Supporting Information for:

Mechanism of Cdc25B phosphatase with the small molecule substrate *p*-nitrophenyl phosphate from QM/MM-MFEP calculations

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Model Calculations

Table S1: Experimental P-O_{lg} Distances (in Å) from the Cambridge Structural Database [1, 2, 3]

CSD ID	P-O_{lg} Distance
HIQXEK	1.61
VICNIR	1.61
XEQNIR	1.64

Experimental distances (Table S1) between P and the leaving group O atom (P-O_{lg}) for the *p*-nitrophenyl phosphate (pNPP) monoanion are significantly shorter than the P-O_{lg} distances in the reactant (1.69 Å) and intermediate state (1.73 Å) obtained in this work. Thus, we were interested in assessing the accuracy of the B3LYP/6-31G(d) method and determining whether the computed P-O distances were an artifact of the method and/or basis set. We performed optimizations of the phenyl phosphate monoanion using a series of basis sets with both the B3LYP functional and MP2 calculation. The P-O_{lg} distances are shown in Table S2. From the Table, it is evident that the convergence with basis set is rapid with B3LYP. For MP2, the convergence is slightly more erratic, but both methods are consistent with each other, even with the relatively small 6-31G(d) basis set.

Table S2: Calculated P-O_{lg} Distance for the Phenyl Phosphate Monoanion

	6-31G(d)	6-31G(d,p)	6-31+G(d,p)	6-31++G(d,p)	6-311++G(d,p)
B3LYP	1.722	1.717	1.717	1.717	1.717
MP2	1.722	1.718	1.719	1.718	1.713

Care must be taken when comparing semi-infinite crystal structures that have neutral charge to computed structures of charged, isolated molecules. A more reasonable means of comparison would be to neutralize the charge in the calculation by including a counterion in the calculation or by simply adding a proton to the phosphate. When a Na^+ counterion is included in a B3LYP/6-31G(d) optimization of the phenyl phosphate monoanion, the P-O_{lg} distance decreases to 1.645 Å, and when the phosphate is neutralized with an additional proton, the P-O_{lg} distance is 1.620 Å.

Thus, while the P-O_{lg} bond in the pNPP monoanion appears somewhat long in our simulations when compared to experimental values, this is not because of the B3LYP functional (or the basis set). A larger basis set would improve the results slightly, but the overall conclusions are not expected to change if another method and/or basis set were used. The lengthening of the P-O_{lg} bond may be due to insufficient stabilization of the negative charge in the active site and/or binding of a transition-state-like structure to facilitate dephosphorylation.

Cdc25 system preparation

The structure of the Δ25B1 catalytic domain (residues 374-551) of Cdc25B phosphatase co-crystallized with sulfate was obtained from the Protein Data Bank (PDB ID 1qb0). The MolProbity server [4] was used to add hydrogens to the protein and assess the assignment of sidechain rotamers. Protonation states were further investigated with the H++ server.[5, 6, 7] In this approach, the Poisson–Boltzmann equation is solved within a continuum dielectric representation of the solvent to compute the pK_a values of ionizable protein sidechains and assign protonation states. An internal dielectric of 6.0, external dielectric of 80.0 and ionic strength of 0.15 M were used.

The H++ server predicted that Cys473, Glu474 and Glu478 were all anionic. Based on histidine protonation assignments from MolProbity and H++, we tested a few different histidine protonation state combinations. MD simulations (∼2 ns) were performed on the solvated protein and the RMSD relative to the crystal structure was monitored. The system was then equilibrated at 300 K and 1 bar while harmonic restraints were gradually reduced. The combination of protonation states yielding the lowest RMSD was then used for further study. The histidine assignments provided by MolProbity yielded the lowest RMSD, so they were used for all subsequent simulations. All six histidine residues were modeled as having neutral charge. Hydrogens were placed at the ϵ-position for His375, His395, His519, and His533, and at the δ-position for His436, His472.

Substrate binding

To investigate the effect of substrate protonation state on binding, classical molecular dynamics (MD) simulations were performed on Cdc25B and two typical PTPases, low molecular weight PTPase from the yeast *S. cerevisiae* (LMW), and bovine heart phosphotyrosyl phosphatase (BPTP). For simplicity, the substrate used for these simulations was the phenyl phosphate dianion (PPH2). The phenyl phosphate monoanion (PPH1) was also used for Cdc25 only.

