Quantum Chemical Study on the Catalytic Mechanism of the C-Subunit of cAMP-Dependent Protein Kinase

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Quantum chemical calculations were performed to clarify the catalytic mechanism of the catalytic subunit (C-subunit) of cAMP-dependent protein kinase (cAPK). The Schrödinger equation for the model reaction system was solved using the density functional theory (DFT). A Mg^{2+} ion resides at the active site and maintains a six-coordinated structure with a ternary complex, which consists of a C-subunit, Mg^{2+}−ATP, and a substrate (protein kinases). It was found that the phosphorylation of the substrate (protein kinases) by the C-subunit of cAPK was a one-step reaction and that the phosphorylated substrate was spontaneously released from the C-subunit. The activation energy required for this phosphorylation was estimated to be 36.23 kcal/mol. Hence, this reaction is expected to proceed at a body temperature of about 310 K. The final product was more stable than the initial reactant, and the energy difference between them was 10.85 kcal/mol.

1. Introduction

A main role of protein phosphorylation in a living cell is regulation of protein activity, in other words, control of information transmission. Phosphorylation was discovered in studies on glycogen metabolism.[1−3] In order to maintain homeostasis, hormones are released from organs and operated to the G-protein. The G-protein activates adenylatecyclase, which produces cAMP as a second messenger of hormones. Most of the messages carried by cAMP are transmitted by cAMP-dependent protein kinase (cAPK).[4] cAPK transmits these messages by transferring a γ-phosphate group from ATP to Ser [or Thr] of the target proteins. In the absence of an activating signal (cAMP), cAPK remains in an inactive state with the conformation of a holoenzyme, in which the active site of the catalytic subunit (C-subunit) is capped by the regulatory subunit (R-subunit). When the concentration of cAMP is elevated, four cAMPs are bound to the R-subunit, and two free active C-subunit monomers and one $R_2(cAMP)_4$ complex are released.[5] The C-subunit recognizes the amino acid sequence of −Arg−Arg−X−Ser[or Thr]−Y−, where X is any small residue and Y is a large hydrophobic group in the target proteins and induces phosphorylation of Ser [or Thr] residue of the target protein kinases.[6,7] Thus, protein kinases are activated by a phosphorylation reaction. This is a good example of information transmission in a living cell.

The C-subunit of cAPK has a simple structure (about 350 residues) compared to that of other protein kinases and can be easily purified experimentally, which has enabled extensive biochemical studies to be performed.[8,9] The three-dimensional (3D) structure has already been determined by X-ray crystallographic analysis.[10] There are many theoretical and experimental approaches to clarify the reaction of cAPK.[11−14] Although, the mechanism of the C-subunit of cAPK was a main target of the researches, it was not possible in experiments to directly observe the phosphate transfer step. Kinetic studies to measure the "catalytic rate" by Adams, Taylor, and their colleagues[15] always contained the time-lags because of conformational changes of the protein before and after the chemical reaction. Therefore, the atomic level reaction mechanism of the C-subunit is required to be clarified. In this study, the reaction process of the C-subunit of cAPK has been examined by performing theoretical computations, and the results would provide an atomic level insight into the understanding of information transmission for maintaining homeostasis in a living cell.

2. Methods

2.1. Molecular Mechanics (MM) Calculation. To clarify the catalytic mechanism of the C-subunit of cAPK, a model reaction system was constructed for the quantum chemical calculation. First of all, the structure of the ternary ES complex, consisting of the C-subunit of cAPK, Mg^{2+}−ATP, and protein kinases (substrate), was determined as follows. An X-ray crystallographic structure of the C-subunit of cAPK−$(Mn^{2+})_2$−ATP−the protein kinase inhibitor (PKI) complex (pdb code: 1ATP) was used as a basis geometry.[10] Because PKI had been produced by substituting Ala for Ser at the phosphorylated part of the substrate, PKI was changed into the normal substrate by replacing Ala with Ser. $(Mn^{2+})_2$−ATP is located at the active center of the ternary complex of 1ATP. Because Mg^{2+}−ATP is present at the active center of the ternary ES complex in a living cell,[16−18] Mg^{2+} was substituted for Mn^{2+} and was set to be coordinated with β- and γ-phosphoric acids. Another Mn^{2+} was removed from the model reaction system, because it was reported that Mg^{2+}, coordinated with α- and β-phosphoric acids, inhibited the phosphorylation.[19−21] Then, the enzyme was placed in a large box (71×55×53 Å) of TIP3P water molecules generated by the Monte Carlo method.[22] The number of water molecules is 4597. For the model, energy minimization using the molecular mechanics (MM) method was executed under periodic boundary conditions. In the computation of energy

