Dissection of the Nucleotide and Metal−Phosphate Binding Sites in cAMP-Dependent Protein Kinase†

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Received November 10, 1998; Revised Manuscript Received February 25, 1999

ABSTRACT: The catalytic (C) subunit of cAMP-dependent protein kinase (cAPK) is more stable by several criteria when it is part of a holoenzyme complex. By measuring the thermal stability of the free C subunit in the presence and absence of nucleotides and/or divalent metal ions, it was found that most of the stabilizing effects associated with the type I holoenzyme could be attributed to the nucleotide. The specific requirements for this enhanced stability were further dissected: Adenosine stabilized the C subunit up to 5 °C; however, divalent cations (i.e., Mg^{2+}, Ca^{2+}, and Mn^{2+}) do not increase heat stability in combination with adenosine and adenine (1). Divalent cations as well as ATP and ADP have no effect by themselves (2). The enhanced stability derived from both ATP and ADP requires divalent cations. MnATP (12 °C) shows a much stronger effect than CaATP (7 °C) and MgATP (5 °C) (3). In the holoenzyme complex or the protein kinase inhibitor/C subunit complex, metal/ATP is also required for enhanced stability; neither the RI or RII subunits nor PKI alone stabilize the C subunit significantly (4). For high thermal stability, the occupation of the second, low-affinity metal-binding site is necessary (5). From these results, we concluded that the adenine moiety works independently from the metal-binding sites, stabilizing the free C subunit by itself. When the β- and γ-phosphates are present, divalent metals are required for positioning these phosphates, and two metals are required to achieve thermostability comparable to adenosine alone. The complex containing two metals is the most stable. A comparison of several conformations of the C subunit derived from different crystal structures is given attributing open and closed forms of the C subunit to less and more thermostable enzymes, respectively.

The metal ion requirements for structure and function of protein kinases are complex, and the extent to which metal ions may contribute to regulation of kinase activity is still unclear. To transfer the γ-phosphate from ATP[1] to a protein substrate, all protein kinases require a metal ion. The serine/threonine-specific protein kinases require a MgATP complex while MnATP is the specific substrate of the tyrosine-specific protein kinases (*1*, *2*). At the same time, many protein kinases are also inhibited by excess metal ions so that active and inactive conformations can both exist in solution depending on the stoichiometry of metal binding (*3*). The role that metals play in catalysis and inhibition has been studied most thoroughly for the two protein kinases that were discovered first, phosphorylase kinase (*4*) and cAMP-dependent protein kinase (cAPK), using a variety of methods (*3*, *5*−*9*). However, the importance of the metal- and nucleotide-binding sites for the structure and function of protein kinases is difficult to dissect.

The catalytic subunit of cAPK phosphorylates Ser or Thr residues preceded by basic amino acids. The metal requirements for cAPK were first described in detail by Armstrong and Kaiser who showed that there are two Mg^{2+}-binding sites (*6*), one required for catalysis and one associated with inhibition. The crystal structures of the C subunit (*10*, *11*) confirmed NMR studies of the C subunit with inert complexes of ATP, from which Kaiser, Mildvan, and their coworkers concluded that the activating metal bridges the β− and γ-phosphates and involves an enzyme-ATP-metal bridge (*6*). The second inhibitory metal site was predicted to be an enzyme-metal-ATP bridge between the α- and γ-phosphate

† This work was supported by Grants He1818-2/1 and SFB 394 B4 from the Deutsche Forschungsgemeinschaft (F.W.H.) and by Grant GM19301 from the National Institutes of Health (S.S.T.).

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[1] Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); AMP, adenosine monophosphate; C, catalytic subunit of cAPK; cAMP, adenosine 3′-,5′-cyclic monophosphate; cAPK, cAMP-dependent protein kinase; DTT, dithioerythreitol; EDTA, *N*,*N*,*N*′,*N*′-ethylenediaminetetracetic acid; GTP, guanosine triphosphate; Me^{2+}, divalent metal ions; Mops, 4-(*N*-morpholino)propanesulfonic acid; MW, molecular weight; Pipes, 1,4-piperazinebis(ethansulfonic acid); PKI, heat stable protein kinase inhibitor; $PKI_{(5-24)}$, synthetic peptide covering amino acid 5−24 of PKI; PPi; sodium-pyrrhophosphate; R, regulatory subunit of cAPK; SDS, sodium dodecyl sulfate.

10.1021/bi982672w CCC: $18.00
Published on Web 04/20/1999

(*12*). Leonard and co-workers using *lin* benzo ADP established the requirement for metals for nucleotide binding using fluorescence displacement titration (*8*, *13*).

The crystal structure of cAPK provides a molecular framework for understanding the metal-binding sites in the catalytic (C) subunit of cAPK. The original crystals of a ternary complex of the mouse recombinant enzyme containing ATP and an inhibitor peptide, $PKI_{(5-24)}$, were grown under conditions where nucleotide was in excess of Mg^{2+} so that only a single site was occupied (*10*). By soaking the crystals in Mn^{2+}, two metal-binding sites could be unambiguously identified (*14*). A similar approach was used to define the metal-binding sites in crystals of the porcine C subunit crystallized in the presence of inhibitor peptide and AMP−PNP (*11*). These structures revealed a primary Mn^{2+}-binding site bridging the β- and γ-phosphates of ATP and interacting with an invariant aspartate, Asp184 (*11*, *14*). This site is predicted to be the binding site for the activating metal. A second metal-binding site, presumed to be at the inhibitory site, bridged the α- and γ-phosphates of ATP. This metal interacted with another conserved residue, Asn171, and also with Asp184.

Physiologically, cAPK not only requires MgATP for catalysis but also for inhibition. cAPK in the absence of cAMP is inhibited by forming a tight complex with regulatory (R) subunits type I and type II. A second class of inhibitors are the heat-stable protein kinase inhibitors [PKI (*15*)]. Inactivation by two of the physiological inhibitors of cAPK, the type I R subunit and PKI, has an absolute requirement for the presence of Me^{2+}/ATP to maintain the inactive complex (*16*, *17*). In contrast, high-affinity binding to the type II R subunit has no requirement for Me^{2+}/ATP. While MgATP binds with low affinity to the free C subunit with an equivalent K_m and K_d [10 μM (*8*)], it binds with a high affinity (60−100 nM) to the inhibitor complexes [RI subunit and PKI (*17*)]. All of these physiological inhibitors of the C subunit serve as competitive inhibitors with respect to protein substrates. Each contains a substrate-like sequence that binds to the active site of the enzyme. In the case of the type II R subunits, this site contains a Ser at the P-site, and the type II holoenzymes are autophosphorylated readily at this site (*18*). In contrast, PKI and the RI subunits have a pseudophosphorylation site with the P-site Ser being replaced with Gly or Ala (*19*, *20*) and require MgATP to form a stable complex (*21*).

