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On Possible Pitfalls in ab Initio Quantum Mechanics/Molecular Mechanics Minimization Approaches for Studies of Enzymatic Reactions

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Reliable studies of enzymatic reactions by combined quantum mechanics/molecular mechanics (QM/MM) approaches, with an ab initio description of the quantum region, presents a major challenge to computational chemists. The main problem is the need for a very large computer time for the evaluation of the QM energy, which in turn makes it extremely challenging to perform proper configurational sampling. A seemingly reasonable alternative is to perform energy minimization studies of the type used in gas-phase ab initio studies. However, it is hard to see why such an approach should give reliable results in protein active sites. To examine the problems with energy minimization QM/MM approaches, we chose the hypothetical reaction of a metaphosphate ion with water in the Ras·GAP complex. This hypothetical reaction served as a simple benchmark reaction. The possible problems with the QM/MM minimization were explored by generating several protein configurations from long MD simulations and using energy minimization and scanning of the reaction coordinates to evaluate the corresponding potential energy surfaces of the reaction for each of these different protein configurations. Comparing these potential energy surfaces, we found major variations of the corresponding minima. Furthermore, the reaction energies and activation energies also varied significantly even for similar protein configurations. The specific coordination of a magnesium ion, present in the active center of the protein complex, turned out to influence the energetics of the reaction in a major way, where a direct coordination to the reactant leads to an increase of the activation energy by 17 kcal/mol. Apparently, using energy minimization to generate potential surfaces for an enzymatic reaction, while starting from a single protein structure, could lead to major errors in calculations of activation free energies and binding free energies. Thus we believe that extensive samplings of the configurational space of the protein are essential for meaningful determination of the energetics of enzymatic reactions. The possible relevance of our conclusion with regard to a recent study of the RasGAP reaction is discussed.

I. Introduction

QM/MM approaches have provided a general scheme for studies of chemical processes in proteins.[1−11] Significant progress has been made with calibrated semiempirical QM/MM approaches.[2,7,10,11] However, one would like to move to an ab initio representation with a QM/MM treatment since such QM representations have been shown to provide "chemical accuracy" in studies of gas-phase reactions of small molecules.[12] In principle, when one uses a reliable large ab initio QM region, one can expect to obtain a reliable description of the potential surface of the reaction region. Unfortunately, this does not mean that the actual free energy barriers are estimated correctly, and in fact there may be many serious problems. First, it is essential to provide a proper long-range treatment and effective boundary conditions for the protein + solvent environments.[13] Second, it is important to use a polarizable force field for the MM region; and finally, it is important to perform sufficient sampling to obtain the actual free energy.[2,14]

The present work will not address the sampling problem, which was discussed recently[2,15,16] in terms of possible simulation strategies. Instead, we will focus on the issue of using energy minimization for a few protein configurations, e.g. refs 17 and 45. Obviously this strategy provides an advance over gas-phase or single-point calculations. However, this approximated strategy was not subjected to systematic scrutiny. In this respect we note that the problem is, that in case of enzyme active sites, the challenge of finding the transition state region and evaluating the activation barrier is quite different than in gas-phase energy search. The problem is that the protein landscape is very complex and different configurations cannot be found by standard energy minimization approaches. Furthermore, results obtained from different minima can be very different, as already observed in a previous work.[17]

To illustrate the above problems we take a relatively simple case, a segment of a possible path in the reaction of Ras, where an H_2O attacks a metaphosphate, which in principle can be formed by a dissociative mechanism. This type of reaction has been considered in a recent QM/MM energy minimization study[45] where it was concluded that the barrier for the phosphorus−oxygen bond breaking is very low and the rate-determining step is a proton transfer to the metaphosphate through a concerted path that involves both Gln61 and the catalytic water. It has also been concluded that a direct proton transfer from the catalytic water to the metaphosphate involves a very high barrier.[45] The present paper does not attempt to explore the possible involvement of such a reaction path in the reaction of the Ras·GAP system, since this should involve a very careful evaluation of the free energy surface for the

