The CDK-activating Kinase (Cak1p) from Budding Yeast Has an Unusual ATP-binding Pocket*

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Cak1p is an essential protein kinase that phosphorylates and thereby activates the major cyclin-dependent kinase in budding yeast, Cdc28p. The sequence of Cak1p differs from other members of the protein kinase superfamily in several conserved regions. Cak1p lacks the highly conserved glycine loop motif (GXGXXG) that is found in the nucleotide binding fold of virtually all protein kinases and also lacks a number of conserved amino acids found at sites throughout the protein kinase core sequence. We have used kinetic and mutagenic analyses to investigate whether these sequence differences affect the nucleotide-binding properties of Cak1p. Although Cak1p differs dramatically from other protein kinases, it binds ATP with a reasonable affinity, with a $K_M$ of 4.8 $\mu$m. Mutations of the putative invariant lysine in Cak1p (Lys-31), homologous to a residue required for activity in virtually all protein kinases and that interacts with the ATP phosphates, moderately reduced the ability of Cak1p to bind ATP but did not dramatically affect the catalytic rate of the kinase. Similarly, Cak1p is insensitive to the ATP analog 5'-fluorosulfonylbenezoyladenosine, which inhibits most protein kinases through covalent modification of the invariant lysine. We found that Cak1p is tolerant of mutations within its glycine loop region. Remarkably, Cak1p remains functional even following truncation of its first 31 amino acids, including the glycine loop region and the invariant lysine. We conclude that the Cak1p nucleotide-binding pocket differs significantly from those of most other protein kinases and therefore might provide a specific target for an inhibitory drug.

Members of the protein kinase superfamily are related by several highly conserved amino acid motifs that make up the catalytic core (1, 2). The degree of sequence identity found among residues within this core is remarkable, and several of the motifs are considered to be essentially invariant. The threedimensional structures of these core regions are predicted to be fundamentally the same (3), and for the protein kinases that have been crystallized to date, this turns out to be the case (4–11).

One extremely well conserved protein kinase motif is the glycine loop. This motif, which contains the consensus sequence GXGXXG (where X is any amino acid), is located in subdomain I near the amino terminus of the kinase domain (12–14). The glycine residues provide flexibility and allow the loop to fold over the nucleotide, thereby excluding solvent from the active site and anchoring the ATP molecule. This interaction with the nucleotide occurs via the backbone amides of the loop and the $\beta$- and $\gamma$-phosphates of the ATP (13, 14). The importance of this motif has been demonstrated by mutagenic analysis of the cAMP-dependent protein kinase (PKA).1 Mutation of either of the first two glycine residues in the loop increased the $K_M$ for ATP by 10-fold and reduced the catalytic rate of the enzyme by severalfold, whereas mutation of the third glycine had only minor effects (14). Similarly, substitution of the second glycine of this motif in phosphorylase kinase reduced the $V_{max}$ for the enzyme by more than 30-fold, although the first glycine residue was less sensitive to substitution (15). The importance of this motif is further underscored by the fact that a mutation of the third glycine residue in the tyrosine kinase domain of the insulin receptor impairs protein kinase activity and has been implicated in one form of human diabetes (16).

A second highly conserved feature of protein kinases is the so-called invariant lysine residue, which is located 14–23 amino acid residues carboxyl-terminal to the glycine loop within the ATP-binding pocket. This residue interacts with the $\alpha$- and $\beta$-phosphates of ATP and is critical for the proper alignment of the triphosphate chain in the active site. This lysine has been shown to be required for protein kinase activity in both serine/threonine and tyrosine kinases (2) and is a standard site of mutation in the construction of catalytically inactive kinases. For example, mutation of this residue in PKA reduced the catalytic rate of the enzyme by 99.9%, whereas the $K_M$ for ATP was only moderately affected (17). Thus, the invariant lysine generally seems to function primarily in catalysis, rather than in nucleotide binding (17–19).