Several approaches were used to evaluate the effect of metals and nucleotides on the stability and function of the catalytic subunit. Heat denaturation studies, based on the catalytic activity of the C subunit, and circular dichroism accessing overall structural changes were used to characterize the intrinsic stability of the catalytic subunit and to determine the extent to which nucleotide, metals, and/or the two classes of regulatory subunits and PKI contribute to overall thermostability. By using a variety of nucleotides and metals, it was possible to discriminate between the metal interaction site and the nucleotide interaction site.

EXPERIMENTAL PROCEDURES

Reagents. The peptide substrate, LRRASLG, was synthesized using standard FMOC chemistry and purified by reversed-phase HPLC (Kontron Instruments) or was purchased from Bachem Biochemicals. Other reagents were purchased as follows: ATP, ADP, AMP, cAMP, adenine, adenosine, and GTP (Sigma); PMSF (Boehringer Mannheim); media supplies (Difco). All other reagents were obtained in the purest grade available.

Protein Purification. Following overexpression in *Escherichia coli* (*22*), the rC subunit was first purified by phosphocellulose chromatography (P11, Whatman) and then resolved into discrete isoelectric variants on a Mono S HR10/10 column using FPLC (Pharmacia) (*23*). Isozymes I and II were used for all experiments. Purity was confirmed by SDS−polyacrylamide gel electrophoresis (*24*) and by analytical gel filtration using Superose 75 (Pharmacia) in buffer A (20 mM Mops, pH 7.0, 150 mM KCl, and 0.5 mM DTT). The specific activity of the rC subunit was 25 μmol/(min mg) as measured by the coupled spectrophotometric method of ref *3* in buffer D (100 mM Hepes, pH 7.0, 5 mM $MgCl_2$, 1 mM ATP, and 200 μM heptapeptide LRRASLG as a substrate). The purified rC subunit was stored at 4 °C in buffer B (20 mM potassium phosphate, 110 mM KCl, and 5 mM 2-mercaptoethanol, pH 7.0).

The rR subunits (type Iα and IIα) were overexpressed in *E. coli* E222 (*25*) and purified by ion-exchange chromatography on DEAE-cellulose (*26*). To obtain cAMP-free R subunits, the R subunits were unfolded with 8 M urea as described by Buechler et al. and refolded in buffer A (*27*). The RII subunit (10 μM) was phosphorylated by incubation with 10 nM rC subunit for 10 min at 22 °C in buffer A containing 100 μM ATP and 1 mM $MgCl_2$ and then unfolded and refolded with urea as described above.

Holoenzyme Formation. Holoenzyme was formed by mixing C subunit and cAMP-free (urea stripped) wild-type RI subunit in a 1:1 molar ratio in buffer C containing 100 μM ATP and 1 mM $MgCl_2$, pH 7.0.

Thermal Denaturation. Heat denaturation was performed according to ref *28* using a spectrophotometric assay. C subunits were diluted into buffer A to a final concentration of 0.1 mg/mL. This diluted enzyme (30 μL) was incubated at various temperatures ranging 22−60 °C. Control experiments exchanging the C subunit into buffer A with a Superdex 75 gel filtration column (Pharmacia) yielded a protein with marginally reduced stability of 0.5 °C in comparison to the protein diluted in buffer C. Aliquots of the heat-treated and -untreated C subunits were transferred into buffer D to a final concentration of 50 nM C subunit. The percentage decrease in phosphotransferase activity was calculated by comparing the activity of the heat-treated samples to the control samples using the software package Prism (Graphpad Inc., San Diego, CA). The apparent T_m corresponds to the temperature at which 50% residual activity remained after heat treatment. The analysis of the thermostability of the holoenzymes formed with the recombinant RI subunit and the rC subunit as described above was performed under the same conditions. The holoenzyme complex was activated by the addition of 10 μM cAMP. All nucleotides were dissolved in buffer A with the pH adjusted to 7.0. The total ionic strength was adjusted between 165 and 168 mM except for high concentrations of divalent metals or adenine nucleotides (i.e., 30 mM $MgCl_2$ or 50 mM ATP).

Since the pH of buffer A containing Mops ($\Delta pK_a/°C = -0.006$) changes with the temperature, control experiments

were performed measuring the heat denaturation at pH 7.0 and 7.3. No significant difference could be observed (data not shown).

Calculations of the Free and Complexed Metal Ion Concentrations. The concentrations of free and complexed metals were determined using the program BAD (Bound And Determined) by (*29*). This software allows the entrance of multiple parameters which influence metal/ATP complex formation. Included are all ion species in the buffer and the resulting ionic strength as well as the pH. Equilibrium-binding constants were taken from ref *29*. Since these constants are temperature dependent, the constants for the temperatures that were used ranging 5−60 °C were calculated assuming a linear decrease of these constants with increasing temperature using BAD and the program stacons by ref *30*. These extrapolations were done separately for each metal. The total ionic strength was adjusted between 165 and 168 mM by addition of KCl.

Analytical Gel Filtration. Analytical gel filtration was carried out using a Superose 12 HR10/30 column or a Superdex 75 HR10/10 column both on a FPLC-system with a flow rate of 0.8 mL/min at 22 °C in buffer A as described previously (*17*).

Ka[cAMP]. Holoenzyme at a concentration of 15 nM was incubated for 5 min at room temperature in buffer D with concentrations of cAMP varying from 1 nM to 2.5 μM. The activity of the free C subunit was then determined using the spectrophotometric assay.

Circular Dichroism. Thermal stability measurements by circular dichroism were measured with a Jasco J-710 spectropolarimeter. Ellipticity was monitored at 230 nm at a scan rate of 50 °C/h. The path length was 0.1 cm. Unfolding curves were analyzed with a two-state unfolding model to estimate the half-unfolded temperature, T_m, as described previously (*31*). Unfolding of C subunit was not reversible under the conditions studied.