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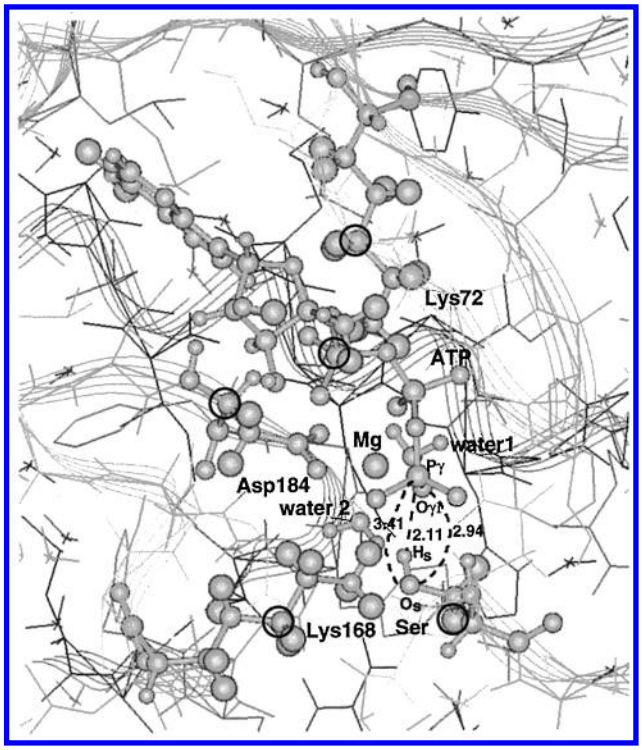

Figure 1. Energy-minimized structure obtained by molecular mechanics calculation. Lys72, Lys168, Asp184, waters 1 and 2, Mg^{2+}, ATP, and Ser of the substrate were close circles. In the model reaction system for MO calculations, the atoms indicated by open circles are replaced by H atoms.

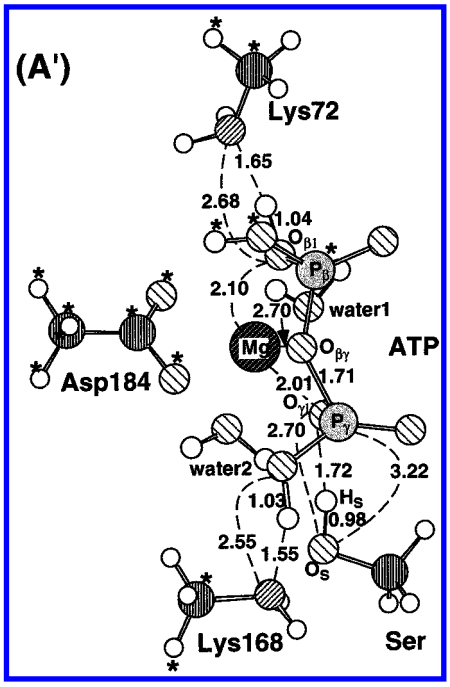

Figure 2. Structure of the ES complex obtained by ab initio MO calculation starting from the atomic configuration shown in Figure 1. ATP, Mg^{2+}, two waters, and amino acids (Lys72, Lys168, Asp184, and Ser) were extracted to compose the model system. Asterisked atoms are fixed through the calculation.

minimization, the steepest descent method was used for the first 1000 cycles, and the conjugate gradient method was used for next 1000 cycles. An all-atom force field[23] was applied to every amino acid. The SHAKE method[24] was used to simplify the calculation, in which every bond distance in the molecule was kept to an equilibrium distance. The minimized structure is shown in Figure 1. The program package used was Amber version 4.1.[25]

