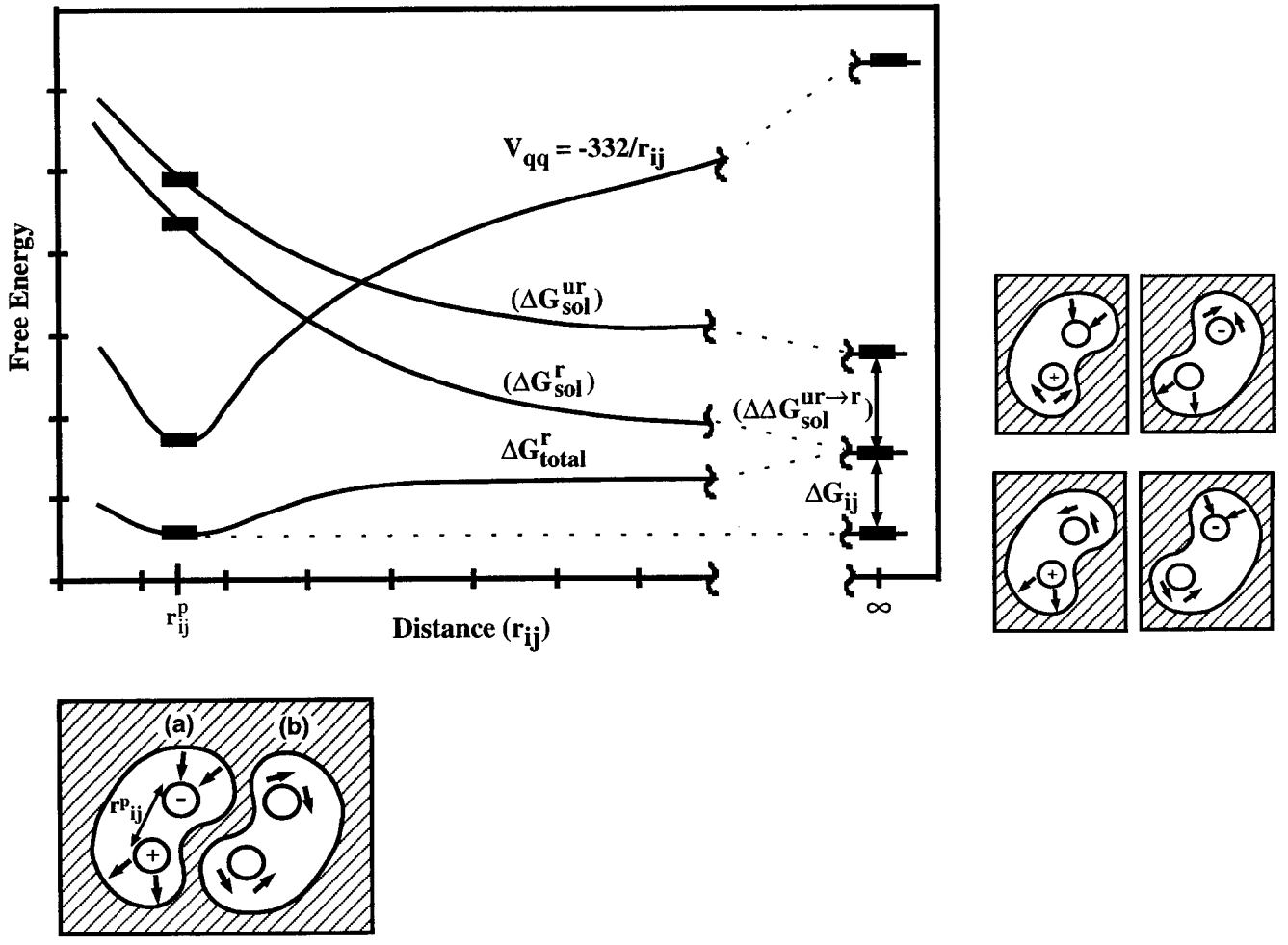


FIGURE 4 A schematic illustration of the effects of the reorganization of the protein dipoles on the free energy of interaction between a positive and a negative ionized residue. The figure describes the contributions to the total free energy, ΔG_{total}, from the vacuum charge-charge interaction, V_{qq}, and the solvation energy due to the protein/solvent system, ΔG_{sol}. The figure describes the solvation free energies for the relaxed (r) and unrelaxed (ur) systems. The figure gives only ΔG^{r}_{total}, but the corresponding unrelaxed contribution ΔG^{ur}_{total} can be obtained by combining ΔV_{qq} and ΔG^{ur}_{sol}. As discussed in the text, we consider the process of charge separation by transferring one charge from the original protein to a second identical protein and then separating the two proteins. The figure illustrates the fact that the relaxation effect is larger for the separated proteins at $r = \infty$ than for the actual distance r^{p}_{ij} in the original protein. As a result, the change in ΔG^{r}_{total} is smaller than in ΔG^{ur}_{total} and ϵ_{eff} is larger for the relaxed model.

Fig. 1. That is, the energy of ion pairs relative to their energy in water depends drastically on the orientation of the protein permanent dipoles, whereas (as stated in the previous section) the interaction term of Eq. 6 depends only on the reorganization of the permanent dipoles and not on the average orientation. Because this paper does not deal with the important problems of the stability of ion pairs, which depends strongly on the presence and the orientation of the permanent dipoles, we focus here on the first definition. In doing so, we have to resort to a process that involves two identical proteins (Fig. 4) and move a charge from one protein to another. When this process involves an ion pair, we find that the largest effect of protein relaxation occurs at the charge separated state ($R\rightarrow\infty$), because the field from the ion pair exerts a smaller electrostatic force on the protein than the field from the isolated ions. This point can be realized by noting that in general (both in solutions and in proteins), the solvation free energy of ion pairs is smaller than the corresponding total solvation free energy of the separated ions (Warshel and Russell, 1984). Thus the protein permanent dipoles around ion pairs do not have to reorient as much as the dipoles around isolated ions. The situation is reversed when we consider two negatively charged groups in nearby sites. In this case, the largest relaxation is expected in the state where the two ionized groups are in their actual positions in the protein, rather than in the state where they are taken to an infinite distance from each other ($R\rightarrow\infty$). In both cases we find that the relaxation process reduces the absolute value of $\Delta\Delta G$ and leads, in most cases, to an increase in ϵ_{eff}.

The relationship between ϵ_{in} and the protein relaxation

