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Influence of key residues on the reaction mechanism of the cAMP-dependent protein kinase

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Abstract

The reaction mechanism of the catalytic phosphoryl transfer of cAMP-dependent protein kinase (cAPK) was investigated by semi-empirical AM1 molecular orbital computations of an active site model system derived from the crystal structure of the catalytic subunit of the enzyme. The activation barrier is calculated as 20.7 kcal mol^{-1} and the reaction itself to be exothermic by 12.2 kcal mol^{-1}. The active site residue Asp166, which was often proposed to act as a catalytic base, does not accept a proton in any of the reaction steps. Instead, the hydroxyl hydrogen of serine is shifted to the simultaneously transferred phosphate group of ATP. Although the calculated transition state geometry indicates an associative phosphoryl transfer, no concentration of negative charge is found. To study the influence of protein mutations on the reaction mechanism, we compared two-dimensional energy hypersurfaces of the protein kinase wild-type model and a corresponding mutant in which Asp166 was replaced by alanine. Surprisingly, they show similar energy profiles despite the experimentally known decrease of catalytic activity for corresponding mutants. Furthermore, a model structure was examined, where the charged NH_3 group of Lys168 was replaced by a neutral methyl group. The energetic hypersurface of this hypothetical mutant shows two possible pathways for phosphoryl transfer, which both require significantly higher activation energies than the other systems investigated, while the energetic stabilization of the reaction product is similar in all systems. As the position of the amino acid side chains and the substrate peptide is virtually unchanged in all model systems, our results suggest that the exchange of Asp166 by other amino acid is less important to the phosphoryl transfer itself, but crucial to maintain the configuration of the active site in vivo. The positively charged side chain of Lys168, however, is necessary to stabilize the intermediate reaction states, particularly the side chain of the substrate peptide.

Keywords: cAMP-dependent protein kinase; phosphoryl transfer; reaction mechanism; semi-empirical molecular orbital calculation

Most cellular processes are controlled by reversible phosphorylation of enzymes. The cyclic 3′,5′-adenosine monophosphate (cAMP)-dependent protein kinase, which is ubiquitously found in eukaryotic cells, modulates substrate peptides by phosphorylating serine and threonine residues (Hanks et al., 1988; Johnson et al., 1996). Like the vast majority of other members of the protein kinase family, it contains a DLK motif (Asp166–Leu167–Lys168) in the catalytic subunit (Hanks et al., 1988; Bossemeyer, 1995). Various X-ray structures of cAPK containing ATP and inhibitor peptides have shown how these residues are arranged in the active site (Shaltiel et al., 1998) (see Fig. 1). Asp166 was suggested to act as catalytic base during the phosphoryl transfer to the serine side chain of the substrate peptide (Johnson et al., 1996; Zhou & Adams, 1997), and similarly for the active site of phosphorylase kinase (Owen et al., 1995). Abstraction of the hydroxylic proton of the substrate's serine would produce a hydroxyl anion that is a much more powerful nucleophile than the hydroxyl group itself, thus promoting the attack on the terminal phosphorous atom of ATP. This suggestion is supported by experimental observations like the pH dependence of the second-order rate constant for kemptide phosphorylation (Yoon & Cook, 1987), the strong decrease of catalytic activity upon exchange of aspartate by alanine in yeast PKA (Gibbs & Zoller, 1991), and the presence of a hydrogen bond-like interaction toward the hydroxyl group of serine found in the crystal structure of the catalytic subunit complexed with a substrate protein (Madhusudan et al., 1994). In contrast to the situation in aqueous solution where this anion would be solvated, there are, however, no polar residues in the local enzymatic environment that are able to energetically stabilize the ionized serine. The large difference in the acidic strengths between the hydroxyl

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Abbreviations: ADP, adenosine diphosphate; AM1, Austin Model 1; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; cAPK, cAMP-dependent protein kinase; PM3, Parameterized Method 3; QM/MM, quantum mechanical/molecular mechanical.