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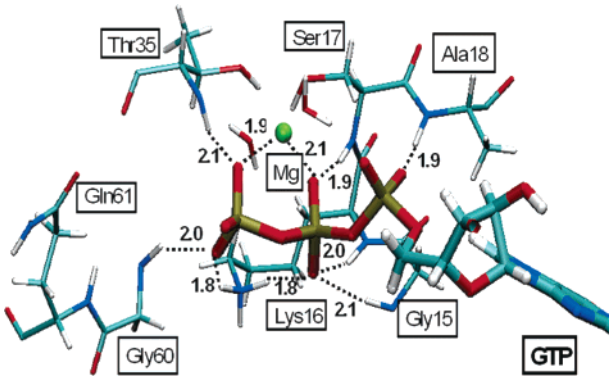

Figure 1. Substrate GTP and its hydrogen bonds to residues of the Ras·GAP complex as derived from the crystal structure of the hydrolysis transition state analogue of Scheffzek et al.[19] (PDB code 1WQ1).

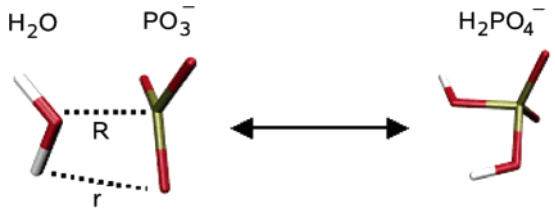

Figure 2. Reaction coordinates *R* and *r* used in the present study.

associative and dissociative mechanisms, a careful calibration, and a systematic comparison to relevant experimental results. Instead we merely chose the hypothetical water−metaphosphate system as a simple benchmark for QM/MM calculations, and we will make only some general comments about the new proposal of ref 45.

Section II describes our benchmark and outlines the computational methods. Section III describes the results of the calculations and demonstrates their sensitivity to the protein conformational states and the position of the Mg^{2+} ion. Finally, we discuss in section IV the implications of our findings and emphasize the importance of a proper average over the protein configurations.

II. Computational Methods

This work considers some aspects of the rather complex mechanism of the Ras·GAP system, whose active site and a model of the bound GTP is depicted in Figure 1. However, instead of focusing on the actual reaction and its biological implications we focus on a relatively simple model reaction, namely, the attack of H_2O on a metaphosphate formed in the active site of the Ras·GAP system, following a hypothetical cleavage of the GTP substrate (Figure 1). The metaphosphate−water system was used as a benchmark in exploring the validity of QM/MM transition state searches in condensed phases in general and in proteins in particular.

The potential energy surface (PES) of our reaction was explored by considering the two reaction coordinates described in Figure 2, the distance of the water oxygen to the phosphorus, *R*, and the distance of the transferred proton to proton acceptor oxygen of the metaphosphate, *r*.

We also examined the possibility that a possible third reaction coordinate (i.e., the distance of the transferred proton to the water oxygen) can be neglected and its inclusion does not change our results quantitatively.

To evaluate the potential surface for the reaction in solution, we started by scanning the surface in the gas phase as a function of *R* and *r*, where at each point we constrained the system to the specific *R* and *r* and minimized the energy with regard to all other coordinates. Next we considered the effect of the solvent by using the conductor reaction field approach COSMO[20] and performed a single-point COSMO calculation at each of the scanning points. The resulting PES served as an approximation for the energetics of the reaction in solution. In doing so, we assumed implicitly that minimization with respect to the coordinates orthogonal to *R* and *r* will give similar results in the gas phase and in solution. This assumption was verified in some specific cases.[21] It seems to us that the above scanning procedure is at present more effective than alternative options of performing a transition state search on the solution surface using analytical derivatives. That is, in our experience there are problems with the analytical derivatives of most current ab initio solvation models, at least with respect to the dependence of the cavity surface and the reaction field on the solute coordinate. Here the use of a systematic scanning procedure is the simplest way of obtaining a reliable mapping of the transition state region.

In addition to the COSMO calculations, we also considered the previously reported results obtained by the Langevin dipole model.[22] The gas-phase optimizations were performed by use of the Hartree−Fock method and a 6-31G(d) basis set, whereas the single-point calculations that considered the solvent effect used the density functional theory (DFT) method with the hybrid functional B3LYP and an extensive 6-311++G(d,p) basis set. The results of this minimization/scanning approach will be described in the next section.