RESULTS

The role of physiological inhibitors as well as the metal- and nucleotide-binding sites of the C subunit of cAPK were characterized on the basis of their capability to provide enhanced thermal stability. These studies were performed by incubating the C subunit in buffer containing the nucleotide and/or metal of interest for fixed time at various temperatures. The residual phosphotransferase activity was then determined in a coupled spectrophotometric assay. The time dependency of the heat denaturation at 46 °C is shown in Figure 1a. To check if any ligand added would display an effect on the inactivation kinetics, the time depencency of the heat denaturation was also monitored in the presence of MgATP, R^I/MgATP (Figure 1a), MnATP, and R^{II}/MgADP (data not shown). Under all conditions, similar inactivation kinetics were monitored (Figure 1a), and based on this initial studies, a fixed denaturation time of 2 min was chosen for all experiments. The temperature value at which the C subunit had half-maximal activity was defined as the apparent T_m (°C). In an independent set of experiments, the time for the thermodenaturation was varied. Heat denaturation experiments were also performed for 1 and 10 min (Figure 1b). Although the apparent T_m values vary significantly with the denaturation time chosen, the slopes

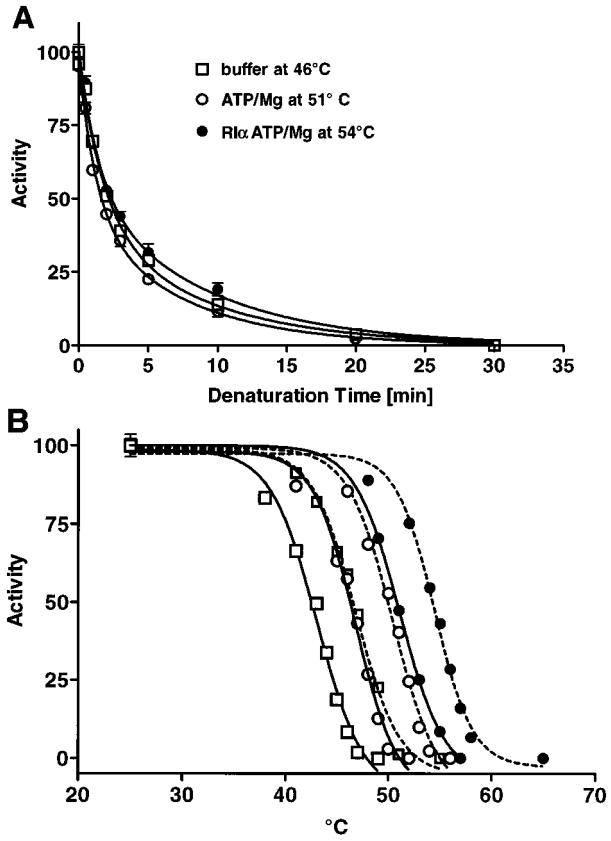

FIGURE 1: Thermal stability of the free catalytic subunit and the type I holoenzyme in the presence and absence of MgATP. (A) Time dependency of the heat denaturation over a period of 30 min for rC subunit in the presence of MgATP and R^I/MgATP at a respective temperature given on the plot. The inactivation was performed in 20 mM Mops, 150 mM KCl, and 1 mM DTT, pH 7.0, for 1 min (solid line) and 10 min (dashed line). The activity of the C subunit was determined with the spectrophotometric assay described in the Material and Methods. (B) Recombinant C subunit and type I holoenzyme in the presence and absence of MgATP were incubated for 2 min at the temperatures indicated and the residual phosphotransfer activity (activity of the free rC subunit = 1) was measured. The app. T_m was defined as 50% residual activity. Open symbols = free C subunit in the absence (□) or presence of 1 mM ATP/5 mM $MgCl_2$ (○); closed symbols = type I holoenzyme in the presence of 1 mM ATP/5 mM $MgCl_2$ (●). All determinations were done in duplicate.

of the denaturation curves taken at 1, 2, and 10 min for the free C subunit and the C subunit in the presence of MgATP and R^I/MgATP are almost identical (Figure 1b). This indicates that the thermal inactivation is independent from the denaturation time.

Previous results established that the C subunit was significantly more resistant to thermal denaturation when it was part of the type I holoenzyme complex (*28*). The T_m of the wild-type C subunit under these conditions in buffer alone was 46 °C. The T_m for the holoenzyme saturated with MgATP was 54 °C and thus represents a substantial enhancement of thermostability. Surprisingly, a stabilizing effect almost as strong was observed in the presence of 10 mM $MgCl_2$ and 1 mM ATP with a T_m of 51.0 °C. The R subunit provided only marginally significant additional stability compared to MgATP alone (Figure 1 and Table 1).

Analytical gel filtration was used to probe whether the loss of activity could be attributed to a change in the overall conformation of the C subunit. It was shown unambiguously

Table 1: Thermostability Properties of the Recombinant C Subunit of cAPK[a]

protein	nucleotide (1 mM)	metal (5 mM)	T_m (°C)
C			46.0 ± 0.2
C	ATP	$MgCl_2$	51.0 ± 0.4
$R^I_2C_2$	ATP	$MgCl_2$	54.2 ± 0.2
$R^{II}_2C_2$	ATP	$MgCl_2$	51.0 ± 0.5
P-$R^{II}_2C_2$	ATP	$MgCl_2$	53.8 ± 0.4
$R^{II}_2C_2$	ADP	$MgCl_2$	55.0 ± 0.2
P-$R^{II}_2C_2$	ADP	$MgCl_2$	57.2 ± 0.5

[a] Thermodenaturation of 50 nM rC subunit was performed for 2 min in a buffer with an ionic strength of 160 mM, and the residual phosphotransfer activity was determined with a spectrophotometric assay. Nucleotide and metal concentrations are total concentrations. Errors are standard errors of the mean of 3 different experiments. P-$R^{II}_2C_2$ denotes the type II holoenzyme with phosphorylated R^{II} subunit as described in the Material and Methods.