The crystal structures for LMW (PDB ID: 1d1p) and BPTP (PDB ID: 1pnt) were obtained from the Protein Data Bank and hydrogens were added with MolProbity. The active site Cys residues of each protein were modeled in the deprotonated form. Force field parameters for PPH1 and PPH2 were obtained from the Charmm22 parameter set [8] and the TIP3P water model was used for the solvent.[9] For each system, the phosphate group of phenyl phosphate was superimposed onto the analogous group of the existing substrate in the active site of the crystal structure.

Each system was solvated in a periodic box of at least 70 × 70 × 70. All bonds were constrained using the SHAKE algorithm.[10] The particle mesh Ewald method [11] was used for computing long-range electrostatic interactions. A multiple timestep algorithm was used in which the integration step sizes were 2 fs for short-range, 6 fs for medium-range, and 12 fs for long-range forces. The nonbond pairlist was updated every 24 fs. The temperature and pressure were maintained at 300 K and 1 bar, respectively, with a Berendsen thermostat and manostat.[12]

After conjugate gradient minimization, the solvent and all hydrogen atoms were equilibrated for 80-360 ps at 300 K with all other heavy atoms held fixed. Then, MD was performed for 360 ps with a 40.0 kcal mol^{-1}Å^{-2} harmonic restraint potential on all backbone and C_β atoms of the protein as well as P1, O2, O3, and O4 of phenyl phosphate and S_γ of the deprotonated Cys nucleophile. Next, an additional 360 ps of MD was performed with a 20.0 kcal mol^{-1}Å^{-2} harmonic restraint on the alpha carbon atoms of the protein and P1 of phenyl phosphate and S_γ of the Cys nucleophile. Finally, all restraints were removed and sampling was performed.

A simple and convenient measure of the degree of substrate binding is the distance between S_γ of the Cys nucleophile and the phosphorus atom of phenyl phosphate. This distance was monitored during the MD trajectory of each system once all restraints were removed. For LMW and BPTP, PPH2 remained stably bound in the active site for more than 2 ns. The P-S_γ distance was 3.996 +/- 0.097 Å for LMW and 3.948 +/- 0.212 Å for BPTP. For Cdc25B, PPH2 did not remain bound, but instead dissociated from the active site immediately upon removal of the harmonic restraints holding the substrate in the active site. However, the phenyl phosphate monoanion PPH1 did remain bound to Cdc25 for the full duration of the MD simulation. The binding orientation of the substrate was significantly different from the structure obtained from QM/MM-MFEP optization. This is likely because of errors in the force field parameters of PPH1, specifically the O-P-O-H dihedral terms that are not present in PPH2. We did not investigate this further because the substrate was represent quantum chemically in subsequent free

energy simulations. These simulations demonstrate that the typical PTPases are able to bind dianionic phosphate substrates, whereas Cdc25 is not. This finding may be due to the lack of a thiolate-stabilizing Ser/Thr residue immediately following the CX_5R motif in the active site of Cdc25.

QM/MM-MFEP procedure

The pNPP substrate was positioned into the active site by superimposing the phosphate onto the crystallographic sulfate and water molecules overlapping with the substrate were removed. After equilibration of the periodic system, a spherical system was constructed in which all water molecules less than 32 Å from the S_γ atom of Cys473 were retained and all others were discarded. In all subsequent simulations, atoms within 20 Å were allowed to move, and all others were held fixed. For the monoanion system, the QM subsystem consisted of the sidechains of Cys473 and Arg479, the monoanionic pNPP substrate and one water molecule. For the dianion system, the QM subsystem contained the sidechains of Cys473 and Arg479 and the dianionic pNPP substrate. As in previous simulations, the protein and solvent were represented by the Charmm22 force field [8] and the TIP3P water model.[9] The pseudobond method [13] was used to describe the interface between the QM and MM subsystems and all QM/MM calculations were performed with the program Sigma [14, 15, 16] interfaced with a modified version of Gaussian 03.[17] The B3LYP exchange–correlation functional [18, 19] and the 6-31G(d) basis set were used for all QM calculations, and the Merz-Singh-Kollman [20] scheme was used for ESP charge fitting. Loose convergence criteria in g03 was used for the geometry optimizations of the QM subsystems within each QM/MM-MFEP *cycle*, and stationary point optimizations were considered converged when the free energy change between consecutive cycles was less than 0.1 kcal mol^{-1}. A multiple time step algorithm was used in the MD simulations during the QM/MM-MFEP optimizations. Step sizes of 1 fs and 4 fs were used for short and medium range forces, respectively. The nonbonded pair list was updated every 20 fs. The temperature was maintained at 300 K using a Berendsen thermostat.[12] For each MD simulation in a given cycle, an initial equilibration period of 16 ps was followed by 64 ps of sampling. Nonbonded cutoffs of 8.0 and 65.0 Å were used for short and long range interactions, respectively.