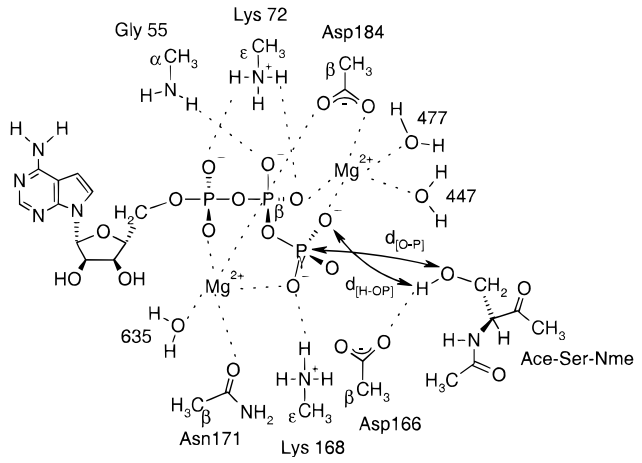

Fig. 1. Quantum mechanical model system used for the active site derived from the crystal structure of cAMP-dependent protein kinase.

(p$K_a \approx 13$) and the carboxyl (p$K_a = 3.9$) groups furthermore favors a neutral serine and a deprotonated carboxylate group at Asp166 (Zhou & Adams, 1997). On the other hand, these pK_a values represent bulk values and do not necessarily reflect their actual pK_a in the specific protein environment (Warshel & Aqvist, 1991; Frey, 1995).

An alternative role of aspartate was proposed by Hart et al. (1998, 1999) based on the results of hybrid quantum mechanical/molecular mechanical (QM/MM) calculations. They suggested that the carboxylate group is able to stabilize the product of the reaction, the protonated phosphoryl serine in the active site, while alternative reaction products are energetically unfavorable. It is, however, not clear if the function of aspartate is predominantly to promote the phosphoryl transfer, or to maintain the configuration of the active site, or to facilitate ligand binding (Hanks et al., 1988; Owen et al., 1995). Of further significance is Lys168. Its positively charged side chain is in contact with the γ-phosphate before and after the phosphoryl transfer as shown by X-ray crystallography (Madhusudan et al., 1994). It is supposed to compensate the negative charge on the phosphate and thus stabilizes the intermediate reaction states (Bossemeyer, 1995).

The aim of this study is therefore to investigate the reaction mechanism of the phosphoryl transfer with respect to alterations of key residues by using a model system for the active center. Our findings should be of general relevance for the mechanistic understanding of enzymatic phosphoryl transfer.

Results

Energetic optimization of the geometry of the active site model system shown in Figure 1 yielded a distance of 2.496 Å between the hydroxyl oxygen of the model substrate and the γ-phosphorous of ATP. This is about 0.2 Å shorter than predicted on the basis of crystal structures of various inhibitor, substrate, and product complexes (Madhusudan et al., 1994). In this reactant complex, the hydroxyl hydrogen is at 1.992 Å distance from the oxygen atom of the terminal phosphate group, which is coordinated to the magnesium ion that bridges P_β and P_γ. These two distances are referred to as $d_{[O\text{-}P]}$ and $d_{[H\text{-}OP]}$ further on. The closest distance between the hydroxyl hydrogen and the carboxylate oxygen atoms of Asp166 is 2.693 Å. Thus, the serine side chain of the model substrate can be regarded as being hydrogen bonded to the phosphate group of ATP rather than to the aspartate. The proposed function of Asp166 as catalytic base, however, would require the abstraction of this hydroxylic proton. The corresponding reaction product with a protonated Asp166 and a dianionic phosphorylated serine was calculated to be 10.3 kcal mol^{-1} higher in energy than the reactant complex, and requires an activation energy of 31.9 kcal mol^{-1}. Alternatively, the nucleophilic attack on the terminal phosphorous atom may be carried out by the neutral serine. This reaction is exothermic by -12.2 kcal mol^{-1} and leads to a phosphorylated serine monoanion, while the hydroxyl hydrogen is synchronously transferred to the oxygen of the γ-phosphate. The resulting product complex is shown in Figure 2.