Table 2: Effect of the Adenine Moiety, Phosphate, and GTP (°C) on the Free C Subunit[a]

additions		+Mg^{2+} (5 mM)	+Mn^{2+} (2 mM)
none	46.0	45.5	47.0
adenine (1 mM)	47.5	47.0	47.5
adenosine (1 mM)	51.0	50.0	51.5
AMP (1 mM)	46.5	47.0	49[b]
ADP (1 mM)	45.5	50.5	52.5
ATP (1 mM)	46.0	51.0	56.0
ATP (50 mM)	46.0	nd	nd
Na-PPi (50 mM)	48.5	nd	49.0
KPO_4 (50 mM)	49.0	nd	49.0
GTP (1 mM)	45.5	45.5	47.0

[a] T_ms were determined using 50 nM of rC subunit as described for Figure 1 using nucleotides and metals as indicated. Ionic strength was adjusted to 160 mM except for sodium pyrrhophosphate (Na-PPi), potassium phosphate and 50 mM ATP. nd = not determined. [b] 5 mM $MnCl_2$.

that the heat inactivated protein was irreversibly unfolded. Either it precipitated after centrifugation at 10000*g* or was eluted on a Superose 12 gel filtration column with the void volume, indicating an unfolded protein. The nondenatured fraction of the heat-treated protein showed an unchanged Stokes' radius of 27 Å characteristic for the wild-type protein (*23*) and displayed full specific phosphotransferase activity (k_{cat} = 25 s^{-1}, data not shown).

Subsequently, the two components of the nucleotide/metal complex were analyzed further in an effort to determine whether either or both factors were responsible for the observed enhanced stability.

Nucleotide Requirements. The effect of various nucleotides, adenine, and adenosine were compared. ADP (+5 °C) had a similar effect to ATP (+5 °C) in that metal ions were required to achieve any enhanced stability; however, no enhanced stability could be induced even by high concentrations (50 mM) of ATP (Table 2). AMP binds very poorly to the C subunit, and only in the presence of high concentrations was there some evidence of enhanced stability. GTP in the presence or absence of Mg^{2+} was used to control for the specificity of the nucleotide-binding site. No stabilizing effect could be observed (Figure 2).

When the effect of the nucleoside alone, devoid of any phosphates, was determined, enhanced stability was still observed, but under these conditions, there was no longer any requirement for metal ions. Free adenosine (1 mM) stabilized the rC subunit by 5 °C in the presence or absence of Mg^{2+} and was almost as effective as MgATP. Adenine was less effective but still showed some enhanced thermal stabilization. Addition of metal did not further increase the stabilizing effects of adenine and adenosine (Figure 2 and Table 2).

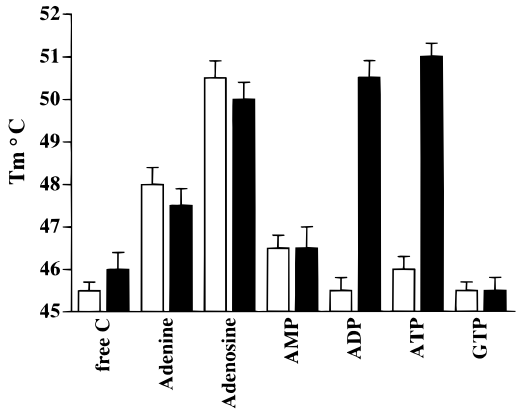

FIGURE 2: Effects of nucleotides and Mg^{2+} ions on thermostability. The thermostability of rC subunit was measured in the absence (open bar) or the presence (solid bar) of 5 mM $MgCl_2$.

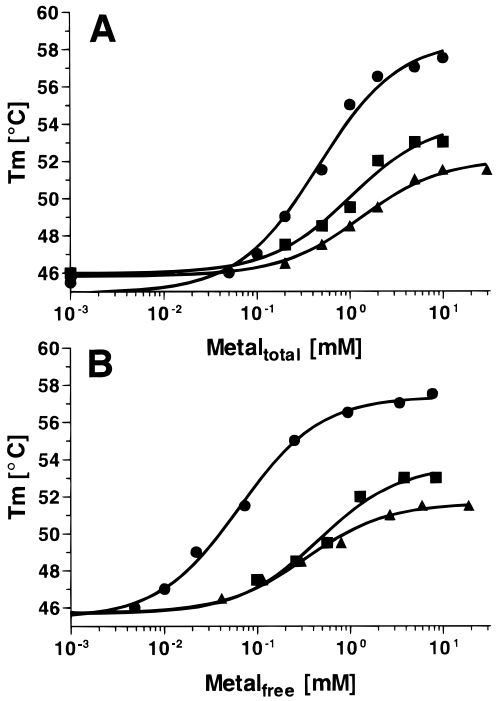

FIGURE 3: Metal requirements for thermostability. The total concentrations (upper panel) and the free concentrations (lower panel) of Mn^{2+} (●), Ca^{2+} (■), and Mg^{2+} (▲) are plotted versus the app. T_m values determined at each metal concentration. Experiments were performed in the presence of 1 mM ATP. Free metal concentrations and apparent K_ds for the binding of the second metal ion were calculated as described in the Experimental Procedures.

Metal Requirements. To examine the role of the metal ions, the thermal stability conferred by ATP was measured as a function of the divalent metal ion concentration. From the three metal ions tested, the most effective was Mn^{2+} followed by Ca^{2+} and then Mg^{2+} in stabilizing the C subunit against thermal denaturation. Figure 3b and Table 2 show the stabilizing effects of the metals relative to the calculated free metal ion concentrations. The half-maximum stabilization

Table 3: A Second Metal Site Is Required to Stabilize the C Subunit in the Presence of ATP[a]

	T_m (°C)				
metal	0 mM	1 mM (total)	10 mM (total)	$^{app}K(Me)^{2+}_{D\ free}$ (mM)	$^{app}K(Me)^{2+}_{D\ total}$ (mM)
Mg^{2+}	46.0	48.5	51.5	0.26	1.36
Mn^{2+}	46.0	55.3	58.0	0.008	0.44
Ca^{2+}	46.0	49.5	53.2	0.16	0.94

[a] T_ms were determined in the presence of 1 mM ATP and indicated metal concentration determined with the software BAD as described in the Material and Methods. Apparent stabilization constants (K_D) were calculated from Figure 3 using the software package Prism.