QM/MM simulation

A common approach for computing enzyme reaction paths involves optimizing a chain-of-replicas, or discrete set of conformations that incrementally transform reactants to products. Often, reaction paths are explored on the potential energy surface of the system. Free energy simulation techniques such as free energy perturbation (FEP) or thermodynamic integration (TI) are often performed to include dynamic contributions from the MM subsystem. However, potential energy based methods are frequently encumbered by large numbers of local minima which may result in discontinuous reaction paths. It is also common to obtain different activation energies when different starting geometries are used for the reactants and products.[21, 22] This conformational bias may call into question the accuracy of the results if these differences are large.

In an effort to compute reaction free energies directly and circumvent the difficulties associated with optimization on potential energy surfaces and bias stemming from single starting conformations, we recently developed the QM/MM Minimum Free Energy Path (MFEP) method for simulating reactions in enzymes and in solution.[23, 24] In this approach, the QM subsystem is optimized in the environment of the fluctuating MM subsystem. A finite ensemble is generated via molecular dynamics simulations and the QM conformations are optimized on the free energy, or potential of mean force (PMF), surface. Once the QM subsystem is optimized, its geometry and charges are then used to obtain more accurate sampling for the MM subsystem. This process is then iterated until convergence is achieved.

QM/MM-MFEP method

In this section, we provide a brief review of the QM/MM-MFEP method. In our implementation, the (Helmholtz) free energy of the system is defined in terms of the QM conformation.

$$A(\mathbf{r}_{QM}) = -\frac{1}{\beta}\left[\int \exp(-\beta E(\mathbf{r}_{QM}, \mathbf{r}_{MM}))d\mathbf{r}_{MM}\right]$$

where $E(\mathbf{r}_{QM}, \mathbf{r}_{MM})$ is the total energy of a system expressed as a function of the coordinates of the QM and MM subsystems, $\mathbf{r}_{QM}$ and $\mathbf{r}_{MM}$, respectively. Often, the free energy difference between two QM conformations is determined by free energy perturbation (FEP) techniques.[25] In the QM PMF representation, the gradient of the PMF is simply the ensemble average of the QM gradient

$$\frac{\partial A(\mathbf{r}_{QM})}{\partial \mathbf{r}_{QM}} = \left\langle \frac{\partial E(\mathbf{r}_{QM}, \mathbf{r}_{MM})}{\partial \mathbf{r}_{QM}} \right\rangle_{E,\mathbf{r}_{MM}}$$

which can be obtained from molecular dynamics (MD) simulations of the MM atoms while the QM atoms remain fixed. To improve computational efficiency without significant loss of accuracy, the total energy of the system may be described in terms of an electrostatic potential (ESP) fitted charge approximation for the QM subsystem.[25] In other words, the energy of the QM subsystem in the presence of the MM electrostatic potential may be expressed as

$$\langle\psi|H_{eff}|\psi\rangle = E_1(\mathbf{r}_{QM}, \mathbf{r}_{MM}) + E^{ESP}_{QM/MM}(\mathbf{r}_{QM}, \mathbf{r}_{MM})$$

where H_{eff} is the effective QM Hamiltonian that includes the MM electrostatic potential as a set of external point charges, $E_1(\mathbf{r}_{QM}, \mathbf{r}_{MM})$ is the QM internal energy polarized by the MM electrostatic potential, and $E^{ESP}_{QM/MM}(\mathbf{r}_{QM}, \mathbf{r}_{MM})$ is the electrostatic interaction energy between the QM and MM subsystems.

