Functional cGMP-Dependent Protein Kinase Is Phosphorylated in Its Catalytic Domain at Threonine-516[†]

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ABSTRACT: The phosphorylation of threonine residues in the catalytic core of several protein kinases is important for the functional integrity of these enzymes. The corresponding residues of cGMP-dependent protein kinase Iα (cGMP kinase) are Thr-514 and/or Thr-516. The *in vivo* phosphorylation and functional role of these residues was studied. cGMP kinase was overexpressed and purified as a catalytically active and inactive enzyme in Sf9 insect cells and in *Escherichia coli*, respectively. The enzymological and physicochemical properties of the Sf9 cGMP kinase were indistinguishable from that of the purified bovine lung enzyme. The cysteines of cGMP kinase including Cys-518 were labeled with vinylpyridine. Amino acid sequencing and mass spectroscopy of the labeled peptides showed that Thr-516 was phosphorylated in the enzyme purified from Sf9 cells but not in that from *E. coli*. The functional importance of phosphothreonine-516 was investigated by substitution of Thr-516 by alanine (T516A) or by glutamate (T516E). Expression in insect cells of the T516A mutant resulted in a protein lacking detectable kinase activity, whereas the T516E mutant retained basal phosphotransferase activity. In *E. coli*, the exchange of Thr-516 by glutamate did not lead to the synthesis of a catalytically active enzyme. These results demonstrate that phosphothreonine-516 of cGMP kinase is crucial for the formation of an enzymatically active protein kinase.

Cyclic GMP-dependent protein kinase (cGMP kinase)[1] is a ubiquitous eukaryotic serine/threonine protein kinase, which is involved in the regulation of diverse physiological processes such as smooth muscle relaxation, platelet aggregation, and neuronal long-term potentiation [for reviews, see Hofmann et al. (1992), Lincoln and Cornwell (1993), and Francis and Corbin (1994)]. The holoenzyme is a dimer of identical 75 kDa subunits each consisting of an amino-terminal regulatory domain and a carboxy-terminal catalytic domain. The regulatory region contains two successive cyclic nucleotide binding sites that bind cGMP with different affinities. cGMP dissociates from these sites with fast (low-affinity site A) and slow (high-affinity site B) kinetics.

Purified bovine lung cGMP kinase Iα contains 1.4 mol of phosphate per mol of subunit (Hofmann & Flockerzi, 1983). Interestingly, only 0.4 mol of phosphate was removed by added phosphatases. The remaining 1 mol of phosphate had the acid/base characteristics of a threonine phosphate, which was metabolically stable. During the last years, it was shown that the phosphorylation of a distinct threonine residue in the highly conserved catalytic core of some protein kinases is of fundamental importance for their catalytic function. The cAMP kinase is stably phosphorylated at Thr-197 in mammalian and prokaryotic cells (Shoji et al., 1979; Yonemoto et al., 1993). The phosphorylation at this residue is thought to be essential for the folding of the active catalytic core of cAMP kinase (Taylor & Radzio-Andzelm, 1994). Thr-514 and Thr-516[2] are located in the catalytic core of cGMP kinase and could serve a similar function as Thr-197 of cAMP kinase. The phosphorylation of either of these threonines may be necessary to fold a functional kinase domain.

An active cGMP kinase Iα has been successfully expressed in insect cells using a recombinant baculovirus vector (Feil et al., 1993a) but not in *Escherichia coli* (Feil et al., 1993b). A possible reason for this difference was that the enzyme was not phosphorylated at either threonine in *E. coli*. The *in vivo* phosphorylation of cGMP kinase at Thr-514 or Thr-516 was investigated using the purified enzyme from both cell systems. The recombinant active insect cell cGMP kinase is phosphorylated in its catalytic domain at Thr-516. In contrast, Thr-516 is not phosphorylated in the biologically inactive protein purified from *E. coli* inclusion bodies. Site-directed mutagenesis showed that the post-translational phosphorylation of cGMP kinase at Thr-516 plays an essential role in the catalytic activity of this protein kinase but that the synthesis of an inactive enzyme in *E. coli* is not primarily caused by the absence of the phosphate group at Thr-516.