This paper examines the effect of the protein relaxation on ϵ_{in} needed to reproduce a consistent energy for charge-

charge interactions in proteins. As seen from Table 3, we find that ϵ_{in} should be larger than 4 in many cases, but this is not the point of our work. That is, as we clarified repeatedly (e.g., Warshel and Åqvist, 1991), there is no universal ϵ_{in}, and this "dielectric constant" depends exclusively on what is being treated explicitly. ϵ_{in} is also not equal or related in a simple way to the true macroscopic protein dielectric, $\bar{\epsilon}$ (King et al., 1991). It is important to clarify this point in view of the repeated attempt to find a general universal ϵ_{in} or to obtain a site-dependent ϵ_{in} (e.g., Simonson and Perahia, 1995; Demchuk and Wade, 1996). In our view such an ϵ_{in} cannot be determined in a general way. For example, Simonson and Perahia (1995) proposed to evaluate ϵ_{in} by determining the microscopic energy associated with the reorganization of the protein upon formation of test charges. Unfortunately, the dielectric constant evaluated in this way cannot reproduce the actual free energy of ionized groups in PB models that include the protein dipole explicitly (if the dipoles are not included, such models are entirely inappropriate). That is, there is no general consistent treatment that allows one to use the dielectric associated with the reorientation of the solvent permanent dipoles for screening the field between these same dipoles and solvated charges. This point can easily be clarified by devising a simple model of a spherical protein that contains a charge and two dipoles, where one dipole (on the left) is completely fixed with its head pointing toward the charge, while a second dipole (on the right) is completely free to rotate. The dielectric constant obtained from the relaxation energy of the second dipole will overestimate the screening of the first dipole (the $V_{Q\mu}/\epsilon_{in}$ term will be too small). Thus it is impossible to reproduce the actual electrostatic energy of a charge in this system. In other words, the dielectric constant deduced from the reorganization energy cannot be used to reproduce the energy of an ionized group even in the above model, which is simple and well defined. To further clarify this point, we note that the relaxation of the protein upon formation of two interacting charges cannot be identical in general to that associated with the formation of a single charge. This is clearer in the most dramatic way in some of the cases studied here, where both ϵ_{eff} and ϵ_{in} are reduced rather than increased when the protein relaxation is included explicitly. This abnormal effect is due to the fact that the two terms in Eq. 5 have different responses to the relaxation. On the other hand, for a single charge the relaxation will always lead to an increase in the solvation energy. Thus we would always obtain a larger ϵ_{in} in models that consider this relaxation implicitly.