The next step of our study involved a QM/MM evaluation of the potential surface of our system in the protein active site. Before describing the actual calculations we will provide below a brief description of our QM/MM approach, emphasizing the specific technical points of the current implementation. The starting point in QM/MM models is the separation of the system into QM and MM regions.[1] In systems where the quantum region is composed of a molecule or molecules that are not bound to the classical region, as is often the case in solution-phase reactions, separation of the two regions is rather trivial. The separation into quantum and classical regions is not as straightforward in enzyme reactions, because the quantum region is frequently bonded to the classical region, and it is not clear how to define uniquely the boundary conditions for the electronic structure calculations of the quantum region, nor how to incorporate the electrostatic and van der Waals effects of the classical region into the quantum region energy expression. An effective way of connecting the two regions can be provided by use of hybrid orbital[1] and related techniques.[14,23] Unfortunately, these approaches are somewhat difficult to implement, and because the current QM(ai)/MM method aims to be an efficient method of linking standard programs (in this case GAUSSIAN[24] and MOLARIS[25]), we chose the simpler method of using link atoms. A link atom (LA)[26] is an atom inserted along the bond between the quantum and classical regions. Such an approach was introduced,[1] in addition to introducing the use of the hybrid orbital approach. In the nomenclature of ref 27, the quantum mechanical atom to which the link atom is bonded is referred to as the link atom bond partner (LABP), and the classical atom replaced by the link atom is referred to as the link atom host (LAH). Warshel and Levitt[1] introduced the link atom treatment in their AMI/MM model of the catalytic reaction of lysozyme, while the more reliable hybrid orbital treatment[1] was used in their QCFF/ALL study of lysozyme. Although the name LA was not introduced, the method was incorporated into the ERFN program[29] and thus constitutes the first LA QM/MM treatment. The LA treatment was also introduced in QM/MM

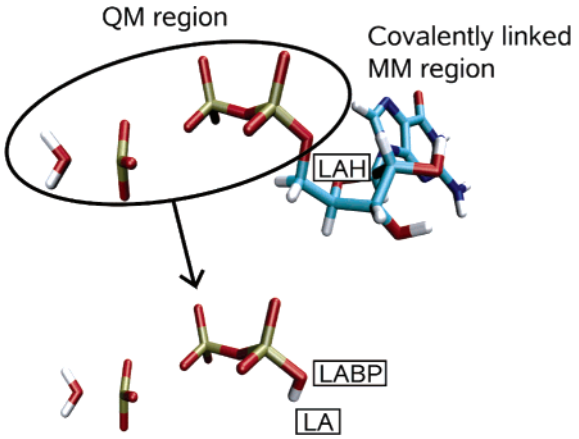

Figure 3. A possible division of the simulated system into QM and MM regions. The upper part shows the QM region and a part of the MM region that contains the guanosine moiety. The rest of the MM region, which is not shown in the figure, contains the surrounding protein and solvent molecules. The lower part shows the QM fragment capped with a hydrogen link atom. LABP and LAH designate, respectively, the link atom bond partner and the link atom host. The link atom host is replaced here by the hydrogen link atom (LA).

studies of π-electron systems.[46] Warshel and Levitt[1] and Ostlund[30] developed methods that adjust the ionization potential of the LAs to reproduce properties of the given molecule in the presence of the actual LAHs. In our and previous studies,[30,27] the LA is a hydrogen atom. Figure 3 illustrates the process of defining the quantum region and inserting LAs.

This figure shows the reacting system separated into the quantum region and a small part of the MM region, which is covalently linked to the QM region. The structure below the quantum fragment shows this fragment capped with LAs to produce the quantum region. The LAs are inserted along the LAH−LABP bond at a distance determined by gas-phase ab initio energy minimization. Despite the intense current interest in proper treatment of link atoms and the availability of more effective hybrid orbital and related localized bond approaches,[1,23] we feel that this problem is not so crucial in studies of enzymatic reactions. That is, errors introduced by use of a small fragment are generally similar in solution-phase and protein simulations. Thus, such errors are largely canceled when one is concerned with enzyme catalysis, which reflects the difference between the activation energy in the enzyme and in the reference solution reaction. In the present case we treated the metaphosphate and the water quantum mechanically using the Hartree−Fock approach with a 6-31G(d) basis set, as was done for the reaction in solution. We also estimated a correction for the reaction energies and activation energies by calculating the energies at the reactant, product, and transition states by use of the B3LYP functionals with a 6-311++G(d,p) basis set.

