**Structural Basis for Adenylate Kinase Activity in ABC ATPases**

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ATP-binding cassette (ABC) enzymes are involved in diverse biological processes ranging from transmembrane transport to chromosome cohesion and DNA repair. They typically use ATP hydrolysis to conduct energy-dependent biological reactions. However, the cystic fibrosis transmembrane conductance regulator and the DNA repair protein Rad50 can also catalyze the adenylate kinase reaction (ATP + AMP $\rightarrow$ 2ADP). To clarify and provide a mechanistic basis for the adenylate kinase activity of ABC enzymes, we report the crystal structure of the nucleotide-binding domain of the *Pyrococcus furiosus* structural maintenance of chromosome protein (pfSMCnbd) in complex with the adenylate kinase inhibitor P$_1$P$_5$-di(adenosine-5$'$)pentaphosphate. We show that pfSMCnbd possesses reverse adenylate kinase activity. Our results suggest that in adenylate kinase reactions, ATP binds to its canonical binding site while AMP binds to the Q-loop glutamine and a hydration water of the Mg$^{2+}$ ion. Furthermore, mutational analysis indicates that adenylate kinase reaction occurs in the engaged pfSMCnbd dimer and requires the Signature motif for phosphate transfer. Our results explain how ATP hydrolysis and adenylate kinase reactions can be catalyzed by the same functional motifs within the structural framework of ABC enzymes. Thus, adenylate kinase activity is likely to be a latent activity in many ABC enzymes.

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**Introduction**

ATP-binding cassette (ABC) enzymes are a large and versatile family of ATPases that conduct diverse biological processes.$^1$–$^4$ Most prominently, the membrane spanning ABC transporters utilize ATP to transport a large variety of substrates across biological membranes. ABC transporters are composed of two ABC-type nucleotide-binding domains (NBDs) that bind to two transmembrane domains.$^5$–$^6$ In the presence of ATP, the two NBDs engage into a tight head-to-tail dimer, promoted by binding of two ATP molecules to two composite ATP-binding sites in the NBD dimer interface.$^7$–$^8$ In the closed ATP-bound conformation, Walker A motifs bind the $\alpha$-, $\beta$-, and $\gamma$-phosphates from one side, while the Signature motifs of the opposing NBDs bind the $\gamma$-phosphates from the opposite side. This $\gamma$-phosphate-dependent conformational engagement and disengagement is thought to be part of the functional power stroke of ABC transporters.$^9$

Besides the membrane-bound ABC transporters, there is a large variety of nuclear and cytoplasmic ABC enzymes. Among these, the structural maintenance of chromosome (SMC) protein family is involved in sister chromatid cohesion, chromosome condensation, and genome maintenance.$^{10}$–$^{11}$ SMC proteins consist of an ABC-type NBD and a dimerization domain, both connected by a long coiled-coil linker. Two SMC proteins assemble into homodimers (in prokaryotes) or heterodimers (in eukaryotes) via their dimerization domains.$^{12}$ Two NBDs in the SMC protein dimer engage in the presence of ATP to form a tight NBD:2ATP:NBD sandwich, similar to that observed for the NBDs
from the ABC transporter. Together with additional subunits, ATP-binding-induced engagement of the two NBDs in the SMC protein dimer is required for chromosome association in vivo, possibly by facilitating the formation of large proteinaceous rings.

However, the sole dependence on ATP hydrolysis of all ABC enzymes is currently challenged by the analyses of the ABC transporter CFTR (cystic fibrosis transmembrane regulator) and the DNA repair enzyme Rad50. CFTR forms a transmembrane channel that regulates the passive flow of anions down an electrochemical gradient. Channel opening is linked to ATP-dependent engagement of its two NBDs. Although CFTR can be regulated by ATP hydrolysis in vitro, it can be also regulated by the reversible adenylate kinase reaction (Mg$^{2+}$·ATP + AMP $\rightleftharpoons$ Mg$^{2+}$·ADP + AMP). The adenylate kinase and reverse adenylate kinase reactions release virtually no free energy under physiological nucleotide conditions, consistent with the passive ion gating function of CFTR. Which of the two ATP-dependent reactions is utilized by CFTR in its physiological context is currently debated.