2.2. Molecular Orbital (MO) Calculation. The Schrödinger equation for the model reaction system was solved with the DFT method including the effect of electron correlation. Becke's three parameter functional[26] incorporating the LYP correlation term[27,28] was used in the DFT calculation. The minimum state and the transition state (TS) on the potential energy hypersurface were obtained by geometry optimization using the energy gradient method. Frequency analysis was executed for the optimized structure of TS, because the vibration of the molecular system in TS must contain only one imaginary frequency. The steepest descent path from TS was calculated for both the forward and reverse directions of the normal vibrational mode of the imaginary frequency, and the minimum points on both sides of TS were determined. The above procedure provided the lowest energy reaction path connecting a reactant and product via TS. The basis set used was 6-31G**. The program package used was Gaussian 98.[29]

Because of the limitation of the model size for the density functional theory (DFT) calculations, the active site must be represented by a model reaction system. To reproduce the atomic geometry involved in phosphorylation, Mg^{2+}, two water molecules, Lys72, Lys168, Asp184, ATP, and Ser (substrate) were extracted from the ternary ES complex obtained by MM calculations (see section 2.1). The main chains of the residues were terminated by a hydrogen (H) atom at the positions marked by open circle in Figure 1. As for ATP, the phosphate (P) atom of α-phosphoric acid was terminated by a H atom. Tsigelny et al. and Shaltiel et al. reported that Mg^{2+} was 6-fold coordinated, making bonds to two O atoms of Asp184, each O atom of β- and γ-phosphoric acids, and two water molecules.[30,31] Therefore, two water molecules were incorporated into this model reaction system. In this paper, we call these water molecules "water 1" and "water 2". To maintain 3D conformation of the ternary ES complex, the positions of carbon atoms of Lys72 and Lys168, Asp184, Mg^{2+}, P atom of β-phosphoric acid, the O atom originally located between the α- and β-phosphoric acids, and the terminating H atoms were fixed through the calculations. Some reports suggested a role of Asp166 to enhance the phosphorylation by increasing the affinity between a substrate and C-subunit.[14,31] However, other groups (Hart et al. and Hutter et al.) indicated that the proton transfer to Asp166 was energetically very unlikely[11−13] and concluded that a hydroxyl hydrogen was synchronously transferred to the oxygen of the γ-phosphate. So our model reaction system does not contain Asp166.

3. Results

3.1. Structure of the ES Complex. In the energy minimized structure by MM calculations (Figure 1), the distances between the oxygen atom (O_S) of the OH group of Ser (substrate) and the phosphorus atom (P_γ) of γ-phosphoric acid, the hydrogen atom (H_S) of the OH group of Ser and the oxygen atom ($O_{\gamma 1}$) coordinated to Mg^{2+}, and O_S and $O_{\gamma 1}$ are 3.41, 2.11, and 2.94 Å, respectively. This suggests a presence of interactions between these atoms.

Geometry optimization of the model system by molecular orbital calculation gave the initial structure (A′) as shown in Figure 2. In (A′), the distances between O_S and P_γ, H_S and $O_{\gamma 1}$, and O_S and $O_{\gamma 1}$ are 3.22, 1.72, and 2.70 Å, respectively. These values are compatible with the results obtained by MM calculation and the other interatomic distances also show a high degree of compatibility. However, the position of protons in

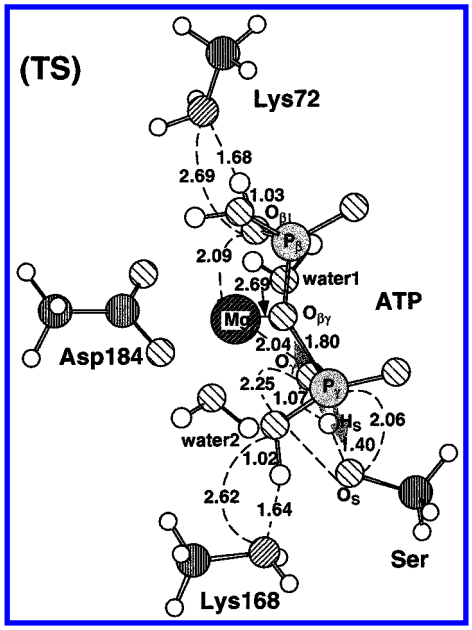

Figure 3. Optimized structure for the transition state (TS) in the reaction system. The arrows indicate the direction of the vibration mode with an imaginary frequency.