Before applying the above QM/MM approach, we generated starting protein configurations. This was done by constructing an empirical valence bond (EVB) potential for the QM system and using this potential to propagate trajectories in the reactant state. The trajectories started from a model structure of the Ras·GAP complex derived from the crystal structure of Scheffzek et al.[19] Because we have been interested here only in the hypothetical reaction of metaphosphate (that is already dissociated from the GDP moiety) with a water molecule, we separated the metaphosphate from GDP by applying a soft harmonic potential with a minimum at 3 Å distance between the two compounds. This model system was first equilibrated by an MD run of 500 ps and then the simulation continued for another 500 ps, generating five representative protein configurations. These configurations were taken as the starting points for the QM/MM studies described in the next section.

III. Results and Discussion

Although it is convenient to assume that ab initio QM/MM approaches should give reliable results, this is far from being certain. Thus before QM/MM calculations of chemical processes in enzymes are performed, it is important to validate the method used by calculating the energetics of the given reaction in solution. That is, since QM/MM calculations in enzymes do not yet give chemical accuracy, it is essential to calibrate (or validate) the specific method used by examining the agreement between the calculated and observed energetics in solution reactions (see discussion in section II). Such calculations are also needed in order to assess the catalytic effect of the enzyme. At any rate, we established the validity of the quantum mechanical model by evaluating the free energy surface for the solution reaction using the COSMO solvent and the mapping procedure described in section II. The corresponding results are summarized in Table 1 (together with the previously reported results with LD solvent model) and Figure 4.

Figure 4 depicts the equipotential lines of the PES, the transition state, and the corresponding reaction path. The figure shows that the PES in the reactant region is shallow and that the water molecule can reach a very close distance ($R = 2.1$ Å) to the metaphosphate before the proton is transferred. The transition state is found at $R = 1.9$ Å and $r = 1.3$ Å. The reaction free energy, ΔG_0, is -23.2 kcal/mol and the corresponding activation free energy is $\Delta G^{\ddagger} = 13.3$ kcal/mol. These energy differences are in very good agreement with the values $\Delta G_0 = -24.0$ and $\Delta G^{\ddagger} = 11.0$ kcal/mol obtained by Florian and Warshel[21] from ab initio/LD calculations and from analysis of relevant experimental data.

We also examined the influence of electron correlation and the basis set on the PES. That is, in addition to our chosen B3LYP/6-311++G(d,p) calculations we also evaluated the activation free energy and free reaction energy with the HF/LanL2DZ and B3LYP/LanL2DZ methods. The results of this study are also summarized in Table 1.

From this comparison we conclude that the neglect of Coulomb electron correlation in the Hartree−Fock approach leads to rather inaccurate estimates for both the reaction and the activation free energy, as expected. Both energy differences deviate around 50% from our original results. The deviations of the smaller basis set, LanL2DZ, compared to the triple-ζ basis set are smaller, but use of this basis set somewhat underestimates the reaction free energy.

After establishing the reliability of the potential surfaces for the solution reaction, we explored the reaction in the active site of the Ras·GAP complex. In this case we performed the transition state search with several initial protein configurations. The potential energy surface for each protein configuration was evaluated by the same mapping procedure described in the discussion of the solvent surface and the method section, but this time with the QM/MM description of the protein. It is important to notice that even in the case of a single protein configuration there is a risk of obtaining an incorrect transition state. This issue is considered in Figure 5, where we depict an example of the PES for a specific protein configuration. As seen from the figure, starting from different initial reactant coordinates and using an incomplete configurational search can lead us to different transition states. This problem, which can also occur in gas-phase studies, is rather well-known[31,32] and is not the topic of this work.