Recently, the ABC ATPase Rad50 involved in DNA repair has been shown to catalyze a similar adenylate kinase reaction in vitro.

The presence of the two distinct ATP hydrolysis and adenylate kinase activities within the ABC enzyme family is puzzling, and the molecular mechanism for adenylate kinase activity remains unclear. To help clarify this issue for this central and important enzyme family, we biochemically analyzed the adenylate kinase activity of the NBD of the SMC protein from Pyrococcus furiosus (pfSMC$^{\text{nbd}}$) and determined the crystal structure of pfSMC$^{\text{nbd}}$ in complex with an inhibitor P1,P5-di(adenosine-5$'$)-pentaphosphate (Ap5A). The R. furiosus SMC protein for adenylate kinase activity is hereinafter) binds to the ATP site, while the other adenine (now denoted adenosine 2) binds to the ADP/ATP-binding site.

To further examine the adenylate kinase activity of pfSMC$^{\text{nbd}}$, we used the adenylate kinase inhibitor Ap5A. Indeed, Ap5A inhibited the reverse adenylate kinase activity of pfSMC$^{\text{nbd}}$ but had no substantial effect on the ATPase reaction (Fig. 1c), similar to what was reported for Rad50. These findings suggest that SMC proteins preferentially conduct ATP hydrolysis. However, our data show that the ability to catalyze the adenylate kinase reaction is not restricted to CFTR and Rad50 but may exist in other ABC enzymes. This observation raises two important questions. Why are two distinct biochemical activities observable within the same ABC enzymes and how are these two types of activities mechanistically related? Thus, the mechanistic characterization of adenylate kinase activity in ABC enzymes is important not only from an evolutionary point of view but also to better understand the mechanism of CFTR and Rad50.

**Crystal structure of pfSMC$^{\text{nbd}}$ in complex with Ap5A**

To reveal the structural mechanism of adenylate kinase activity of ABC enzymes, we crystallized pfSMC$^{\text{nbd}}$ in complex with Mg$^{2+}$ and Ap5A (for nomenclature, see Fig. 1d). Mg$^{2+}$-Ap5A is a bi-substrate inhibitor of adenylate kinases that blocks two sites: one adenosine (denoted adenosine 1 hereinafter) binds to the ATP site, while the other adenosine (now denoted adenosine 2) binds to the AMP site.

Crystals of the Mg$^{2+}$-Ap5A-SMC$^{\text{nbd}}$ complex diffracted to 1.6 Å resolution (Table 1). Molecular replacement and refinement yielded clear electron density for a whole Mg$^{2+}$:Ap5A:SMC$^{\text{nbd}}$ moiety in one of the two molecules in the asymmetric unit. A simulated annealing omit map countered at 2σ for Ap5A, Mg$^{2+}$, and four important water molecules calculated from the final model is shown in Fig. 2a.