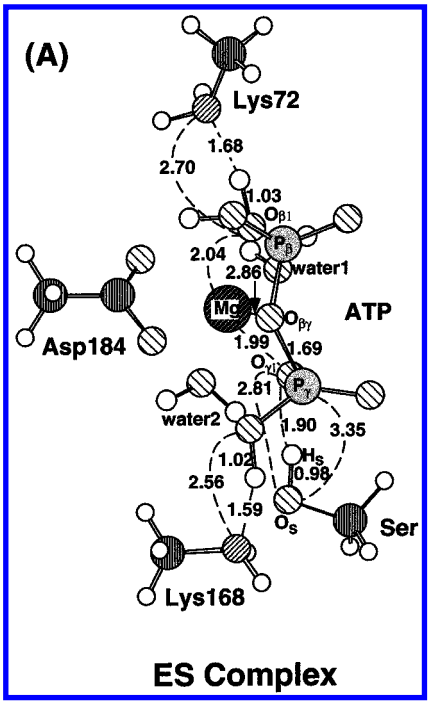

Figure 4. Stable structure (ES complex) obtained at the last point of the steepest descent path for the reverse direction of the vibrational mode of TS (Figure 3). This structure is considered identical to that of the ES complex of Figure 2.

amino group of Lys72 and Lys168 were different between Figures 1 and 2. This means the proton transfer from Lys72 and Lys168 residues to β- and γ-phosphoric acids. To confirm the occurrence of this proton transfer, two kind of model reaction systems based on MM calculation in Figure 1 were constructed; (1) amino groups of two residues of lysines (Lys72 and Lys168) are protonated and (2) amino groups of lysines are neutral and those β,γ-phosphoric acids are protonated. Furthermore, potential energies are compared between them. At each model, two types of computations with and without the surrounding effects using the SCRF method were executed. As a result, case 2 was about 20 kcal/mol more stable than case 1 in both types of computations. Therefore, it is confirmed that the model reaction system seen in Figure 2 is adequate for the configuration of the active site of the ternary ES complex.

3.2. Structure of the Transition State. The final product of phosphorylation by the C-subunit of cAPK should have a covalent bond between the P atom of phosphoric acid from ATP and the O atom of OH group in Ser. Hence, two routes for phosphorylation would be probable. (1) Ser−OH changes into Ser−O^-, and it attacks P atom of γ-phosphoric acid. (2) Ser−OH approaches the P atom, and at the same time, the O−H bond is breaking and the P−O bond is creating. Previous reports[11−13] indicated that the proton transfer to Asp166 was energetically unfavorable, and our present model does not include Asp166. Accordingly, there is no appropriate acceptor for the H atom of the OH group, and route 1 is impossible. In this study, we selected the route 2 as the probable reaction route. The TS was determined by approaching Ser−OH to the P atom of γ-phosphoric acid. The TS determined in this study is shown in Figure 3. This TS structure has only one vibration mode with an imaginary frequency. It should be emphasized that this TS structure was obtained through geometry optimization, only under the condition that the fixed atoms were the same as those in the model system shown in Figure 2. The arrows in Figure 3 show the direction for the vibration mode corresponding to an imaginary frequency, which indicates the pathway of the reaction, namely, a concerted antiparallel motion of H_s and P_γ. In TS, the distances between O_S and P_γ, between H_S and $O_{\gamma 1}$, and between O_S and H_S are 2.06, 1.07, and 1.40 Å, respectively. These values suggest that the OH group is breaking and H_S is transferring from the OH group of Ser to P_γ of γ-phosphoric acid. Hutter & Helms's AM1 calculation indicated that the distances between O_S and P_γ and between H_S and $O_{\gamma 1}$ at the TS are 2.239 and 1.206 Å.[13] These values are slightly longer than ours. It may be due to the difference of the calculation method, because semiempirical values tend to be longer.[32] The distance between the oxygen atom ($O_{\gamma 1}$) and P_γ is 1.80 Å, which is slightly longer than the value in the ES complex, 1.69 Å. Mg^{2+} is 6-fold-coordinated by two oxygen atoms of Asp184, two oxygen atoms of β- and γ-phosphoric acids ($O_{\beta 1}$ and $O_{\gamma 1}$), and two oxygen atoms of waters 1 and 2.