Due to the exceptional degree of sequence conservation among members of the protein kinase superfamily, enzymes that lack one or more conserved motifs are relatively unusual. One such protein kinase is Cak1p, the Cdk Activating Kinase from Saccharomyces cerevisiae. This essential protein is responsible for activating the major cyclin-dependent kinase in budding yeast, Cdc28p, by phosphorylating a conserved threonine residue (Thr-169) in the activation loop (20–22). This phosphorylation is absolutely required for the activity of Cdc28p in the yeast cell cycle (23–25), and the activity of Cak1p is likewise essential in vivo (21, 22). Cak1p, however, is only

1 The abbreviations used are: PKA, cAMP-dependent protein kinase; AMPPPN, 5'-adenylylimidodiphosphate; Cak1p, CDK-activating kinase; CDK, cyclin-dependent kinase; Me$_2$SO, dimethyl sulfoxide; DTT, dithiothreitol; FSBA, 5'-$p$-fluorosulfonylbenezoyladenosine; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis.

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distantly related to the identified CAK in higher eukaryotes, which is a multi-subunit complex consisting of a kinase subunit, p40MG15, a regulatory partner, cyclin H; and an assembly factor, MAT1 (26). In contrast, CAK1 functions as a monomer (21) and is only 20–25% identical to p40MG15, its closest relative in vertebrates.

Several lines of evidence indicate that CAK1 contains an unusual nucleotide binding region. Sequence alignment of CAK1 with other protein kinases shows that it does not contain a glycine loop motif near its amino terminus. We are aware of only two other protein kinases, Vps15p and Mik1, that lack a glycine motif in this region (27, 28). In addition, mutational analysis of CAK1 has shown that the “invariant lysine” residue (Lys-31) is not required for Cak1p function (9). Analysis of Cak1p has shown that the “invariant lysine” residue (Lys-31) is not required for Cak1p function (9).

In this paper, we characterize the nature of the nucleotide-binding pocket of CAK1 by kinetic and mutagenic analyses. We have determined the basic kinetic parameters for wild type CAK1 and for CAK1 containing substitutions at the invariant lysine residue and in the glycine loop region. We describe experiments that probe the role of the invariant lysine residue of CAK1 using the nucleotide analog FSBA. The conclusions drawn from these experiments indicate that the ATP-binding site of CAK1 is functionally diverged from other members of the protein kinase superfamily, especially with respect to the role of the invariant lysine residue. Unlike other protein kinases, CAK1 is relatively insensitive to substitutions in the glycine loop region.

EXPERIMENTAL PROCEDURES

Buffers—CAK buffer is composed of 50 mM Tris, pH 7.5, 15 mM MgCl2, 1 mg/ml ovalbumin, 10 mM DTT, 0.5% Tween 20, and 1× protease inhibitors. FSBA buffer is composed of 50 mM K+ Hepes, pH 7.5, 50 mM NaCl, 10 mM MgCl2, 1 mg/ml ovalbumin, 1× protease inhibitors. 1× protease inhibitors are composed of 10 μg/ml each of leupeptin, chymostatin, and pepstatin.

GST-CAK1 Mutants—QuikChange mutagenesis (Stratagene, La Jolla, CA) was performed to introduce mutations into the CAK1 sequence. The following primers were used to create the K31A and glycine loop mutants (only the sense primer is indicated (5′ to 3′)). Altered codons are underlined. K31A nucleotides 61–111 of CAK1 (underlined). QuikChange mutagenesis (Stratagene, La Jolla, CA) was performed using the template of an existing clone in YCp50 (kindly provided by Ann Sutton).

Expression and Purification of Proteins—Wild type and mutant GST-CAK1 proteins were expressed in Escherichia coli and purified as described previously for wild type GST-CAK1 (21). The typical yield was 100 μg of GST-Cak1p per liter of culture. The concentrations of purified GST-CAK1p were determined by comparing the intensity of the Coomassie-stained proteins on an SDS-PAGE gel to stained bovine serum albumin standards. Recombinant baculovirus containing budding yeast CAK1 was generated by inserting a BamHI-EcoRI fragment of GST-Cak1p (21) into transfer vector BacPAK8 (CLONTECH, Palo Alto, CA). The transfer vector was co-transfected into SF21 cells with BacPAK6 viral DNA (CLONTECH, Palo Alto, CA) according to the manufacturer’s instructions. The recombinant virus was plaque-purified in three consecutive rounds and amplified. 2.7 × 107 High Five Cells (Bacblue 22, Pall, Inc., IL) was transfected with a 10 ml Bio-Rad GS-250 PhosphorImager (Bio-Rad). Image units were converted to counts/min by exciting bands from a phosphorimaged gel and scintillation counting. Based on this data, a conversion factor was calculated to convert PhosphorImager units to counts/min. The PhosphorImager units were shown to be linear with increasing signal and exposure times. In all assays, unless otherwise noted, less than 10% of total substrate was phosphorylated.