The mechanism of the phosphoryl transfer was elucidated by a two-dimensional grid calculation that yields the potential energy surface as a function of the distances $d_{[O\text{-}P]}$ and $d_{[H\text{-}OP]}$ (see Fig. 3). The reaction path proceeds from the reactant complex (denoted as cricle) over the transition state (denoted as diamond) with an activation barrier of 20.7 kcal mol^{-1} toward the product (denoted as circle). The transition state was characterized by an imaginary frequency of $-1{,}173$ cm^{-1}, which predominantly involves the movement of the hydroxylic proton. The proton is moving constantly during the reaction, while the bond formation between P_γ and the serine oxygen occurs at a very late stage: at the transition state the corresponding distance $d_{[O\text{-}P]}$ is still 2.381 Å, whereas the proton has come within 1.132 Å of the phosphate oxygen. After the phosphoryl transfer, the distance between the oxygen of the aspartate and the shifted proton is 2.922 Å. During the reaction, the truncated amino acid side chains hardly move at all. The largest displacement (0.212 Å) was found at the transition state for the carboxylic oxygen of Asp166 closest to the shifted proton. All other displacements are negligibly small.

To investigate the function of the carboxylate group of Asp166 on the reaction mechanism, this group was replaced by CH_4, which corresponds to a mutation of aspartate to alanine. The two-dimensional reaction profile for this model system revealed surprisingly similar values for the activation barrier (21.5 kcal mol^{-1}), the stabilization of the product complex (-10.5 kcal mol^{-1}), and essentially the same energetic hypersurface (data not shown). The interatomic distances $d_{[H\text{-}OP]}$ and $d_{[O\text{-}P]}$ at the transition state

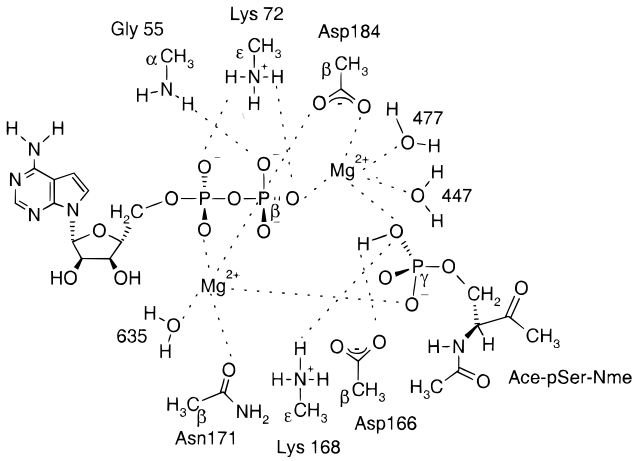

Fig. 2. Product complex of the phosphoryl transfer.