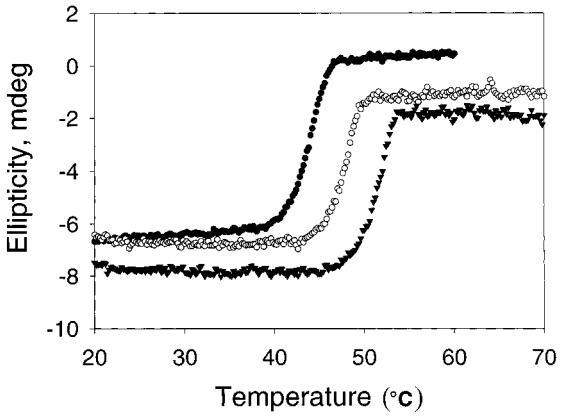

FIGURE 4: Thermal stability of C subunit measured by circular dichroism (CD). The thermal stability of C subunit measured by CD is shown as ellipticity at 230 nm versus temperature °C in 10 mM Pipes and 150 mM NaCl, pH 7.0. The following concentrations were used: C subunit alone at 4 μM (●), with 5 mM $MgCl_2$ + 1 mM ATPγS (○), and 2 mM $MnCl_2$ + 1 mM ATPγS (▼).

by Mn^{2+} was achieved at much lower concentrations than for the other two metals following the stability constant of the given metal/ATP complex. Apparent free metal concentration for half-maximal thermal stabilization in the presence of 1 mM ATP were 0.34, 0.64, and 0.063 mM for Mg^{2+}, Ca^{2+}, and Mn^{2+}, respectively (Table 3).

When the apparent T_ms were calculated as a function of the metal ion concentration, it was found that ATP complexed with Mn^{2+}, Mg^{2+}, or Ca^{2+} had no stabilizing effect under conditions where only one metal site was occupied (Figure 3). To stabilize the C subunit, metal ions were required in concentrations high enough so that *both* metal-binding sites could be occupied.

Circular Dichroism. The effect of ATP and divalent metal ions on the thermostability of the C subunit was also demonstrated independently by circular dichroism (CD, Figure 4 and Table 4). The apparent T_ms based on CD were very consistent with those calculated from thermodenaturation using the kinetic assay. Furthermore, the metal dependency was also consistent with Mn^{2+} being much more effective than Mg^{2+}. Metal ions alone destabilized rather than stabilized the C subunit showing a slightly reduced T_m. MgATPγS behaves similarly to MgATP and contributes to enhanced thermostability to the C subunit. The $^{app}K_D$s calculated based on CD were significantly lower; however, the differences were very consistent using these two independent methods (M. Doyle, unpublished results).

Effect of the R Subunits on Thermostability. The effect of both type I and II R subunits was next compared (Figure 5).

Table 4: PKI Stabilizes the C Subunit Only in the Presence of ATP/Metal[a]

components added	T_m (°C)
none	44.3
5 mM $MgCl_2$	44.0
2 mM $MnCl_2$	43.0
5 mM $MgCl_2$ + 1 mM ATPγS	47.5
2 mM $MnCl_2$ + 1 mM ATPγS	51.2
0.4 mM $MgCl_2$ + 0.35 mM ATPγS	45.6
12 μM $PKI_{(5-24)}$	45.3
12 μM $PKI_{(5-24)}$ + 0.4 mM $MgCl_2$ + 0.35 mM ATPγS	47.4
24 μM PKI	44.0
12 μM PKI + 0.4 mM $MgCl_2$ + 0.35 mM ATPγS	50.4

[a] Thermal stabilization of rC subunit by the heat stable protein kinase inhibitor (PKI) in the presence of metals and nucleotides as indicated in the table measured by circular dichroism determined in 10 mM Pipes and 150 mM NaCl, pH 7.0.

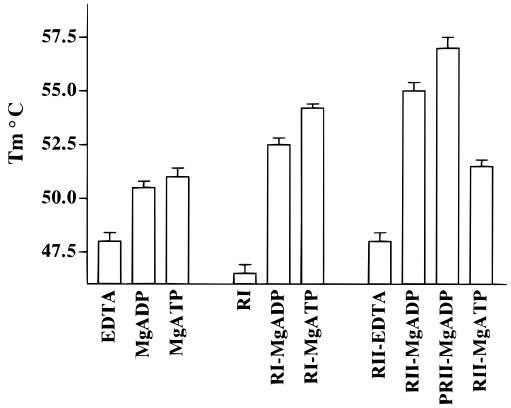

FIGURE 5: Effects of the type I and type II R subunits on the thermostability of the rC subunit. Experiments were performed in the presence or absence of MgATP. Holoenzymes were formed according to the Material and Methods.

The type II R subunit binds to the C subunit with a subnanomolar binding constant in the absence of divalent metals and ATP. In contrast, the type I R subunit has an absolute requirement for divalent metals and nucleotides for high-affinity subunit interaction (*16*, *17*).

In the absence of MgATP the $^{app}K_D$ for interaction of the RI subunit and the C subunit is 125 nM as opposed to 0.1 nM in the presence of MgATP as determined previously (*17*), and no enhanced thermostability was achieved when the type I holoenzyme was formed in the absence of ATP. As was seen in Figure 5, the holoenzyme in the presence of MgATP was slightly more stable than the free C subunit in the presence of MgATP, and maximum stability was achieved in the presence of MnATP. The T_m for the C subunit was increased by 13 °C under these conditions. The effect of the metal ion concentration on the stabilization of the holoenzyme was also determined. These results showed that one metal ion was not sufficient to stabilize the holoenzyme complex against thermal denaturation. Even though ATP binds more tightly to the holoenzyme complex [K_D = 60−100 nM (*17*)], there was still a requirement for a second metal-binding site for thermostability just as was seen for the free rC subunit (Table 1). At a concentration of 200 μM total Mg^{2+} (equivalent to 8 μM free Mg^{2+}), no stabilization could be observed. These results suggest that the high-affinity binding of MgATP requires both metal ions.