In the QM/MM-MFEP method, we compute relative free energies between conformations within a finite, fixed-size ensemble. The PMF of a system at iteration n is then defined as

$$A^n(\mathbf{r}_{QM}) = A_{ref} - \frac{1}{\beta}\ln\left\{\frac{1}{N}\sum_{\tau=1}^{N}\exp\left\{-\beta\left[\tilde{E}\left(\mathbf{r}_{QM}, \mathbf{r}^n_{MM}(\tau)\right) - E_{ref}\left(\mathbf{r}^{n)}_{MM}(\tau)\right)\right]\right\}\right\}$$

where A_{ref} is the PMF corresponding to an initial reference QM conformation, β is $1/k_BT$, N is the number of molecular dynamics steps in the finite sampling window, $\tilde{E}\left(\mathbf{r}_{QM}, \mathbf{r}^{n)}_{MM}(\tau)\right)$ is the reference sampling energy used to represent a QM conformation in the mean-field environment of MM conformations at the nth iteration, and $E_{ref}\left(\mathbf{r}^n_{MM}(\tau)\right)$ is a reference total energy in which the MM conformation is fixed at the initial conformation.

The free energy gradient is then

$$\frac{\partial A^n}{\partial r_{QM}} = \sum_{\tau=1}^{N}\frac{\partial\tilde{E}(\mathbf{r}_{QM}, \mathbf{r}^n_{MM}(\tau)/\partial\mathbf{r}_{QM,i}\ \exp\left\{-\beta\left[\tilde{E}\left(\mathbf{r}_{QM}, \mathbf{r}^n_{MM}(\tau)\right)\right]\right\} - E_{ref}\left(\mathbf{r}^n_{MM}(\tau)\right)}{\sum_{\tau=1}^{N}\exp\left\{-\beta\left[\tilde{E}\left(\mathbf{r}_{QM}, \mathbf{r}^n_{MM}\right) - E_{ref}\left(\mathbf{r}^n_{MM}(\tau)\right)\right]\right\}}$$

Path optimization procedure

The QM/MM-MFEP process proceeds as follows. First initial structures for reactants and products are generated. The QM energy and ESP charges (in the presence of the MM environment) are then computed and a classical MD simulation is performed on the MM atoms with the QM subsystem held fixed. The positions of the MM atoms are recorded every few steps for use as a more accurate, delocalized representation of the MM electrostatic environment during subsequent QM geometry optimization steps. The MM trajectory is also recorded so that the QM/MM interaction energy averaged over the entire trajectory can be computed for each updated QM geometry. Once the MD trajectory is complete, the QM geometry optimization begins and proceeds until converged. This process is referred to as a *cycle*. Once a cycle has completed, the process repeats, but with an improved QM geometry and ESP charges. As a result, the MM sampling improves, which then results in more optimal QM geometries in subsequent steps. The use of a finite, fixed-size ensemble in the iterative, sequential QM/MM-MFEP

method enables the potential of mean force and its gradient to be precisely determined on a well-defined energy surface.

Once the endpoints have been obtained, an initial interpolated path smoothly connecting them is generated. The QM subsystems of the endpoints remain fixed while all other points are optimized using the quadratic string method (QSM)[26]. Once a reasonable transition state structure has been obtained from QSM, it may then be used to perform an optimization to a true first-order saddle point using an approach similar to the endpoint optimizations. The paths connecting the exact transition state structure to the reactants and products may then be reinterpolated and reoptimized to obtain the final free energy path.

Quadratic string method

An important aspect of reaction path calculations involves obtaining a smooth, continuous path connecting the relevant reactant and product structures and providing accurate transition states along the path. In this work we have used the Quadratic String Method (QSM)[26] to accomplish the task of optimizing the reaction paths. In the current implementation all atoms in the QM subsystem make up the collective reaction coordinate for the reaction, although this is not necessarily required. In fact, it is often advantageous to choose certain chemically relevant degrees of freedom to act as the reaction coordinate. Here, we make no assumptions about the nature of the reaction coordinate and simply allow the optimization to proceed.

The QSM procedure proceeds as follows: An initial linear interpolation is first generated. Then, the energy and gradient of each point are evaluated and the corresponding approximate Hessian matrices and trust radii are updated. The energy of each point is then minimized by integrating in the descent direction perpendicular to the path. The individual points are then redistributed using a cubic spline interpolation to enforce equal geometric spacing along the path. The process is iterated until the maximum projected gradient norm is less than a given tolerance, or the maximum number of iterations has been reached.

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