3.3. IRC Calculations. Next, the steepest descent paths were calculated for forward and reverse directions of the vibration mode shown in Figure 3, and this eventually provided the lowest potential energy path connecting the reactant and the product. As a consequence of the computation for the reverse direction, a stable structure (A), which is shown in Figure 4, was obtained. In this structure (A), the distances between O_S and P_γ, O_S and H_S, and $O_{\beta\gamma}$ and P_γ are 3.35, 0.98, and 1.69 Å, respectively. Therefore, this structure in Figure 4 was confirmed to be identical to the structure (A′) in Figure 2. This means that the optimized structure in Figure 3 is an appropriate TS leading from the initial reactant. The potential energy difference between ternary ES complex and TS is 36.23 kcal/mol.

Finally, the intrinsic reaction coordinate (IRC) calculation was performed for the forward direction following an imaginary vibration mode of TS. The potential energy decreased spontaneously, and the structure (B) shown in Figure 5 was obtained. In this structure, the direction between $O_{\beta\gamma}$ and P_γ is 3.56 Å, demonstrating that the phosphorylated substrate has separated from the C-subunit, and a new coordinate bond was produced between Mg^{2+} and $O_{\beta\gamma}$, instead of $O_{\gamma 1}$. This structure is a stable structure after the phosphorylation of Ser (substrate); therefore, this structure is the final product of phosphorylation. The final product is more stable than the ES complex by 10.85 kcal/mol.

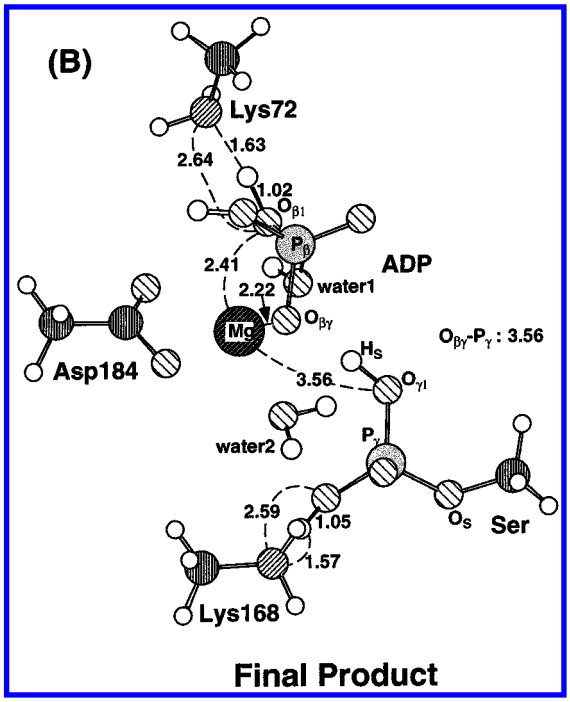

Figure 5. Stable structure (final product) obtained at the last point of the steepest descent path for the forward direction from TS (Figure 3). A coordinate bond was created between Mg^{2+} and $O_{\beta\gamma}$, whereas there was no bonding connection between Mg^{2+} and $O_{\gamma 1}$.