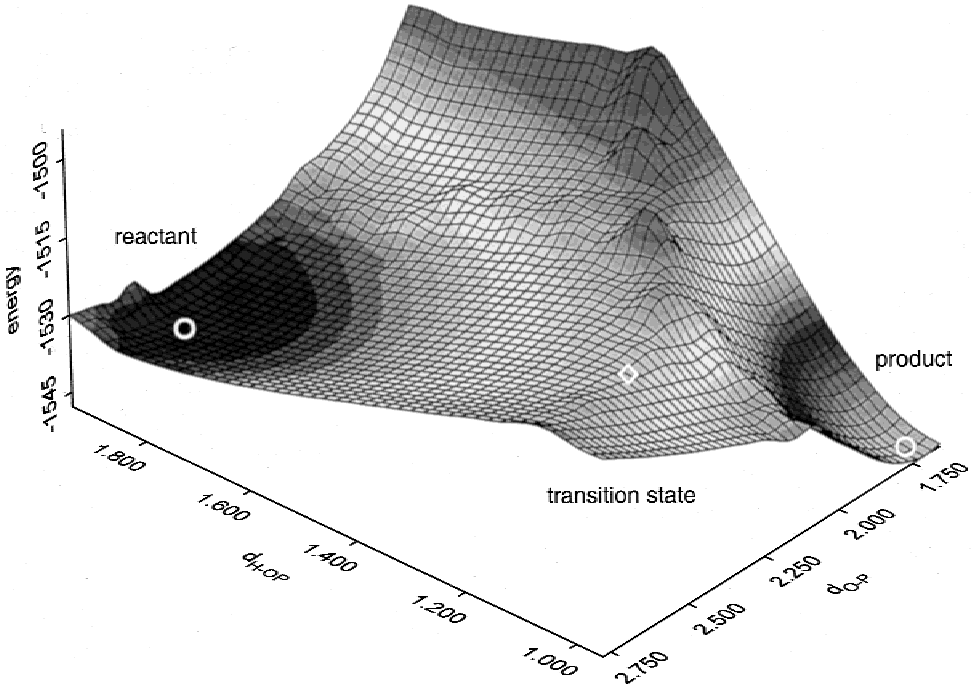

Fig. 3. Two-dimensional energy hypersurface of the phosphoryl transfer in the wild-type model of cAPK. The distances $d_{[O\text{-}P]}$ and $d_{[H\text{-}OP]}$ refer to the corresponding interatomic distances as shown in Figure 1. The energy is given in kcal mol^{-1} and the distances in Å.

are 1.206 and 2.239 Å, respectively. Comparison of the energetic terms that arise from the harmonic potentials on the carbon atoms of the amino acid fragments shows that the CH_4 fragment experiences a steric repulsion of around 0.3 kcal mol^{-1} in the region of the transition state. The corresponding potential experienced by the carboxylate fragment is only lowered by a similarly small value. Thus, the COO^- group does not significantly facilitate the phosphoryl transfer in our model system.

In the cAPK crystal structure, Asp166 is tightly coordinated by a salt bridge with Lys168. Together with Leu167 they form the highly conserved DLK motif of all kinases with specificity toward serine and threonine (Bossemeyer, 1995). The positively charged lysine side chain furthermore coordinates the negatively charged γ-phosphate before, during, and after phosphoryl transfer and may therefore stabilize intermediate reaction states (Bossemeyer, 1995). The effect of altering this charged side chain was studied by replacing it with an uncharged CH_3 group in our model system. The energetic hypersurface of this system shows two possible pathways for the phosphoryl transfer (Fig. 4). The reaction path, which is similar to the other model systems, has an activation barrier of 24.4 kcal mol^{-1}, while the alternate path involves an even higher barrier of 27.8 kcal mol^{-1} (transition state denoted as triangle). The exothermicity of the reaction itself (-10.2 kcal mol^{-1}), however, agrees well with all other model systems.

To obtain an estimate for the overall protein effect, we calculated the activation energy of phosphoryl transfer from ATP to the same Ace-Ser-Nme substrate when ATP was only complexed by two magnesium ions and five water molecules. This computation yielded an activation barrier of 25.8 kcal mol^{-1}. The product, which is analogous to the results of the active site models, is, however, 29.0 kcal mol^{-1} more stable than the reactant. During the reaction the coordination spheres of the two magnesium ions change to an approximately octahedral coordination by oxygen atoms in the product, while there are only five ligands in the cAPK crystal structure and in our model systems. The corresponding addition of a single water molecule to a magnesium penta aquo complex is exothermic by more than 20 kcal mol^{-1} in an AM1 calculation. Thus, the completion of the coordination sphere of magnesium can account for the stronger exothermicity of the phosphoryl transfer in the absence of a protein-like environment.