The effect of the RII subunit on the stabilization of the C subunit with various nucleotides was then investigated. As

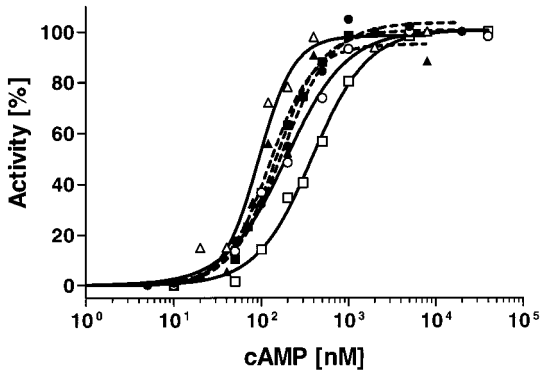

FIGURE 6: Activation constants for cAMP for the type I holoenzyme were determined in the presence of 1 mM ATP and 2 mM of each metal according to the Material and Methods. K_a values were 123, 172 , and 148 nM for holoenzymes formed with the RIα subunit in the presence of Mg^{2+} (▲), Ca^{2+} (●), and Mn^{2+} (■), respectively, dotted line, and 92, 192, and 348 nM for holoenzymes formed with the RIIα subunit in the presence of Mg^{2+} (△), Ca^{2+} (○), and Mn^{2+} (□), respectively, solid line.

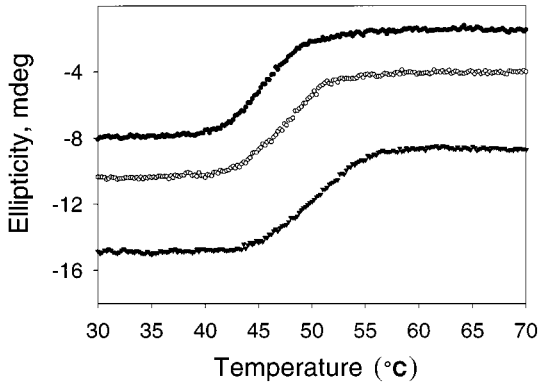

FIGURE 7: Thermal stability of C subunit in complex with PKI measured by circular dichroism. Data are shown as ellipticity at 230 nm versus temperature in 0.4 mM $MgCl_2$, 0.35 mM ATPγS, 6 mM Pipes, and 150 mM NaCl, pH 7.0. The C subunit alone was tested at a concentration of 6 μM (●) in the presence of 12 μM $PKI_{(5-24)}$ (○) or 24 μM PKI (▼). Addition of $PKI_{(5-24)}$ and PKI to the sample increased the negative ellipticity at 230 nm because $PKI_{(5-24)}$ and PKI themselves display a significant ellipticity. However, the amplitude changes are the same for all three curves (6.3 ± 0.2 mdeg).

for the RI subunit, no stabilizing effect was observed which could be attributed to the type II R subunit in the absence or presence of MgATP. This was surprising since the RII subunit binds to the C subunit with a subnanomolar binding constant in the absence of MgATP. However, in the presence of MgADP, the C subunit was significantly more stable. The apparent T_m was 55 °C for the dephosphorylated RII subunit and 58 °C for the phosphorylated RII subunit. Once again, to achieve this significant enhancement in thermostability, two metal ions were required. Thus, for both RI and RII, it is the occupancy of the nucleotide-binding pocket that conveys thermostability not the R subunit.

To determine whether the activation of the wild-type recombinant holoenzyme with cAMP was altered by the presence of Mg^{2+} versus Mn^{2+} or Ca^{2+}, the activation constants ($K_{a,cAMP}$) were determined (Figure 6). A $K_{a,cAMP}$ of 148 nM cAMP was measured for the type I holoenzyme formed with MnATP similar to 123 nM for the holoenzyme formed with MgATP. A similar $K_{a,cAMP}$ was found for the type I holoenzyme formed with CaATP. The $K_{a,cAMP}$ for the type II holoenzyme formed with MgATP was also 92 nM; however, the $K_{a,cAMP}$ for the RII holoenzyme formed in the presence of MnATP was significantly increased to 380 nM. The cooperativity of the activation in the presence of MnATP was slightly stronger than in the presence of MgATP (Hill coefficient 2.3 for MnATP versus 1.8 for MgATP).

Thermostability of C/PKI Complex Determined by CD. Thermal unfolding followed by circular dichroism revealed that, similar to the type I and type II R subunits, PKI alone had no effect on the thermostability of the C subunit with a $^{app}T_m$ value of 44.3 °C. The addition of a 20 residue peptide derived from the sequence of PKI (PKI_{5-24}) slightly stabilized the C subunit in the presence of MgATPγS (Table 4). However, addition of PKI and MgATPγS significantly stabilized the C subunit by 6 °C using 0.4 mM $MgCl_2$ and 0.35 mM ATPγS (Figure 7). Experiments performed with ATP instead of ATPγS were not significantly different (data not shown).

DISCUSSION

From the crystal structures of the C subunit have emerged snapshots that define a conformationally very dynamic protein. On the basis of the crystal structure, the core of the kinase consists of two lobes: a smaller nucleotide-binding lobe, residues 40−120, a linker strand (residues 121−127), and a large lobe (residues 128−300) (*32*). The opening and the closing of the active-site cleft between the two lobes are an essential part of catalysis with the closed conformation presumably reflecting the transition state for the rapid transfer of the γ-phosphate from ATP to the protein or peptide substrate. Opening of the cleft is essential for the rate-limiting step, i.e., the release of ADP following phosphoryl transfer (*33*).

The opening of the active-site cleft is a consequence of two motions. Of the multiple conserved loops that converge at the active-site cleft, only the glycine-rich loop in the small lobe is highly mobile. The tip of that loop interacts with the γ-phosphate of ATP, and this is an essential requirement for catalysis (*34*). The large lobe serves as a stable docking surface for the peptide. It does not appear to undergo major conformational changes. The opening is achieved by a leverage of the glycine-rich loop residues 47−56, which on its own is sufficient to achieve partial opening (*35*). Further opening is achieved by a rotation of the small lobe relative to the large lobe which is more a sliding motion than a hinging motion (*36*). Rigid body analysis reveals an extensive network of interactions that cross between the small and the large lobes. The rigid body motions of the protein are unveiled by a series of crystal structures of binary and ternary complexes (*37*, *38*). Although they do not define the boundaries for openness, they do show a flexibility.