**The ADP/ATP-binding site**

Ap5A binds to pfSMC$^{\text{nbd}}$ in an “S”-like conformation (Fig. 2a–c). The two adenosine moieties are attached to two ~15 Å separated binding sites. Mg$^{2+}$, adenosine 1, plus α-, β-, and γ-phosphates bind to the canonical Mg$^{2+}$-ATP-binding site on Lobe I of pfSMC$^{\text{nbd}}$. Adenine 1 is recognized by three specific hydrogen bonds to the main chain of residues 65, 67, and 69 (Fig. 2b). The α- and β-phosphates are bound to the P-loop (Walker A motif) and among
SMC proteins conserved “arginine finger” (R59)\(^{15}\) (Fig. 2b). The \(\beta\) - and \(\gamma\)-phosphates form two coordination ligands of the active-site Mg\(^{2+}\) ion, with the coordination sphere of Mg\(^{2+}\) completed by the side-chain oxygen of Q145 (Q-loop), the side chain of S40 (Walker A motif), and two water molecules (Fig. 2b). The arrangement of Mg\(^{2+}\), adenosine 1, and \(\alpha\)-, \(\beta\)-, and \(\gamma\)-phosphates of Ap5A is virtually identical with that of ATP in the Mg\(^{2+}\)-ATP-pfSMC\(^{nbd}\) complex\(^{15}\) (Fig. 3a). The structural data are in agreement with the biochemical evidence that Ap5A blocks the ATP-binding site in the asymmetric unit, crystal packing prevents the Ap5A from adopting the same bound conformation. The stacking interaction with the Q145 carboxamide moiety is similar to the interaction of adenine 1 with the K13–S14 peptide bond. Furthermore, N7 nitrogen of adenosine 2 forms a hydrogen bond to a Mg\(^{2+}\) water ligand (W1). Finally, the adenosine N6 amine is specifically recognized via hydrogen bonds to the same water of the Mg\(^{2+}\) coordination sphere (W1) and a second water (W2) interacting with a water (W3) bound by the main-chain carbonyl of Q145 and N1 nitrogen (Fig. 2b). Together, these hydrogen-bonding interactions could explain the observation that GMP cannot substitute for AMP in the CFTR adenylate kinase reactions, as previously tested, and argue for a fairly specific adenine recognition\(^{22,25}\).

To biochemically verify the importance of this interaction, we mutated Q145 to alanine and tested the reverse adenylate kinase activity of pfSMC\(^{nbd}\). As expected, Q145A substantially affected the reverse adenylate kinase activity, ATP\(^{\text{ase}}\) activity is not affected. (d) Chemical structure of the bi-substrate adenylate kinase inhibitor Ap5A with the nomenclature used in this study. A1 denotes adenosine 1 and A2 denotes adenosine 2.

**The AMP/ADP-binding site**

Remarkably, in one of the two molecules in the asymmetric unit, adenosine 2 stacks onto the side chain of Gln145 of the Q-loop at the interface of Lobe I and Lobe II (Fig. 2b). In the second pfSMC\(^{nbd}\) in the asymmetric unit, crystal packing prevents the Ap5A from adopting the same bound conformation. The stacking interaction with the Q145 carboxamide moiety is similar to the interaction of adenine 1 with the K13–S14 peptide bond. Furthermore, N7 nitrogen of adenosine 2 forms a hydrogen bond to a Mg\(^{2+}\) water ligand (W1). Finally, the adenosine N6 amine is specifically recognized via hydrogen bonds to the same water of the Mg\(^{2+}\) coordination sphere (W1) and a second water (W2) interacting with a water (W3) bound by the main-chain carbonyl of Q145 and N1 nitrogen (Fig. 2b). Together, these hydrogen-bonding interactions could explain the observation that GMP cannot substitute for AMP in the CFTR adenylate kinase reactions, as previously tested, and argue for a fairly specific adenine recognition\(^{22,25}\).

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Table 1. Crystallographic data collection and model refinement statistics

<table>
<thead>
<tr>
<th>Data collection</th>
<th>Beamline 14-2 (European Synchrotron Radiation Facility)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength</td>
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<tr>
<td>Space group</td>
<td>P2₁</td>
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<tr>
<td>Unit cell parameters</td>
<td>65.0, 86.1, 70.1, 90, 90, 90</td>
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<tr>
<td>a, b, c (Å)</td>
<td>Resolution (Å)</td>
</tr>
<tr>
<td>α, β, γ (°)</td>
<td>Rmerge</td>
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<tr>
<td>Rfree</td>
<td>50.0–1.6</td>
</tr>
<tr>
<td>I/α</td>
<td>16.3 (3.8)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>95.7 (83.0)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>3.7 (3.2)</td>
</tr>
</tbody>
</table>