To quantitatively elucidate the charge distribution on the key residues and the transferred PO_3 group, the VESPA electrostatic potential derived atomic charges (Beck et al., 1997) were calculated for the reactant, the transition state, and the product structure of the wild-type model (see Table 1). The PO_3 group and the serine oxygen atom experience the largest changes during the reaction. The phosphate's negative charge decreases strongly from the reactant to the transition state, and further to the product, while the

Table 1. *Electrostatic potential derived atomic charges during the phosphoryl transfer in the wild-type model of cAPK*[a]

Fragment	Reactant	Transition state	Product
P_β-P_γ bridging O	−1.27	−1.25	−1.20
Transferred PO_3	−0.90	−0.65	−0.60
Transferred proton	0.32	0.45	0.42
Serine O	−0.28	−0.56	−0.72
Lys168 NH_3	0.78	0.83	0.82
Asp166 COO	−0.93	−0.95	−0.95

[a]Charges in e.

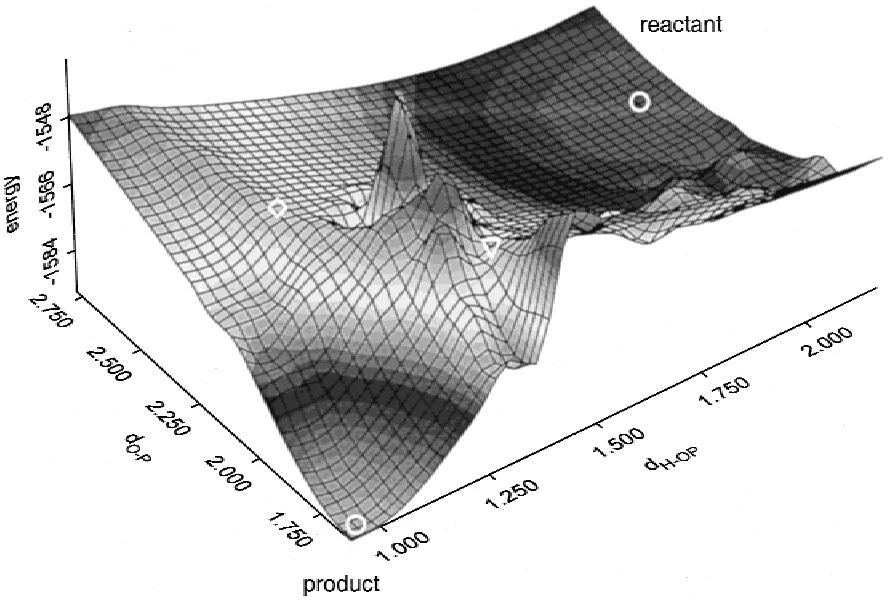

Fig. 4. Two-dimensional energy hypersurface of the phosphoryl transfer in the model system where the NH_3 group of Lys168 was replaced by a neutral methyl group (see text). Note the different orientation of the plot with respect to Figure 3.

negative charge on the serine oxygen atom steadily increases with the formation of the bond to the phosphorous. As the nearest hydrogen atom of Lys168 is in close distance to this oxygen atom (2.124 Å in the transition state), the positively charged NH_3 group compensates for this increasingly negative charge. The total charge of the NH_3 group of Lys168 and that of the carboxylic group of Asp166 show a contrary behavior. Although the charge on Lys168 increases by 0.05 e in the transition state, the charge on Asp166 decreases by 0.02 e. The atomic charge on the oxygen atom that bridges the β- and γ-phosphorous atom shows only a minor decrease by 0.07 e when the bond to the γ-phosphate is broken.

Discussion

Our calculations of the reaction mechanism show that the hydroxylic proton of the substrate serine is moved to the simultaneously transferred γ-phosphate and not to the carboxylate group of Asp166, which would lead to an energetically unfavorable serine phosphate dianion. Instead, the reaction product is the protonated serine phosphate monoanion, while the aspartate stays deprotonated. In agreement with this mechanism, Schweins et al. (1994) found on basis of EVB/free-energy perturbation methods for the Ras p21 protein, which converts guanosine triphosphate to guanosine diphosphate, that no protein residue acts as catalytic base, but the terminal phosphate acts as proton acceptor.