K₉₅ Determinations—To determine the K₉₅ of baculovirus-produced Cak1p, 5 μl of enzyme substrate mix containing 4 mM CAk1p and 6 μM human CDK2 was mixed with 5 μl of ATP or GTP mix ranging in concentration from 1.25 μM to 1.25 mM with a specific activity of 10 μCi/μmol of nucleotide. To determine the K₉₅ of a CAk1p/ATP mix containing 6 μM CAk1p and 4 mM ATP, 0.5 μl of ATP mix was added to 5 μl of CDK2 mix ranging in concentration from 0.06 to 15 μM. Samples were prepared in CAK buffer and contained 150 mM NaCl. Reactions were incubated for 10 min at room temperature and terminated by the addition of 10 μl of 2× SDS-PAGE sample buffer. Phosphorylated CDK2 was separated from free counts by SDS-PAGE. Following PhosphorImager analysis, K₉₅ determinations were made by fitting the data sets to the Michaelis-Menten equation using the Kaleidograph program (Version 2.1.3, Abelbeck Software, Stable Isotope Lab, University of Michigan).

K₉₅ determinations using E. coli produced GST-CAk1p proteins (wild type and mutants) were performed as above with 360 nM GST-CAK1 in the enzyme substrate mix. For K₉₅ determinations, the concentrations of CDK2 were at least 5-fold in excess of the Km,CDK2 for each GST-CAK1 protein unless otherwise indicated. K₉₅ determinations for the K31A and Gly mutants contained 1 mM ATP.

Competition Assays Using ATP Analogs—To determine the ability of FSBA and ADP to inhibit Cak1p, 5 μl of 4 mM CAk1p and 3 μM CDK2 was added to 5 μl of 200 μM ATP, 0.5 μM μl γ-32P]ATP, 10 mM DTT, and FSBA or ADP ranging in concentration from 7.8 μM to 2 mM in 50 mM Tris, pH 7.5. reactions were incubated for 10 min at room temperature. Phosphorylated CDK2 was quantified as described above.

To measure the ability of ADP and AMPPNP to inhibit Cak1p competitively in buffer lacking Me2SO, 5 μl of a mixture containing CAk1p and CDK2 as described above was incubated with 5 μl containing 200 μM ATP, 0.5 μM μl γ-32P]ATP, and ADP, or AMPPNP ranging in concentration from 7.8 μM to 2 mM in 50 mM Tris, pH 7.5. Reactions were incubated for 10 min at room temperature. Phosphorylated CDK2 was quantified as described above.

FSBA Assays—To determine the sensitivity of CAk1p to FSBA, 2 μM CAk1p was incubated with 1 mM FSBA at room temperature in FSBA buffer. At each time point, 5 μl was added to 5 μl of CAK buffer containing 20 mM DTT to inactivate the FSBA, and the sample was then stored on ice. 5 μl of a mixture containing 300 μM ATP, 1.0 μM μl γ-32P]ATP, 200 ng of CDK2, 20 mM DTT in CAK buffer was added. Reactions were incubated at room temperature for 10 min and terminated with the addition of 15 μl of 2× SDS-PAGE sample buffer. Control samples contained FSBA buffer with 10% Me2SO. To measure the sensitivity of human CDK2-cyclin A to FSBA, a sample containing 455 nM CDK2-cyclin A was incubated with 1 mM FSBA as described above. At each time point, 5 μl of the mixture was added to CAK buffer containing 20 mM DTT to inactivate the FSBA. 5 μl of the stopped reaction was added to 5 μl of 1 mM ATP, 0.25 μM μl γ-32P]ATP, and 267 μg/ml histone H1. Reactions were incubated at room temperature for 10 min. Phosphorylated CDK2 was quantified as described above. ATPase Assays—To measure the rate of ATP hydrolysis by baculovirus-produced GST-CAk1p, 3.8 μg GST-CAk1p in 7.5 μl of CAK buffer was added to 7.5 μl of ATP mix containing 50 μM ATP, 2.5 μg/ml γ-32P]ATP in CAK buffer. At each time point, 1 μl of the assay was added to 4 μl of Stop buffer containing 50 mM Tris, pH 7.5, 20 mM EDTA, 10 mM DTT, 1 mM MgCl2, 1 mg/ml ovalbumin, 0.1% Tween, and 1× protease inhibitors. 1 μl of the terminated reaction was spotted onto a polyeth-

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3 P. Kaldis and M. J. Solomon, unpublished results.
yleneimine cellulose plate (Selecto Scientific, Norcross, GA), and ascending chromatography was performed for 2 h in 50 mM HCl. Plates were dried and chromatographed a second time under the same buffer conditions. Plates were then dried under a lamp and subjected to PhosphorImager analysis. Data are expressed as a percent of the total ATP hydrolyzed after subtracting background orthophosphate present in the [γ-32P]ATP mix.