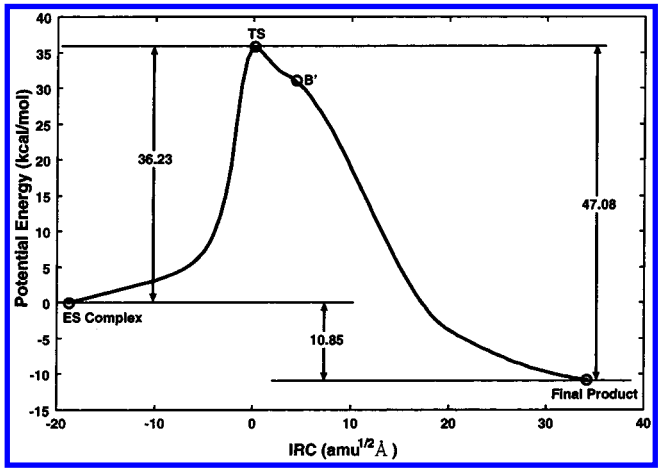

Figure 6. Potential energy change in phosphorylation reaction of Ser by cAPK. The abscissa represents the distance along the reaction coordinate (amu$^{1/2}$ Å), and the ordinate is the potential energy (kcal/mol).

4. Discussion

4.1. Role of ATP. Figure 6 shows the potential energy curve obtained by IRC calculation of the phosphorylation of Ser (substrate) by the C-subunit of cAPK. This phosphorylation reaction is a one-step reaction, and the final product is more stable than the ES complex by 10.85 kcal/mol. That is, the phosphorylation of Ser (substrate) by the C-subunit of cAPK is an exothermic reaction, releasing an energy of 10.85 kcal/mol. This result agrees with experimental findings that the hydrolysis of ATP yields ADP and P_i.[33] Although the reaction requires a much higher activation energy (36.23 kcal/mol), it is believed that the energy necessary for this reaction is supplied from other parts of a living cell. Because the enzyme usually functions at a temperature of around 310 K, a living cell would provide the energy required for this reaction; that is, the cell would act as a heat source for the energy. Semiempirical calculations using different model systems indicated that the activation and stabilization energies were +39 and −24 kcal/mol with PM3 and +21.5 and −10.5 kcal/mol with AM1.[13] These results mean that the main role of ATP is not to supply the energy necessary for the phosphorylation, which is conventionally called "an energy current",[33] but to determine the direction of the information transmission by acting as a trigger through phosphorylation.

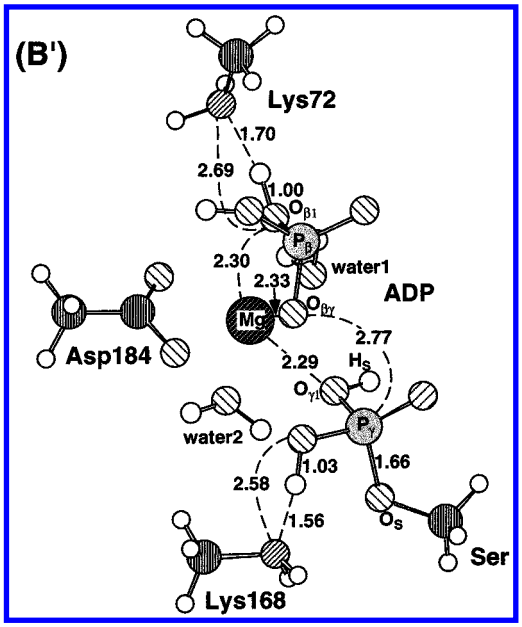

Figure 7. Structure B′, which appears after the TS for phosphorylation as indicated in the potential energy curve shown in Figure 6. A coordinate bond between Mg^{2+} and $O_{\beta\gamma}$ is created, whereas the coordinate bond between Mg^{2+} and $O_{\gamma 1}$ is breaking.