The active site cleft and the extensive interactions of the protein with the nucleotide are summarized in Figure 8. While the crystal structures reveal a detailed picture of the molecular basis for conformational flexibility, they say little about the actual dynamics of that process. They also do not allow for evaluating the specific contributions of the two substrates to the dynamics. The understanding of the dynamics requires solution methods. In this study, we have used the thermodynamic stability of the C subunit to evaluate the importance of both the nucleotides and the two inhibitor proteins of the cAPK, the R subunits type I and II, and PKI

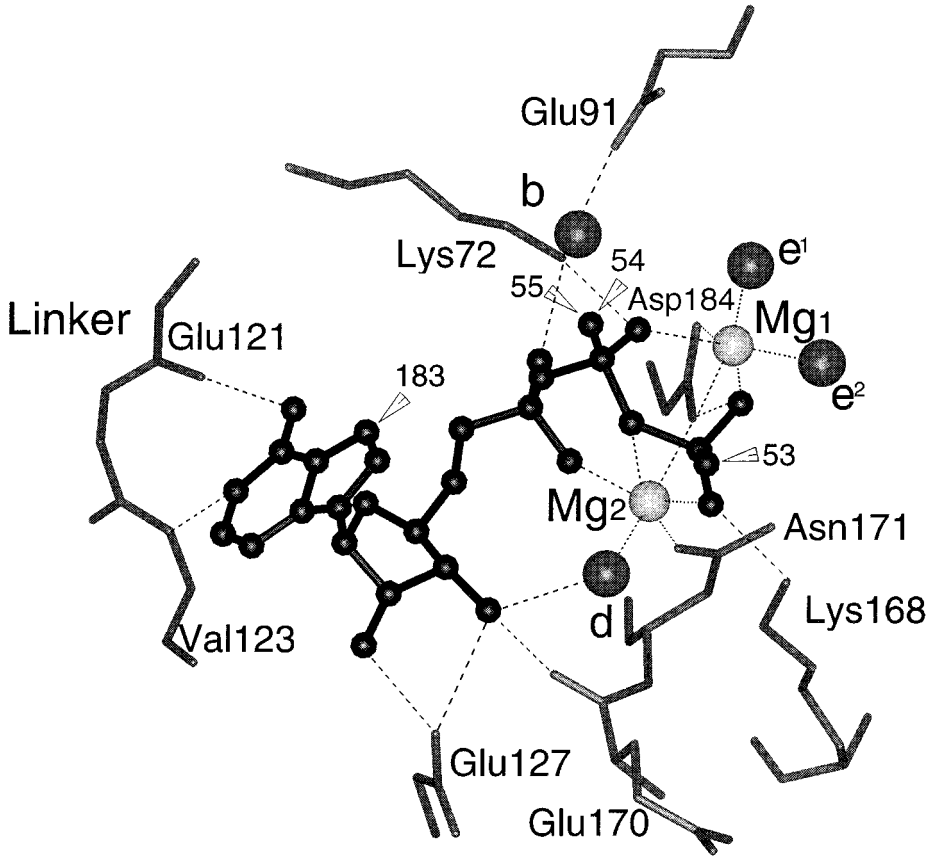

FIGURE 8: Interactions of the protein with the nucleotide, metal ions, and water molecules in the active site cleft. Both, the small and large domains of the conserved core plus the linker region all converge at the active site cleft to form the nucleotide binding site. The glycine rich loop in the small lobe is not shown; however, interactions of the Gly 55 and Phe 54 backbone amides with the β-phosphate of ATP are indicated by arrows as is the Ser 53 amide interaction with the γ-phosphate. Lys 72 interacts with the α- and β-phosphates of ATP and is positioned by Glu 91. The linker region anchors the nucleoside via Glu 121, Val 123, and Glu 127 with additional contacts to the large lobe via Thr 183 and Glu 170. The large lobe mainly orientates the metal ions by hydrogen bonding to Asp 184 from the DFG-motif of all protein kinases and Asn 171 in the catalytic loop. Major contacts are also made to conserved water molecules. The distances of all hydrogen bonds (dotted lines) are 3.1 Å or less. The metal ions are indicated as small white balls. The water molecules (*46*) are shown as large dark balls.

on the stability of the enzyme. Furthermore, once the critical importance of the nucleotide was corroborated, it was possible to dissect the specific contributions of the adenine, ribose, and phosphate subsites as well as the metal ions. A schematic overview is given in Figure 9.

Holoenzyme and PKI/C subunit complexes are known to be more stable than the free subunits based on protection from proteolysis (*39*, *40*). In vivo evidence also supports a longer half-life of the holoenzyme complex than the free subunits (*41*, *42*). When we compared the thermostability of the free C subunit to that of the type I or II holoenzyme complexes, we could show that the thermostability was increased. T_m values of 54.2 and 51.5 °C were determined for the type I and II holoenzyme complexes respectively, with the $^{app}T_m$ for the free C subunit being 46.0 °C. This result was confirmed independently for the C subunit and the C:PKI:MgATP complex using circular dichroism. Surprisingly however, when the individual contributions of the inhibitor proteins and the nucleotide were evaluated, it was found that the stability derived almost exclusively from the nucleotide. Very little stability was contributed by the inhibitor proteins. This was particularly apparent for the type II holoenzyme, which does not require MgATP for complex formation. The presence of the RII subunit does not contribute thermostability to the protein, although the K_D for this subunit is less than 1 nM (*17*, *43*).

What portion of the nucleotide contributes specifically to the thermostability of the C subunit? By looking more closely at the specific importance of the subsites in the nucleotide as well as the metal ions, a more complex picture emerged of the nucleotide:protein complex. Adenine alone contributed some enhanced stability; however, adenosine was as effective in stabilizing the free C subunit as MgATP. Furthermore, unlike ATP, there was no metal requirement for adenosine. The initial studies that defined nucleotide specificity for the catalytic subunit showed that most of the energy for binding could be attributed to the adenosine moiety since the K_i for adenosine was 32 μM compared to a K_m of 10 μM for ATP (*8*). By using a series of nucleotide analogues, one can clearly discriminate between the effects of the nucleotide and the effects of the metal ion and the phosphate on thermostability. The stabilizing effect of the nucleotide is due primarily to occupancy of the adenine-binding site, where the adenine ring is localized precisely at the base of the cleft between the two lobes. The adenine ring is deeply buried with the N6 nitrogen hydrogen binding directly to the backbone carbonyl of Glu 121 in the linker region. The linker region cradles the nucleotide with Glu 127 at the end of this linker hydrogen bonding to the 3′ OH of the ribose ring. The tight packing of the nucleotide into this pocket explains why the