The actual serious problem addressed by this paper starts to become apparent when we consider the effect of the protein

TABLE 1: Comparison of the Free Reaction Energy and Free Activation Energy for Reaction of Metaphosphate with Water in Solution for Different Methods[a]

	B3LYP/6-311++G(d,p) COSMO	MP2/6-31+G(d,p) Langevin dipoles[b]	exp estimates of actual free energy[b]	HF/LanL2DZ	B3LYP/LanL2DZ
ΔG_0	−23	−21	−24	−37	−27
$\Delta G^{\ddagger}$	13	16	11	20	11

[a] All energies are given in kilocalories per mole. [b] Langevin dipole results and estimates of actual experimental free energies are taken from Florian and Warshel.[21]

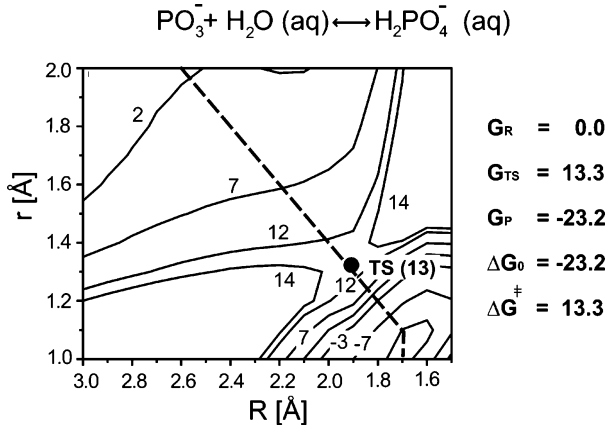

Figure 4. Free energy surface for the reference reaction of metaphosphate with water in solution and dependency on the two reaction coordinates *R* and *r*. Solid lines are the equipotential lines of the surface with the corresponding free energy values given next to them. The dashed line represents the reaction pathway connecting the shallow reactant region (upper left corner) to the product valley (lower right corner) through the transition state (marked with a dot). The free energies of the ground, transition, and product states as well as the reaction and activation free energy are given on the right side of the figure. All energies are given in kilocalories per mole.

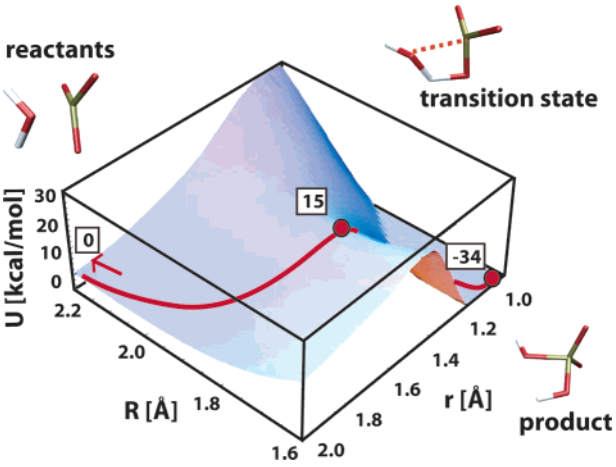

Figure 5. Potential energy surface of the reaction of metaphosphate with water in the active site of Ras·GAP for one representative protein structure. The red line represents the reaction pathway connecting the reactant (on the left side) with the product (right side).

configurations. This issue is demonstrated in Figure 6 and Table 2, where we examine the dependence of the lowest energy profile on the protein configuration used.

As seen from the figure, the absolute energies of the ground state can easily vary by 30 kcal/mol between different protein configurations. This finding makes it rather clear that calculations of binding free energies, which reflect the energy of the reactant state, cannot be performed by QM/MM approaches without very extensive averaging. The situation seems to be less catastrophic when one considers the activation barriers relative to the given ground state energy. Here the variation is about 6 kcal/mol, which is large but can be used in some

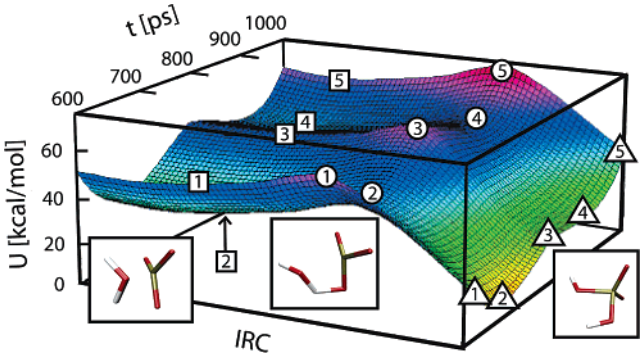

Figure 6. Temporal development of the potential energy surface of the reaction metaphosphate with water in the active site of Ras·GAP. The generic surface is based on the two-dimensional PES, like the one shown in Figure 5, where we present the energy along the intrinsic reaction coordinate (IRC), determined at five different protein configurations taken from the MD trajectory after 600, 700, 800, 900, and 1000 ps. The reactant states are marked with squares; the product states, with triangles; and the transition states, with circles. The inscribed numbers correspond to one of the five protein structures.