4.2. Coordination of Mg^{2+}. In the ES complex, Mg^{2+} is located at the center of the reaction system and is 6-fold coordinated by two O atoms of Asp184, two water molecules, $O_{\beta 1}$, and $O_{\gamma 1}$. In the final product, however, $O_{\beta\gamma}$ is substituted for $O_{\gamma 1}$ as a ligand. The results of IRC calculations in Figure 6 show that this replacement has no potential energy barriers and proceeds spontaneously. This coordination replacement occurs at a point (B′) in the curve of IRC calculation (TS → final product), at which the tendency of the curve is significantly changed. Figure 7 shows the structure (B′). In B′, the distances between $O_{\beta\gamma}$ and P_γ and between P_γ and O_S are 2.77 and 1.66 Å, respectively; that is, the phosphorylation of Ser (substrate) is completed. Mg^{2+} is coordinated by two oxygen atoms of Asp184, two water molecules (waters 1 and 2), and $O_{\beta 1}$ through this reaction. However, the distance between Mg^{2+} and $O_{\gamma 1}$ increases (ES complex: 1.99 Å → TS: 2.03 Å → B′: 2.33 Å), whereas the distance between Mg^{2+} and $O_{\beta\gamma}$ decreases (ES complex: 2.86 Å → TS: 2.69 Å → B′: 2.29 Å). This means that the replacement of $O_{\gamma 1}$ with $O_{\beta\gamma}$ occurs around the point B′ and that the phosphorylated substrate is releasing from the C-subunit. Our previous study reported that Mg^{2+} was still connected to $O_{\gamma 1}$ after phosphorylation and that Mg^{2+} became 5-fold coordinated with $O_{\beta\gamma}$, provided that Mg^{2+} was 4-fold coordinated without two water molecules.[34] These results strongly suggest that water molecules coordinated to Mg^{2+} are necessary to release the phosphorylated Ser (substrate) from the C-subunit.

4.3. Effects of the Surroundings. Because our model reaction system hardly contained effects of the surrounding factors, further computations indicating the surrounding effects have been executed using the SCRF (Onsager model) and two-layer ONIOM method (Table 1). In the SCRF method, single-

TABLE 1: Potential Energy Difference Including Surrounding Effects[a]

method	ES complex	TS	final product
no	0.00	36.23	−10.25
SCRF	0.00	45.40	−2.28
+Asp166	0.00	19.94	−13.38
ONIOM	0.00	18.72	−14.87

[a] "no", "SCRF", and "ONIOM" mean no surrounding effect, SCRF (Onsager model), and two-layer ONIOM method, respectively. "+Asp166" in SCRF method means that Asp166 is added to the model reaction system.

point calculations are performed at every stationary point on the condition that dielectric constant of 20.00 was applied. As a result, the potential energy (PE) differences calculated between ES and TS and between ES and the final product are 45.40 and 2.28 kcal/mol respectively. The reason the energy difference between ES and TS was increased would be that ES was slightly stabilized by the SCRF method relative to TS and final product. These values are compatible with those obtained without the surrounding effects. This result is in agreement with our previous study that there was no significant effects to note between dielectric constant 1 and 4 or 20.[34] Furthermore, SCRF calculations were also perfomed at the model reaction system including Asp166 in every stationary point. These calculations resulted in the lowering of the potential energy difference between ES and TS (Table 1). This result is consistent with the ONIOM method including surrounding effect directly.

In the two-layer ONIOM method, we constructed an ONIOM model system, using the energy minimized structure obtained by MM calculations (Figure 1). All of the residues less than 6.0 Å from Mg^{2+} (Phe54, Lys72, Asp166, Lys168, Asn171, Asp184, Phe185, Gly186, Phe187, ATP, and Ser of the substrate) and four water molecules were included. In every stationary point, the atoms appearing in the model of Figure 2 were assigned for the high-layer of ONIOM method and kept fixed during calculations. The rests were assigned for the low-layer and optimized during calculations. As a result, the PE differences calculated between ES and TS and between ES and final product are 18.72 and 14.87 kcal/mol respectively. Because the ONIOM method largely effects the semiemprical term, the energy profile would become similar to Hutter's results[13] performed with the semiempirical calculation.

5. Conclusions

Quantum chemical calculations were performed to clarify the catalytic mechanism of the C-subunit of cAMP-dependent protein kinase, and the following conclusions were provided.

1. The Phosphorylation reaction is an one-step reaction.

2. Through the phosphorylation, Mg^{2+} locates at the center of the reaction site and is 6-fold-coordinated. Only when Mg^{2+} is in the 6-fold coordination, does the release of the phosphorylated substrate occurs.

3. The activation energy required is 36.23 kcal/mol. At a body temperature of around 310 K, this phosphorylation is expected to proceed in a living cell.

4. The role of ATP is not to supply activation energy as "an energy current" but to determine the direction of information transmission through phosphorylation of a substrate.

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