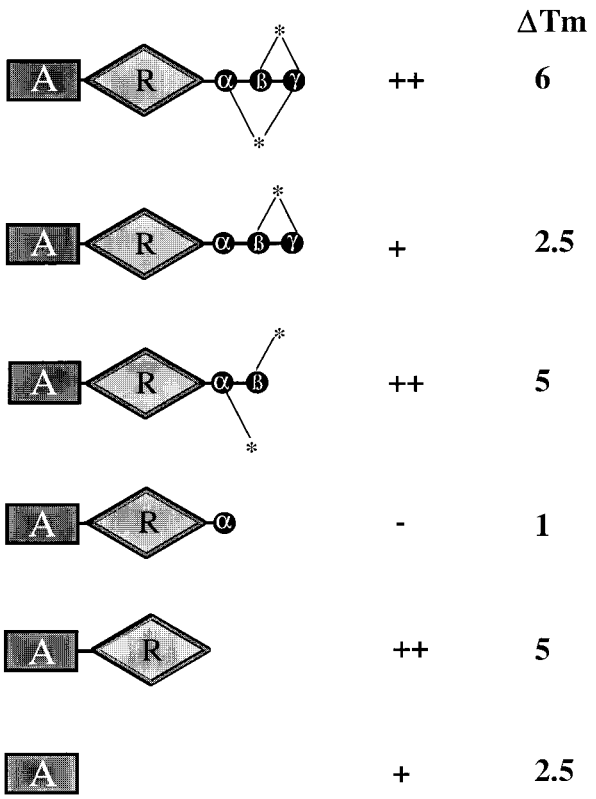

FIGURE 9: Effects of adenine (A), ribose (R), and metal ions (*) on the thermostability of the recombinant C subunit. The effects on the stabilization (ΔT_m in degrees Celsius) in comparison to the rC subunit in buffer C using Mg^{2+} as a metal are presented on the right side of the cartoon.

enzyme is so intolerant of substitutions. GTP, for example, will not serve as a phosphate donor, and GTP, likewise, did not contribute to the stability of the C subunit. GTP binds also poorly to the enzyme (*8*, *44*). Metals had no effect on stabilizing the adenine:C subunit complex; they are only important when the phosphates are present.

In contrast to adenosine, the metal binding was essential for achieving a more thermostable protein in the presence of ATP. However, there was a difference between MgATP and MgATPMg. When the concentrations of free and bound metal were calculated using BAD, there is actually very little MgATPMg present when the Mg and ATP are both present at a concentration of 1 mM. Under these conditions, which are optimal for catalysis (*3*), there was very little enhanced stability. Thus, the addition of the phosphates, while essential for catalysis, is destabilizing relative to adenosine alone. The full stabilization was only observed when ATP was present in sufficient concentrations to allow two Mg ions to bind. While this conformation is optimal for achieving a tight complex with both the RI subunit and PKI, the second metal diminishes the efficiency of catalysis specifically by reducing the off rate of ADP which is the rate-limiting step in catalysis (*33*).

As seen in Scheme 1, the first metal binds with a K_d of approximately 10 μM as an MgATP complex, and this is the catalytically active form of the C subunit.

Metal ions thus have the capability of shifting the equilibrium between the active and inactive conformations.

The critical question raised in this study is whether the metals actually induce this conformational change or whether they simply stabilize the altered structure once it is assumed. It has been postulated that enzymes use their ordered structures to facilitate catalysis; however, enzyme residues involved in function are not optimized for stability as demonstrated for T4 lysozyme (*45*). As a consequence of this relationship between stability and function, it is plausible that the C subunit of cAPK is switching between a more thermostable conformation, secured by two metal sites being occupied, and a catalytically more active, but less stable, conformation with one metal site occupied. The results, furthermore, support the conclusion that the conformational state of the enzyme that is the least stable in thermodynamic terms is the form that is optimal for catalysis.

The presence of multiple conformations of the C subunit in solution was first described kinetically by Cook (*3*). Adams and Taylor showed kinetically that only a small amount of the C subunit is in the catalytically active conformation when the Mg concentration is high (10 mM) and in excess of ATP (*9*). The results described here furthermore demonstrate that the amount of nucleotide and even more important the absolute amount of metal ions can play a critical role in regulating the catalytic efficiency of this enzyme. The levels of nucleotide and metal ion here are physiological and can direct the enzyme into an inhibited form that is poised to form a complex with its inhibitors. One of the most intriguing observations was that Mn^{2+} and Ca^{2+} were more efficient than Mg^{2+} in stabilizing the protein. Whether this is of physiological relevance remains to be established; however, it is known that Mn^{2+} typically inhibits the activity in Ser/Thr protein kinases. It has been shown previously that when Mn^{2+} is the metal ion, a stronger C:MnATP-Mn complex is formed, needing less Mn^{2+} to form a stable complex (*6*). The same is true for C-MnADP-Mn. Ca^{2+} can also stabilize the rC subunit and is even more effective than Mg^{2+}, although Ca^{2+} is not able to support catalysis (*8*). The apparent K_Ds measured for the Mg^{2+} by heat denaturation are similar to those measured by Cook (*3*) using a kinetic approach (1.7−3 mM). Bhatnagar (*8*) using a fluorescence titration technique measured in reasonable agreement ^{app}Ks of 2.82, 0.031, and 3.3 mM for Mg^{2+}, Mn^{2+}, and Ca^{2+}.

Whether high Mg^{2+} stabilizes the closed conformation or not remains to be established since crystals have never been grown in the presence of high Mg^{2+}. The addition of excess Mg^{2+}, in fact, prevents crystallization under conditions that otherwise work well. Until crystals are obtained of the holoenzyme complex, it is also not clear whether the catalytic

Scheme 1

$$\underset{T_m = 45.5\ ^\circ C}{C} \xleftrightarrow{K_d = 10\ \mu M^a} \underset{T_m = 45.5\ ^\circ C\ \text{active}}{C\_MgATP} \xleftrightarrow{K_d = 1.4\ mM^b} \underset{T_m = 54\ ^\circ C\ \text{inactive}}{C\_MgATP\ Mg}$$

[a] K_d is determined kinetically. [b] K_d is determined from heat denaturation experiments.

subunit will be in a closed or open or otherwise different conformation. Further structural studies are required to resolve this question.

ACKNOWLEDGMENT

We thank Rolf Thieleczek, Bastian Zimmermann, and Ludwig Heilmeyer for helpful discussions and Marco de Stefano and Petra Goldmann for excellent technical assistance.

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BI982672W