TABLE 2: Dependence of Energetics of the Reacting System on the Protein Configurations[a]

protein structure	reactant	transition state (*R*, *r*)	product	ΔU_0	$\Delta U^{\ddagger}$
600 (1)	57	75 (1.85, 1.31)	36	−21	18
700 (2)	39	52 (1.89, 1.33)	16	−23	13
800 (3)	52	67 (1.84, 1.29)	18	−34	15
900 (4)	27	46 (1.85, 1.32)	4	−23	19
1000 (5)	46	65 (1.85, 1.31)	25	−21	19

[a] Energies of the reactant, product, and transition states as well as the corresponding reaction energies and activation energies are given in kilocalories per mole. Each row contains the values for one protein structure derived from the MD trajectories after 600, 700, 800, 900, and 1000 ps. The number in parentheses for the protein structure corresponds to the label used in Figure 6. For the transition states, the values for *R* and *r* are given in parentheses in angstroms.

qualitative considerations. Unfortunately this is only the tip of the iceberg. That is, while the error in $\Delta U^{\ddagger}$ obtained in closely related configurations may be tolerable, some configurational changes might lead to much larger effects.

A detailed analysis of the effect of the protein fluctuations on the QM/MM surfaces is usually rather complicated. However, one can focus on some key elements. Here we explored the effect of changes in the position of the Mg^{2+} ion, considering two different coordinations that were both found to be stable (assuming that the Mg^{2+} ion is fixed at the single position found in the transition state analogue is apparently unjustified). The results of this study are summarized in Figure 7 where we compared the reaction profile associated with the two initial configurations depicted on the right-hand side of the figure. As seen there, increasing the distance between the Mg^{2+} ion and the metaphosphate oxygen reduces the barrier by 17 kcal/mol.

The results presented in Figure 7 clarify that the situation of QM/MM calculations in proteins is quite different than the situation of those obtained in gas-phase calculations. That is, while in gas-phase calculations it is reasonable to assume that

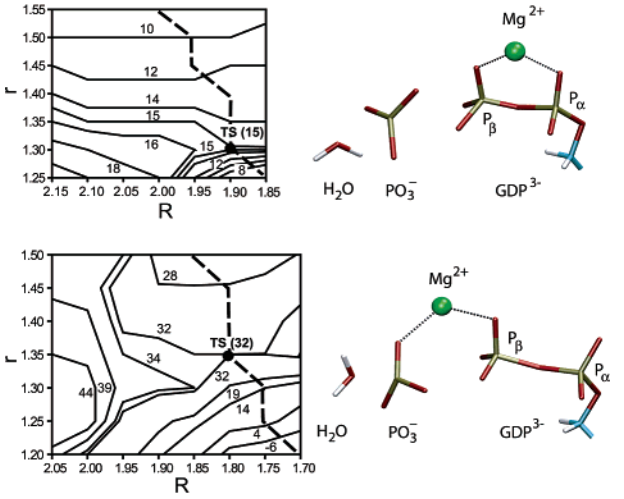

Figure 7. Potential energy surfaces in the transition state region for the reaction of metaphosphate with water in Ras analogous to Figure 4. The lower panel displays the case where the Mg^{2+} ion is coordinated between the metaphosphate reactant and GDP. The upper panel shows the PES for the case where the Mg^{2+} ion is coordinated to two GDP oxygens and not to the metaphosphate.

a convergent minimization approach will lead automatically to a displacement of the magnesium ion, it is unlikely that this will happen in an energy minimization in the protein active site. These results underscore the importance of a proper conformational search in QM/MM evaluations of activation energies. Here one of the best options is to use EVB as a reference potential for QM/MM free energy calculations.[33]