To determine the effect of FSBA on ATPase activity, 5 μl of mix containing 2.9 μM baculovirus GST-Cak1p, 100 μM FSBA, 10 mM DTT in FSBA buffer or 4 μl of mix lacking DTT was preincubated for 30 min at room temperature. 1 μl of 50 mM DTT was then added to the reaction lacking DTT. 5 μl of ATP mix containing 100 μM ATP, 5 μCi/μl [γ-32P]ATP in FSBA buffer was added, and the reactions were incubated for 1 h at room temperature. Chromatography and quantitation were performed as above.

Thermal Stability—To determine the thermal stability of wild type and mutant GST-Cak1p, 700 nm GST-Cak1p in CAK buffer was incubated at a given temperature for 10 min and then stored on ice until assay. 5 μl of 1.5 μM CDK2, 300 μM ATP, 0.5 μCi/μl [γ-32P]ATP in CAK buffer was added, and the samples were incubated for 10 min at room temperature. CDK2 phosphorylation was analyzed as described above.

RESULTS

Determination of the Basic Kinetic Parameters for Cak1p—Because of the extensive differences between Cak1p and other protein kinases, particularly within the ATP-binding site, we decided to characterize the ability of Cak1p to utilize nucleotides (ATP and GTP) and protein substrate. As a substrate, we used the human cyclin-dependent kinase, CDK2, rather than the endogenous yeast CDK, Cdc28p. CDK2 can function in yeast in place of Cdc28p (23, 33, 34) and is thus a physiological Cak1p substrate. It is also widely used as a Cak1p substrate in vitro (20–22). Finally, unlike Cdc28p, CDK2 is soluble at the high concentrations necessary for the analyses reported here. To determine the K_M values for the nucleotides, we performed kinetic assays over a wide range of ATP or GTP concentrations at a fixed concentration of the CDK2 substrate equal to five times the K_M(CDK2) (~3.5 μM). The extent of CDK2 phosphorylation was quantified with a PhosphorImager, and the data were fit directly to the Michaelis-Menten equation (Fig. 1). The K_M(ATP) for wild type Cak1p was 4.8 μM. The K_M(GTP) for Cak1p was over 200-fold higher than the K_M(ATP), whereas the extrapolated V_max value was slightly lower (Fig. 1A). Similarly, we determined the K_M(CDK2) by performing assays at a saturating ATP concentration while varying the concentration of CDK2 over a broad range (Fig. 1B). The K_M(CDK2) was determined to be 0.7 μM. The V_max value was 56.8 pmol of phosphate transferred per min per μg of Cak1p, which corresponds to a kat value of 2.6 per min.

Insensitivity of Cak1p to FSBA—Sequence alignment of Cak1p with other protein kinases (20–22, 29) identified Lys-31 as the invariant lysine that is required for catalytic activity in Cak1p with other protein kinases (20–22, 29) identified Lys-31 as the invariant lysine that is required for catalytic activity in Cak1p. The role of this lysine in Cak1p may be the equivalent lysine in other protein kinases, resulting in either a reduced binding or reactivity to FSBA. We presume shown). Thus Lys-31 in Cak1p is positioned differently from the equivalent lysine in other protein kinases, resulting in either a reduced binding or reactivity to FSBA. We presume that Lys-31 also has an altered interaction with ATP.

It was possible that the insensitivity of Cak1p to FSBA was simply due to the inability of the enzyme to bind the nucleotide analog. To test this possibility, we performed competition assays using either DTT-inactivated FSBA, ADP, or ATP (Fig. 2B). Both ADP and FSBA competed with ATP. FSBA competed at least as well as ATP, indicating that its inability to inactivate Cak1p is not due to lack of binding to the ATP-binding pocket of Cak1p.