Hart et al. (1998, 1999) performed hybrid quantum mechanical/molecular mechanical (QM/MM) calculations on the same reaction in cAPK using the semi-empirical PM3 hamiltonian. Their quantum mechanically treated part of the system, however, was considerably smaller compared to the system used here, i.e., from the key residues only the truncated side chains of aspartate and lysine were present, and no water molecules were included. They also found a simultaneous transfer of phosphate and proton, but a much higher activation barrier (39 kcal mol^{-1}) and a stronger exothermicity (-24 kcal mol^{-1}) of the reaction. It is known that the PM3 hamiltonian leads to higher atomic charges compared to other semi-empirical methods (Clark, 1993), especially for hypervalent compounds, such as phosphorous. The larger energetic values obtained by PM3 thus are possibly due to a stronger polarity of the QM system, which leads to larger electrostatic interactions with the MM system formed by the surrounding protein. Hart et al.'s transition state is somewhat different from this work, as the γ-phosphorous is about 0.3 Å closer to the serine oxygen (2.07 Å), while the proton is about 0.1 Å further away from the oxygen atom of the γ-phosphate (1.22 Å). Consequently, a rather different imaginary frequency ($-2{,}064$ cm^{-1}) was obtained. Nevertheless, both studies propose the same mechanism of phosphoryl transfer.

The actual phosphoryl transfer step in vivo is preceded by the binding of MgATP and substrate peptide, and is followed by the release of the phosphorylated substrate peptide and ADP (Madhusudan et al., 1994). Thus, the catalytic activity measured experimentally comprises all these steps. Detailed values for the catalytic activity of systematic cAPK mutants are only available for the yeast enzyme (Gibbs & Zoller, 1991), which has 72% sequence homology to the mammalian cAPK used as structural basis for this study. The yeast enzyme is, however, considered as being structurally and functionally identical to cAPK and mutational effects are commonly adopted straightforward (Gibbs & Zoller, 1991). Mutations of single residues that lead to a very strong decrease in catalytic activity are the replacement of Asp210 (166 in the mammalian enzyme) by alanine and the exchange of lysine by alanine at position 212 (168 in the mammalian enzyme). Mutation of Asp210 goes along with a strong decrease in catalytic activity as well as reduced binding affinity for the substrate peptide (Gibbs & Zoller, 1991).

Considering these results, it was suggested that this residue is either crucial in catalysis and/or important in maintaining the configuration of the active site. In our model calculations we do not account for any significant structural alterations due to mutations. These calculations solely demonstrate electronic effects of

individual residues on the synchronous proton and phosphoryl transfer step. However, we find that the activation barrier for phosphoryl transfer increased only slightly, which rules out a crucial role of aspartate for the actual transfer step. Consequently, the observed 300-fold reduction of the catalytic rate by the D166A mutation must therefore have other reasons than the ones we investigated. For example, Asp166 may be important to structurally stabilize Lys168 and to pre-orient the reacting ATP and Ser residue. Also, the catalytic step may include structural rearrangements of the enzyme that are affected by mutating Asp166.

Experimentally, the replacement of this particular lysine by alanine at position 212 (168 in the mammalian enzyme) causes a similar strong decrease of activity as observed for the aspartate mutation, but reveals also reduced binding affinities for the substrate peptide as well as MgATP (Gibbs & Zoller, 1991). For the corresponding alteration in the model system, our results show a significant increase of the activation barrier (compared to the wild-type model) in combination with the possibility of an alternative reaction path, which, however, involves an even higher activation barrier.