Refinement

| No. of reflections (test) | 84,044 (4271) |
| Refinement factor (Å²)    | 1.138         |
| Bond lengths (Å)          | 0.007         |
| Bond angles (°)           | 1.138         |
| Ramachandran plot (%)     | 94.7          |
| Additional allowed        | 4.7           |
| Generously allowed        | 0.6           |
| Disallowed                | 0             |
| PDB accession code        | 3KTA          |

Activity (Fig. 1b). However, activity was partially restored at higher concentrations of ADP, suggesting that Q145 is involved in substrate binding, as suggested by the structural analysis, and not direct catalysis.

The δ- and ε-phosphates of Ap5A are not recognized by pSMC<sup>nbd</sup>, and ribose 2 is bound by the C-terminal tail of pSMC<sup>nbd</sup> (Figs. 2c and 3a). Since this tail is not conserved among ABC enzymes, its involvement could be due to the crystal packing, and its position is typically occupied by the D-loop in the engaged state of the NBD dimer. To test whether adenylate kinase indeed occurs in the engaged state of the NBD dimer. To learn more about the relationship between adenylate kinase and ATP hydrolysis activities, we superimposed Ap5A- and ATP-bound pSMC<sup>nbd</sup> via Lobe I. ATP and adenosine 1 plus α-β-γ, and γ-phosphates of Ap5A overlap very well (Fig. 3a). There are also no significant differences in the conformation of the ATP-binding site of Ap5A- and ATP-bound conformations of pSMC<sup>nbd</sup>. This structural similarity indicates that the same type of ATP recognition is used in ATP-hydrolysis and adenylate-kinase-type reactions within the structural framework of ABC enzymes.

However, the superposition indicates that in the presence of Ap5A, Lobe I and II of both molecules in the asymmetric unit are rotated ~15° compared to their conformation in the presence of ATP (Fig. 3b). ATP binding does not induce such a reorientation of the two lobes compared to the apo pSMC<sup>nbd</sup>. The rotation could be induced by

Comparison of Ap5A-bound and ATP-bound pSMC<sup>nbd</sup> and implications for CFTR

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Fig. 2 (legend on previous page)
binding of AMP at the Q-loop glutamine. In fact, the conformation of the helix protruding from the Q-loop is markedly different between Ap5A- and ATP-bound conformations of pSMC (Fig. 3a). Hereby, subtle conformational changes of the main-chain geometry around the Q-loop between ATP- and Ap5A-bound forms could lead to the observed geometrical differences. If this is the case, AMP binding to CFTR could also help to orient Lobes I and II, a process that may follow or facilitate engagement of the NBDs in response to ATP binding. Since the transmembrane domains bind to the NBDs of ABC transporters near the Lobe I/Lobe II interface, both NBD engagement and interlobe rotations could contribute to channel gating in CFTR.

The location of the AMP-binding site in pSMC is not located near the canonical ATP-binding site or the proposed NBD1:NBD2 interface of CFTR. There is no structure available for NBD2 of CFTR, to which N1303 belongs. However, the structure of CFTR NBD1 has been determined and the equivalent N499 in NBD1 binds to the main chain and stabilizes the \( \beta \)-strand that follows the Q-loop. Sequence conservation of NBD1 and NBD2 in this region suggests that N1303 and N499 residues fulfill similar structural roles. Thus, N1303K would be expected to affect main-chain stability at the Q-loop, thereby perturbing the proposed AMP-binding site.

**Discussion**

We report here the first structural analysis of adenylate kinase activity in ABC enzymes. The presence of both adenylate-kinase- and ATP-hydrolysis-type reactions in ABC enzymes has puzzled and raised the question as to how these two types of biochemical reactions have evolved and how they are related within the same structural framework. The identification of adenylate kinase reactions in now three different ABC proteins (CFTR, Rad50, and SMC) indicates that this type of biochemical reaction is perhaps a widespread and inherent feature of many ABC-type enzymes.