Cak1p Binding to ATP Analogs: ADP and AMPPNP—To examine further the nature of the ATP-binding pocket of Cak1p, we compared the ability of Cak1p and CDK2-cyclin A complexes to bind adenine derivatives. Competition assays were performed in which ADP and AMPPNP concentrations were varied while the concentration of ATP was held constant (Fig. 2, C and D). The abilities of both ADP and AMPPNP to compete for ATP binding were similar for the two enzymes. ADP competed more effectively than AMPPNP, which inhibited only 60% of the activity of either enzyme when present in 5-fold excess over ATP. By contrast, ADP competed with [γ-32P]ATP for binding to each enzyme nearly as well as unlabeled ATP itself. A 5-fold excess of ADP inhibited CDK2 by 80% and Cak1p by 60%. Therefore, although the two enzymes had
similar overall binding specificities for ATP, ADP, and AMP-PNP, Cak1p had slightly greater discrimination against ADP.

**Kinetic Analysis of Mutations at the Invariant Lysine of Cak1p—** The results of the above experiments indicated that the orientation and/or reactivity of the invariant lysine within the ATP-binding pocket of Cak1p was unusual. To examine the role of this residue in more detail, mutant Cak1p proteins containing conservative (K31R) and non-conservative (K31A) mutations of Lys-31 were expressed as glutathione S-transferase fusions in *E. coli*. We purified the mutant proteins and determined their kinetic parameters after assaying them over a wide range of ATP and CDK2 concentrations. The results of these experiments for wild type and mutant GST-Cak1p proteins are shown in Fig. 3A and Table I. The $K_{M,ATP}$ for wild type K31R and K31A GST-Cak1p were 5.2, 44.9, and 248.0 $\mu$M, respectively, indicating that mutations of the invariant lysine have a direct effect on nucleotide binding and that loss of this positive charge in the ATP-binding pocket reduces ATP binding by almost 98%. These mutants also displayed modest increases in $K_{M,CDK2}$ from 0.5 $\mu$M for wild type GST-Cak1p to 0.9 and 4.1 $\mu$M for K31R and K31A, respectively (Fig. 3B). The $k_{cat}$ values for the K31R (0.026 min$^{-1}$) and K31A (0.021 min$^{-1}$) GST-Cak1p mutants were between 91 and 113% of the wild type $k_{cat}$ (0.023 min$^{-1}$). (Because the CDK2 concentration in the assays of the K31A mutant was approximately equal to the $K_{M,CDK2}$, the true $V_{max}$ value of the K31A protein is about 2-fold higher than that indicated in Fig. 3A (see also Fig. 3B). ($k_{cat}$ values for the *E. coli*-produced GST-Cak1p proteins were significantly lower than those observed for the baculovirus-produced Cak1p presumably because only a small fraction of the molecules were catalytically active.) Thus, mutation of Lys-31 had little effect upon catalysis but had a significant effect on ATP binding.

**ATPase Activity of Cak1p—** The glycine loop of protein kinases creates a flexible flap that covers the nucleotide and excludes solvent from the active site (13). This protection from solvent is important for reducing the ATPase activity of the protein kinase, preventing transfer of the $\gamma$-phosphate of ATP to water. In PKA, mutation of these glycine residues to serine or alanine increases the ATPase rate by more than an order of magnitude, indicating that the presence of the glycine residues is critical for excluding water from the active site (14). Cak1p,

![Fig. 2. Cak1p is insensitive to FSBA. A, baculovirus-produced Cak1p (2 nM) (circles) or CDK2-cyclin A complexes (455 nM) (squares) were treated with (closed symbols) or without (open symbols) FSBA for various times. Cak1p activity was assayed by phosphorylation of CDK2, and CDK2-cyclin A activity was assayed by phosphorylation of histone H1. Data are expressed as the percentage of activity remaining after FSBA treatment. B, FSBA competes for the ATP pocket of baculovirus-produced Cak1p. The concentration of each competitor was varied, whereas the radiolabeled ATP concentration was fixed at 100 $\mu$M. Competition assays were performed as described under “Experimental Procedures.” C and D, comparison of nucleotide usage between baculovirus-produced Cak1p (C) and CDK2 (D). The concentration of each cold nucleotide was varied, and the radiolabeled ATP concentration was fixed at 100 $\mu$M.](http://www.jbc.org/doi/10.1074/jbc.M100313200/)