The determination of the relative role of the two magnesium ion configurations of Figure 7 is far from being simple. In principle one can start by evaluating the electrostatic free energy of the Mg^{2+} ion in the two configurations by a free energy perturbation (FEP) approach. Then, by assuming that the energy of moving the Mg^{2+} ion between the two sites is small, it would be possible to estimate the relative energy of the corresponding configurations. This treatment can be improved by the linear response approximation (LRA) treatment, which is quite effective in capturing the protein reorganization energy.[34] However, such a study is out of the scope of the present work, since it requires one to resolve the issue of the optimal representation of the Mg^{2+} ion. That is, an MM representation of the magnesium ion considers charge transfer to the substrate by the empirical van der Waals parameters. On the other hand, the seemingly superior treatment that includes the Mg^{2+} ion in the QM region may be problematic if the ligands of this ion are treated classically (in this case one neglects the charge transfer from the ligands to the metal, and the inclusion of the ligands in the QM region is likely to be too expensive.[35,36] At present we simply point out that the conceivable case, where both configurations are accessible, can be used as an instructive example of the risk in QM/MM energy minimization approaches. In view of the problems with simple QM/MM minimizations or scanning procedures, it seems obvious that one should attempt to average such calculations over the relevant protein configurations. The most rigorous way of obtaining such averages is by free energy calculations, but such calculations can be too expensive when one uses a high level ab initio treatment for the QM region and regular free energy perturbation treatments. This problem can be drastically reduced by use of the EVB or other simple potentials as a reference for the ab initio QM/MM free energy calculations.[37,38]

IV. Concluding Remarks

Evaluation of the energetics of chemical processes in proteins by classical force fields requires usually very extensive averaging over the configurational space of the protein. Thus it is quite likely that the same requirement will hold for QM/MM calculations. This means that simple minimization approaches of the type used in gas-phase QM calculations might not be effective in evaluations of activation energies of chemical reactions in proteins. The present work examines this issue and demonstrates that QM/MM energy minimization approaches can lead to significant errors in evaluation of activation barriers of enzymatic reactions. The difficulties in using minimization approaches can be particularly serious when the enzyme−substrate complex involves a rugged landscape with many local minima. An instructive example of the coupling between the protein coordinate and the solute activation barrier is provided here, where the position of the Mg^{2+} ion determines the transition state energy.

While the aforementioned problems with regard to the evaluation of activation barriers might not occur in some cases, the related problems associated with calculations of binding free energies are much more serious. That is, as demonstrated in Figure 6, calculations of absolute ground state energies and thus also binding energies by QM/MM minimization are extremely problematic.[39] Here one expects to obtain very different results in different local minima. Obviously the only way to obtain reasonable binding energies is to perform very extensive averaging of the type performed in QM/MM redox calculations.[40]

The present test case involves the attack of a water molecule on a hypothetical metaphosphate intermediate in the Ras·GAP complex. This intermediate has been proposed recently in the QM/MM energy minimization study of Grigorenko et al.[45] As stated in the Introduction, we did not examine yet the proposal of a very low barrier for the dissociative formation of the metaphosphate, but we find it to be rather unlikely. Furthermore, the idea that a concerted H_2O−Gln61 attack on the metaphosphate as the rate-limiting step is problematic. For example, this concerted reaction cannot occur in elongation factor Tu (EF-Tu), which has histidine instead of Gln61, but this system hydrolyzes GTP quite effectively.[47] Thus we believe that, despite the intriguing insight provided by the studies of Grigorenko et al.,[45] it is crucial to reexamine the corresponding conclusions by a well-calibrated approach (with careful attention to more complete sampling). It would also be crucial to examine the corresponding result in related systems such as Ras alone, as was done in EVB studies.[43]

It is also useful to point out that energy minimization studies might miss the contribution from the protein reorganization energy. That is, the approximately linear response of the protein to the movement along the reaction coordinate might not be captured correctly.

Finally, perhaps the simplest message that emerges from this study is the idea that QM/MM minimization and scanning approaches should involve averaging (and validation) over several protein configurations generated by long MD simulations. This idea has been found to be very useful for theoretical IR spectroscopy of the ground state of GTP in Ras[41,42] and in statistical mechanically more rigorous treatments such as FEP calculations of electrostatic energies[13] and in EVB calculations of enzymatic reactions.[43,44]

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