How does the adenylate kinase reaction occur? The crystallographically observed conformation of Ap5A could resemble the substrate conformation of the reverse adenylate kinase reaction. Adenosine \( \alpha \) and \( \beta \)-phosphates are in a position to mimic the acceptor ADP, while adenosine 2 and \( \delta \) and \( \epsilon \)-phosphates are in a position to approximately mimic the donor ADP, although it is possible that this conformation slightly changes in an engaged NBD dimer. In particular, the \( \beta \)-phosphate oxygen is in a good position for collinear nucleophilic attack on the \( \delta \)-phosphate. The observed conformation of Ap5A is unlikely to resemble the substrate conformation of the forward adenylate kinase, because P\(_{\gamma}\) (AMP) in pSMC is not positioned for nucleophilic attack on P\(_{\gamma}\) (ATP). However, a simple rotation of the sugar-\( \epsilon \)-phosphate moiety around the N-glycosidic bond of adenosine 2 would position the \( \epsilon \)-phosphate oxygen at the expected place of the nucleophile (water W4 in Fig. 2a and b), in bonding distance to the Walker B glutamate. The opposing Signature motif, as deduced from its position in the ATP-bound engaged form of NBD dimers, would be ideally suited to facilitate adenylate kinase activity. In particular, the
Signature motif could bind to transferred phosphate and help to stabilize the transition state with its positive helix dipole charge. Such a role would explain not only why the mutation in the Signature motif disrupts ATP hydrolysis but also why it disrupts reverse adenylate kinase activity (Fig. 1b). Moreover, this model would mechanistically unify the function of the Signature motif in ATP hydrolysis and adenylate kinase activities of ABC enzymes. The conserved nature of the involved motifs in most if not all ABC enzymes now provides a molecular explanation for the puzzling phenomenon that two distinct enzymatic activities have been identified in several ABC enzyme family members. A plausible model that combines the structural and biochemical data for both ATP hydrolysis and adenylate kinase activity is shown in Fig. 4.

For all three ABC proteins able to catalyze the adenylate kinase reaction, the biological relevance of this activity is still under discussion. A clear dissection of the two functions, namely, ATPase and adenylate kinase activity, is hardly possible because both reactions use the same functional motifs. However, the adenylate kinase activity of CFTR and presumably Rad50 is suggested to be physiologically important by some experimental data, raising the question as to how a more robust adenylate kinase activity has evolved from ATP hydrolysis. Since Walker A and Signature motifs are shared between ATP-hydrolysis- and adenylate-kinase-type reactions, optimizing or specifically blocking the sites for AMP (adenylate kinase) or nucleophilic water (ATP hydrolysis) may determine whether adenylate kinase or ATP hydrolysis is preferred by the ABC enzyme. In particular, our model hints at how CFTR evolved out of an ATP-hydrolyzing ABC transporter. Additional loops as well as the precise primary sequence and geometry of Q- and D-loops could additionally strengthen AMP binding of CFTR, making it a much more efficient adenylate kinase than pSMC$_{nbd}$. For instance, CFTR possesses a loop around Lys411, which is suitably located to form a lid for AMP binding.

Fig. 4. Proposed reaction mechanism for adenylate kinase activity in ABC enzymes and its comparison to ATP hydrolysis. Part of the active site in the engaged NBD dimer with nucleotides, selected protein motifs, and the active-site magnesium (plus part of its coordination sphere) is schematically indicated. The proposed nucleophilic attacks of water or phosphate oxygens are indicated by arrows. In ATP hydrolysis (a), a water molecule is positioned and activated for nucleophilic attack by the Walker B of protomer 1 of the NBD dimer and D-loop of protomer 2. The Signature motif of protomer 2 binds the ATP$\gamma$-phosphate and likely stabilizes the transition state as well as promoting phosphate release. In the proposed adenylate kinase reaction, the adenine of AMP (or ADP in the reverse reaction) binds to the Q-loop Q145 and the magnesium coordination sphere (b). The Signature motif likely binds the transferred phosphate, a model that would unify its role in ATP-hydrolysis- and adenylate-kinase-type reactions in ABC enzymes. Parts of the model are speculative and need to be addressed in future studies.
Other ABC enzymes could also specifically block the AMP-binding site. For instance, in the translation-initiation- and ribosome-biogenesis-associated ABC enzyme ABCE1, a conserved tyrosine residue specifically blocks the proposed adenine binding surface of the Q-loop glutamines.30