**Fig. 3. ATP and CDK2 utilization by the Cak1p Lys-31 mutants.** A, the concentration of ATP was varied at a fixed, saturating concentration of CDK2 (3 $\mu$M) in assays containing 360 nM bacterially produced GST-Cak1p. Note that although the concentration of CDK2 is 3–5 times the $K_{M,CDK2}$ for wild type and K31R Cak1p, it is only about equal to the $K_{M,CDK2}$ for the K31A mutant, resulting in a reduced reaction velocity. B, the concentration of CDK2 was varied at a saturating concentration of ATP (200 $\mu$M for wild type and K31R, 1 mM for K31A). The curves in each panel represent the calculated best fits to the Michaelis-Menten equation.
however, lacks an obvious glycine loop and therefore may be unable to exclude water from its ATP-binding site. We tested this possibility by measuring the ATPase rate of baculovirus-produced Cak1p in the absence of protein substrate and comparing it to the ATPase rates determined for other, more conventional, protein kinases. The ATPase rate for Cak1p was found to be 0.13 min\(^{-1}\) under standard assay conditions (Fig. 4), which is somewhat lower than the reported ATPase activity of wild type PKA (0.66 min\(^{-1}\)).

We determined the effect of FSBA on the ATPase activity in order to be sure that it was not due to the presence of a contaminating ATPase. Incubation of GST-Cak1p with FSBA did not decrease the ATPase activity in the assay (data not shown). As FSBA has been shown to inhibit the activity of ATPases (39–41), but not of Cak1p, this result is a strong indication that the ATPase activity in this assay is specific to Cak1p. The presence of any FSBA-insensitive ATPase contaminant would only strengthen the conclusion that Cak1p does not have an unusually high ATPase activity.

**Table I**

Comparison of the \(K_m\) and \(k_{cat}\) values of wild type and mutant Cak1p

| Enzyme   | \(K_m\)\(_{ATP}\) (\(\mu\)M) | \(K_m\)\(_{CDK2}\) (\(\mu\)M) | \(k_{cat}\) (min\(^{-1}\)) |
|----------|----------------------------|-----------------------------|---------------------------|
| Wild type| 5.2                        | 0.5                         | 0.023                     |
| K31R     | 44.9                       | 0.9                         | 0.026                     |
| Gly1     | 16.4                       | 1.9                         | 0.150                     |
| Gly2     | 54.9                       | 2.7                         | 0.143                     |
| Gly3     | 11.0                       | 1.9                         | 0.080                     |
| ΔGly     | 110.9                      | 3.9                         | 0.019                     |

The ATP-binding Pocket of Cak1p

**DISCUSSION**

Cak1p, the cyclin-dependent kinase-activating kinase from budding yeast, is an essential protein that is responsible for catalyzing the activating phosphorylation of Cdc28p. Cak1p is an unusual protein kinase whose primary amino acid sequence is diverged from members of the protein kinase superfamily. In this paper, we have examined the nature of the ATP-binding pocket of Cak1p by kinetic and mutagenic analysis. We show that even though Cak1p binds ATP with an affinity similar to that of other protein kinases, the sequence requirements for this binding are highly unusual.
Determination of Kinetic Parameters—The $K_{M, ATP}$ for Cak1p is 4.8 $\mu$M, which is somewhat lower than the reported $K_{M, ATP}$ values for other protein kinases. For example, the $K_{M, ATP}$ for p40MO15 and p34cdc2-cyclin B complexes are 40 (43) and 75 $\mu$M (44), respectively, and the $K_{M, ATP}$ for PKA, p38 mitogen-activated protein kinase, and pp60c-Src are 17, 23, and 80 $\mu$M, respectively (45–47). We believe that the low $K_{M, ATP}$ for Cak1p is a consequence of its low catalytic rate, as we have determined that the $K_{M, ATP}$ is approximately equal to the $K_d (ATP)$.

Cak1p can utilize GTP as a substrate, although its $K_{M, GTP}$ (1114 $\mu$M) is over 200-fold higher than the $K_{M, ATP}$. A few protein kinases, including p34 Cdc2, CDK2, and casein kinase, can also utilize GTP (35, 44). Cak1p and CDK2 (a more typical protein kinase) bind other nucleotides such as ADP and AMPPNP with similar specificities. Thus, Cak1p binds nucleotides relatively normally, despite its unusual sequence and binding requirements (see below).