The above discussion (which might seem to be unnecessary semantics to some) is essential to clarify conceptual and practical points that cannot be realized without careful microscopic consideration. Thus it appears to us that although ϵ_{in} in PB models can be adjusted to account for the general trend expected from observed electrostatic energies, the best and perhaps the only way to obtain consistent microscopic energetics is to consider the relaxation effect explicitly.

CONCLUDING REMARKS

This paper examines the effect of protein relaxation on charge-charge interaction. This is done by evaluating the charge-charge coupling terms in the bacterial RC of *Rhodobacter sphaeroides*, with and without explicit consideration of the protein reorganization energy. It is found that the protein relaxation leads to a significant reduction of the calculated charge-charge interactions and constitutes a major component of the effective dielectric constant, ϵ_{eff}, for such interactions.

The present work helps to remind us of an important point about the relationship between the energy of ion pairs and ϵ_{eff}. As was already concluded previously (Warshel, 1981) and reiterated in the Methods section, the energy of ion pairs can be expressed in terms of pK_a changes. However, the pK_a change includes both the intrinsic pK_a term (the self-energy) and the interaction term. The self-energy reflects the orientation of the protein permanent dipoles and is, in many cases, the leading term. Thus it is frequently found that pairs with a small ΔG_{ij} are more stable than those with a large ΔG_{ij}. It should also be kept in mind that the ϵ_{eff} obtained from Eq. 6 is much less sensitive to the presence of the protein permanent dipoles than that of Eq. 10, which reflects the actual energy of the ion pairs. This issue will be addressed further in subsequent studies.

The results of the present study have a significant implication with regard to the nature of ϵ_{in} in semimicroscopic and PB calculations and the magnitude of ϵ_{eff} deduced from such calculations. That is, as demonstrated here, the use of a small ϵ_{in} without an explicit consideration of the protein relaxation can lead to an overestimation of the charge-charge interactions and an underestimation of the corresponding ϵ_{eff}. This is particularly serious when one deals with charged groups in protein interiors. In such cases the solvent contribution to ϵ_{eff} (which is correctly taken into account by current PB methods) is rather small, and $\epsilon_{eff} \cong \epsilon_{in}$. Assuming that $\epsilon_{in} = 4$ will lead to $\epsilon_{eff} \cong 4$ when one deals with internal ions (and therefore small solvent contributions) and to a very large ΔG_{ij}. Such a prediction, however, is inconsistent with mutation experiments, where ϵ_{eff} is usually rather large, even in protein interiors (Alden et al., 1995). Here we provided a direct support to an early concept (Warshel and Russell, 1984) that ϵ_{eff} is large, even in protein interiors, because it reflects the reorientation of the protein polar groups and other factors and not only the effect of the solvent around the protein. We also provided further demonstration of the idea (King et al., 1991) that ϵ_{in} represents the contributions that are not included explicitly in the model, rather than the "true" protein dielectric constant. This point is illustrated in Table 3, where we show that larger ϵ_{in} should be used in models that consider the protein relaxation implicitly rather than in models that consider it explicitly. However, it seems to us that there is no position-dependent ϵ_{in} that can describe the effect of the relaxation of the protein in a general way. As discussed in the previous subsection, the ϵ_{in} that allows one to reproduce

relaxation effects on charge-charge interactions is quite different from the ϵ_{in} needed to reproduce such effects in studies of isolated charges. Of course, one may use different values of ϵ_{in} for these different properties (large ϵ_{in} for charge-charge and small ϵ_{in} for charge-dipole). However, a much more consistent picture would be obtained by treating the protein relaxation explicitly at a microscopic level (Langen et al., 1992; Sham et al., 1997), which is exactly what is done by our LRA treatment.

The authors thank Dr. J. Bentzien for critically reading the manuscript and for helpful scientific discussions.

This work was supported by grant GM40283 of the National Institutes of Health.

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