Investigation of the atomic charges of the key residues in the wild type shows that the positively charged Lys168 compensates a distribution of negative charge on the nearby carboxylate group of Asp166 and the serine oxygen. Our results thus clarify the important role of Lys168 that stabilizes the reaction intermediates through favorable electrostatic interactions. The protein environment represented in our model lowers the activation barrier for phosphoryl transfer by $\sim$5 kcal mol^{-1}. About two-thirds can be attributed to Lys168. Although the transition state geometry agrees with an associative mechanism of phosphoryl transfer, we do not obtain a corresponding concentration of negative charge on the γ-phosphate group during the reaction that follows from a formal description of the reaction mechanism. This may be explained by the coupling of the phosphoryl transfer step with the simultaneous proton transfer.

Summarizing our results, the function of Asp166 as a catalytic base can be excluded in accordance with the results of Hart et al. (1998, 1999). Furthermore, we find that the negatively charged Asp166 is of minor importance for phosphoryl transfer, because an aliphatic residue does not significantly increase the activation barrier in our model system. On the other hand, we suggest that Lys168 is significantly involved in the actual reaction mechanism of the phosphoryl transfer in addition to its function of maintaining the configuration of the active site.

Materials and methods

The active site model system used for cAPK in this study as shown in Figure 1 contains ATP, the phosphoryl-accepting Ser, the two Mg^{2+} ions with full coordination shells, and six residues thought to be critical for phosphoryl transfer—a total of 123 atoms, all of which are treated fully quantum mechanically by the semiempirical AM1 method (Dewar et al., 1985). All calculations were performed using a modified version of the program package VAMP (Rauhut et al., 1997). For phosphorous, the existing parameters (Dewar & Jie, 1989) were used, while for magnesium the recently published parameter set (Hutter et al., 1998) was employed, as it provides substantially better reproduction of overall geometrical structures of five- and six-coordinated magnesium species compared to corresponding PM3 results. Test calculations on Mg^{2+} diphosphate compounds with the AM1 Hamiltonian (data not included) show good structural agreement with ab initio calculations (Saint-Martin et al., 1996).

Atomic coordinates of the nonhydrogen atoms in the model system were taken from the X-ray crystallographic structure (Zheng et al., 1993) of the complex of the catalytic subunit with ATP and the inhibitor PKI(5–24). The inhibitor was replaced by a model substrate (Ace-Ser-Nme) using the coordinates of the backbone and the C_β atoms (i.e., the alanine residue of PKI(5–24) at the phosphorylation site was mutated to serine). The crystallographic water molecules number 447, 477, and 635 were included to complete the coordination spheres of the two magnesium ions. Corresponding hydrogen atoms were added initially with bond lengths of 1.08 Å assuming the side chains of aspartate and lysine to be charged.

For the Asp166 to Ala mutant, the CH_3COO^- fragment modeling the aspartate residue was replaced by CH_4 at the position that corresponds to the C_β atom, thus altering the formal charge of the complete model system from 0 to +1. Another modification consisted of replacing the charged NH_3 group of Lys168 by a neutral methyl group, thus changing the formal charge of the model system to −1.

To emulate the structural effect of the protein backbone, the carbon atoms of the truncated side chains of the active site amino acids were harmonically restrained to their crystallographic positions. Thus, stationary points on the energetic hypersurface (minima and transition states) can still be characterized (Clark et al., 1997). Also, the "hardness" of each harmonic potential can be adjusted to suit the environment. To hold the ATP molecule in place in a protein-like fashion, such constraints were applied to its N-1, N-9, C-1′, and C-4′ atoms as they have low crystallographic *B*-factors, instead of adding further residues around the adenosine moiety that would have increased the number of atoms beyond computable scope. Similar restraints were also used on the included waters. For the oxygen atoms of the water molecules a force constant of 2,306 kcal mol^{-1} Å^{-2} was employed, while for the carbon atoms of the truncated amino acid side chains a "harder" potential of 115,302 kcal mol^{-1} Å^{-2} was used to account for the effect of protein backbone (Hutter et al., 1999).

The eigenvector following (Baker, 1986) (EF) algorithm was used throughout all calculations to optimize each of the model systems to a gradient norm below 0.4 kcal mol^{-1} Å^{-1}. For the two-dimensional grid calculations a step size of 0.05 Å was employed.

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