Analysis of the Invariant Lysine of Cak1p—The role of the invariant lysine in other protein kinases is to orient the $\alpha$- and $\beta$-phosphates of ATP to promote the in-line phospho-transfer reaction (2). The kinetic analysis of Cak1p mutants containing substitutions at this residue shows that Lys-31 is involved primarily in nucleotide binding; its role in catalysis is minimal. The conservative mutant, K31R, displayed a 9-fold increase in $K_{M, ATP}$ and a 1.7-fold increase in $K_{M, CDK2}$. The K31A mutant, which removes a positive charge in the ATP-binding pocket, showed a 42-fold increase in $K_{M, ATP}$ over wild type and a 7.6-fold increase in $K_{M, CDK2}$. However, neither mutation significantly affected $V_{max}$.

In contrast, the equivalent mutation in other protein kinases primarily affects $k_{cat}$. For example, the K116A mutation of Tpk1p, a yeast homologue of PKA, results in an 800-fold decrease in $K_{M, ATP}$ and a 1.7-fold increase in $K_{M, CDK2}$. The K31A mutant, which removes a positive charge in the ATP-binding pocket, showed a 42-fold increase in $K_{M, ATP}$ over wild type and a 7.6-fold increase in $K_{M, CDK2}$. However, neither mutation significantly affected $V_{max}$.

In contrast, the equivalent mutation in other protein kinases primarily affects $k_{cat}$. For example, the K116A mutation of Tpk1p, a yeast homologue of PKA, results in an 800-fold decrease in $k_{cat}$ and only a 6-fold increase in $K_{M, ATP}$ (17). Similarly, the K52R and K52A mutations in the mitogen-activated protein kinase ERK2 result in a decrease in $k_{cat}$ to 0.5–5% of wild type (19). Most protein kinases containing substitutions at this residue are completely unable to function in vivo (see, for example, Refs. 48–53). In contrast, mutations of this residue in Cak1p are fully capable of complementing a CAK1 deletion in S. cerevisiae (22, 29). Our biochemical results can rationalize this complementation. Even though the $K_{M, ATP}$ for the K31A mutant (248 $\mu$M) is 50-fold higher than for wild type Cak1p, this value is still far below the physiological concentration of ATP (~2 mM) (42). Thus, the K31R and K31A mutants should...
be bound to ATP and fully active in vivo.

Further evidence for a distinct role of the invariant lysine in the ATP-binding pocket of Cak1p comes from its insensitivity to the nucleotide analog FSBA. FSBA covalently modifies and thereby inactivates a number of kinases including PKA, pyruvate kinase, p34\(^{cdk2}\), EGF receptor kinase, p60\(^{src}\), casein kinase II, calmodulin-dependent protein kinase II, and myosin light chain kinase (35–37, 48–59). FSBA inactivates many other kinases in vitro, presumably covalently (for example, see Refs. 43 and 60). FSBA competes with ATP for binding to Cak1p, indicating that Cak1p can bind the adenosine moiety of FSBA. However, whereas CDK2 is rapidly and completely inhibited by FSBA, Cak1p is virtually insensitive to treatment with the analog either in the presence or absence of protein substrate. Thus, Lys-31 may not be properly oriented or sufficiently reactive to allow modification by the sulfonyl fluoride group of FSBA. A less likely alternative possibility is that Lys-31 may be positioned in the ATP-binding pocket such that modification by FSBA does not inhibit catalysis. A final possibility is that another charged residue is located at the active site and can compensate for the function of the invariant lysine in Cak1p. Such a situation occurs in the protein kinase domain of the large subunit of herpes simplex virus type 2 ribonucleotide reductase (ICP10), where two lysine residues (Lys-176 and Lys-259) can perform the role of the invariant lysine (61). Mutation of either one of these lysines reduces the activity of the kinase by 80–90%, whereas a double mutation inactivates the kinase. FSBA binding is reduced in either single mutant of ICP10 but is not completely inhibited. The effect of FSBA binding on ICP10 protein kinase activity was not examined. There are no obvious candidates for such a second lysine in Cak1p.