In summary, we have identified the previously elusive AMP-binding site for adenylate kinase activity in ABC enzymes. While this binding site is perhaps suboptimal or even blocked for most ABC enzymes that utilize ATP hydrolysis, CFTR and other ABC enzymes with physiologically used adenylate kinase activity may have optimized AMP binding. Thus, it will be important to further analyze whether other ABC proteins also display or use adenylate kinase activity rather than ATP hydrolysis in vitro and in vivo.

Methods

Crystalization, data collection, phasing, and structure refinement

pfSMCnbd was expressed and purified as previously described.15 pfSMCnbd–Ap5A [9 mg/ml in 5 mM Tris–HCl (pH 8.0), 100 mM NaCl, 0.1 mM ethylenediaminetetraacetic acid, 2.5 mM Ap5A, and 25 mM MgCl2] was crystallized by sitting-drop vapor diffusion at 25 °C after mixing 2 μl protein solution with 2 μl precipitant solution [2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol (pH 6.6) and 16% (w/v) polyethylene glycol 3350]. Crystals were flash frozen in liquid nitrogen propane-1,3-diol (pH 6.6) and 16% (w/v) polyethylene glycol 400. Diffraction data were recorded at 100 K at ID14-2 (European Synchrotron Radiation Facility) and processed with XDS31 (Table 1). Phases were obtained by molecular replacement with Phaser,32 using pfSMCnbd–ATP [Protein Data Bank (PDB) ID: 1XEX] as a search model. An initial model was automatically built with ARP/wARP33 and completed by cycles of manual model building with Coot34 and positional, B-factor, and occupancy refinement with CNS35 and PHENIX.36 The coordinate, topology, and parameter files for the ligand Ap5A were obtained from the HIC-Up database.37 Refinement and model statistics are shown in Table 1. Structure figures were generated with PyMOL†.

Adenylate kinase activity assay

Protein (1 μM) was incubated with increasing concentrations of ADP in 50 mM Tris (pH 7.5), 100 mM NaCl, and 10 mM MgCl2 at 55 °C for 2 h. To determine the amount of produced ATP, we mixed 40 μl of the reaction mixture with 40 μl of the “ATP determination kit” reagent from Biaffin (Kassel, Germany). ATP generated in the reverse adenylate kinase reaction is used by firefly luciferase to oxidize D-luciferin. The resulting chemiluminescence was recorded with a Tecan infinite M1000 (Tecan Group Ltd., Männedorf, Switzerland) immediately after mixing.

For inhibitor studies, reverse adenylate kinase activity was assayed at a concentration of 1 μM wild-type protein in the presence of 2 mM ADP and increasing Ap5A concentrations (Sigma-Aldrich, Taufkirchen, Germany).

ATPase assay

Protein (1 μM) was incubated with 2 μM ATP for 2 h at 55 °C in the presence of increasing Ap5A concentration. Released phosphate was determined by mixing 40 μl of reaction with 110 μl BICMOL Green Reagent (Enzo Life Science, Lörrach, Germany). OD260 (optical density at 260 nm) was measured after 30 min incubation at room temperature using a Tecan infinite M1000.

Accession number

Coordinates and structure factors for the crystal structure of pfSMCnbd in complex with Ap5A have been deposited in the PDB with accession code 3KTA.

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References


†www.pymol.org