The Glycine Loop Region of Cak1p—We have determined the ATPase rate for Cak1p. The sequence in Cak1p that aligns with the position of the glycine loop in other protein kinases contains several bulky amino acid side chains (IDTHCQ, see Fig. 5A). We thought that Cak1p might therefore be less able to exclude solvent from the ATP site, resulting in an increased ATPase activity. We found that the ATPase rate for Cak1p is 0.13 min\(^{-1}\). This value is somewhat less than the rate for p38 mitogen-activated protein kinase (0.36 min\(^{-1}\)) and the catalytic subunit of PKA (0.66 min\(^{-1}\)) (14, 38, 62). Therefore, the ATPase rate for Cak1p is not dramatically different from other protein kinases. This result is quite surprising, since substitution of the first or second glycines with serine in the conserved loop in PKA results in a 12-fold increase in ATPase rate, to 8.1 min\(^{-1}\), indicating that the glycines of this loop are required for the low intrinsic ATPase activity of PKA (14). Cak1p, by contrast, can exclude water from the active site in the absence of a glycine loop, presumably because the rest of the molecule has accommodated itself to the absence of this motif.

Analysis of the Amino Terminus of Cak1p: Creation of a Glycine Loop—We introduced a canonical glycine loop motif near the amino terminus of Cak1p in three contexts, anticipating that introduction of a glycine loop into this region might increase the affinity for ATP. However, for each of these mutants, the \(K_{\text{M, ATP}}\) and \(K_{\text{M, CDK2}}\) increased moderately, indicating that the affinity of the protein for its substrates decreased. The most dramatic effect, resulting from the replacement of two isoleucine residues and a cysteine residue with glycines, increased the \(K_{\text{M, ATP}}\) by 10-fold. This result might be explained by examination of the crystal structure of PKA, which indicates that the adenine ring is buried in a hydrophobic pocket containing a leucine located in \(\beta\)-strand 1, whereas the ribose ring associates with a conserved valine in \(\beta\)-strand 2 (63). The glycine loop links these two strands, and the entire structure acts as a lid that locks the nucleotide in place. Perhaps the isoleucine residues in Cak1p are important for binding to the adenosine portion of ATP in a similar manner.

Despite the absence of a glycine loop motif and the tolerance of Cak1p for mutations at the invariant lysine (Lys-31), it was remarkable that proteins completely deleted for one or both of these regions could function in vivo. The \(\Delta Gly\) mutant had only a 10-fold increase in \(K_{\text{M, ATP}}\) and a normal \(V_{\text{max}}\), whereas the \(\Delta 31\) mutant had no detectable activity in vitro. Both a normal glycine loop and the invariant lysine help to position the ATP phosphates for catalysis. Cak1p is a slow enzyme (\(k_{\text{cat}}\) \(\sim 2.6\) min\(^{-1}\)) with a rate-limiting catalytic step. Depending on exactly what chemical step is rate-limiting, it is possible that the less precise positioning of ATP in these mutants could have little effect on the overall rate of catalysis. The Cak1p mutants of the glycine loop (including the \(\Delta Gly\) mutant) and of Lys-31 still bind ATP reasonably well. The reduction in binding presumably reflects elimination of weaker interactions with the ATP phosphates. We are not aware of other protein kinases that retain activity after introduction of mutations comparable to \(\Delta Gly\) and \(\Delta 31\).

Each of the glycine loop mutants had a reduced thermostability compared with wild type Cak1p. The temperature sensitivity of the three mutants was similar, suggesting that the amino-terminal portion of Cak1p confers stability on the protein structure and that Cak1p cannot accommodate the flexibility induced by the introduction of glycine residues into this region. Interestingly, mutation of the first and second glycines to serine in the PKA glycine loop only increased the thermostability of the protein by 1–2 °C (14). This result further emphasizes that whereas other protein kinases may be able to compensate for the flexibility introduced by the glycine loop in this region, Cak1p may require a more anchored structure at its amino terminus.

In summary, Cak1p possesses an unusual ATP-binding motif. Despite the lack of a conserved nucleotide-binding sequence near its amino terminus, Cak1p binds ATP with reasonable affinity and effectively excludes water from the active site. The mechanism by which catalysis is mediated within the ATP-binding site of Cak1p may differ somewhat from that described for other protein kinases, since the invariant lysine appears to have an altered position and/or reactivity. The number of protein kinases with these unusual properties appears to be small, although only a limited number of kinases have been examined in depth. There may well be a number of structural solutions to the problem of excluding water from the active site. Elucidation of the crystal structure of Cak1p will provide a more detailed understanding of the structural differences that are responsible for the ability of Cak1p to function in the absence of conserved motifs and may indicate how general this mechanism is.

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