EXPERIMENTAL PROCEDURES

Purification of Recombinant cGMP Kinase from Insect Cells. cGMP kinase Iα was expressed as described previ-

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[1] Abbreviations: cGMP kinase, cyclic GMP-dependent protein kinase; cAMP kinase, active catalytic subunit of cyclic AMP-dependent protein kinase; 8-AEA-cAMP, 8-[(2-aminoethyl)amino]adenosine 3′,5′-cyclic monophosphate; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; AU, absorbance units.

[2] Amino acid residues are numbered by ignoring the amino-terminal methionine in accordance with the sequence of the mature protein.

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ously (Feil et al., 1993a). Several suspension cultures (500 mL each) of Sf9 cells were infected with recombinant baculovirus (multiplicity of infection = 5) at a cell density of 1.5×10^6 cells/mL and were harvested 3 days after infection. All subsequent steps were carried out at 4 °C. Cells from 5 L of culture were washed twice in phosphate-buffered saline and resuspended in 50 mL of buffer A (20 mM Tris (pH 8.0), 100 mM NaCl, 2.5 mM DTT, 2.5 mM EDTA, 2.5 mM benzamidine, 2.5 mM PMSF, and 1.0 μg/mL leupeptin). Cells were lysed by freeze−thawing and homogenized with a Teflon-coated glass homogenizer. The homogenate was centrifuged at 60000*g* for 15 min. The supernatant (673 mg of protein) was supplemented with 20% (w/v) glycerol (final concentration) and directly loaded onto a 10 mL 8-AEA-cAMP−agarose affinity column at a flow rate of 2 mL/min. The column was washed with 200 mL of buffer B (20 mM Tris (pH 8.0), 100 mM NaCl, 20% (w/v) glycerol, 0.1 mM DTT, 0.1 mM EDTA, 0.1 mM benzamidine, 0.1 mM PMSF, and 0.1 μg/mL leupeptin). cGMP kinase was eluted with buffer B containing 20 μM cGMP and collected in fractions of 5 mL. All protein-containing fractions were pooled and concentrated by ultrafiltration using an Amicon Centriprep unit (10 kDa cutoff). Removal of cGMP from the preparation was achieved by two successive steps of 50-fold dilution in cGMP-free buffer B (without leupeptin) and 50-fold concentration. Glycerol was added to the second concentrate up to a concentration of 40% (w/v). The final preparation was stored at −20 °C and contained less than 10 nM cGMP. The purified enzyme was diluted a further 1000- and 20 000-fold to measure the cGMP-binding and kinase activity, respectively. The final carry-over of cGMP into the assay mixtures was less than 10 and 0.5 pM in the cGMP-binding and the phosphotransferase assay, respectively.

Purification of Recombinant cGMP Kinase from E. coli. cGMP kinase Iα was expressed at 37 °C in *E. coli* BL21-(DE3) using the expression plasmid pET3a/cgk as described previously (Feil et al., 1993b). The following procedure was carried out at room temperature. Cells from 1.5 L of culture were suspended in 5 mL of buffer A and lysed in a French press. The insoluble cell fraction, which mainly contained inclusion bodies, was isolated by centrifugation at 5000*g* for 15 min and washed twice with buffer A. The pellet was solubilized by boiling for 10 min in 5 mL of SDS (sodium dodecyl sulfate) sample buffer (50 mM Tris (pH 6.8), 2.5% β-mercaptoethanol, 1.25% SDS, 10% (w/v) glycerol, and 0.001% bromphenol blue). The solubilized protein was separated by preparative SDS-PAGE (Model 491 Prep Cell instrument from Biorad). Electrophoresis was carried out vertically through a cylindrical gel column (3.7 cm ID) according to the manufacturer's instructions. A 3% stacking gel (1.5 cm height) and an 8% separating gel (7.0 cm height) were used. Protein was eluted in running buffer (25 mM Tris (pH 8.4), 0.01% SDS, and 192 mM glycine) at 0.5 mL/min and collected in fractions of 5 mL. Fractions, which contained only the intact cGMP kinase protein of 75 kDa, were detected by analytical SDS-PAGE and pooled. The purified protein was concentrated via ultrafitration with an Amicon Centriprep unit (10 kDa cutoff) and stored at −20 °C.

Construction of Mutant cGMP Kinases. Threonine-516 of cGMP kinase was exchanged for alanine (T516A) or glutamate (T516E) by PCR-mediated mutagenesis using the primer overlap extension method (Clackson et al., 1993). Outside primers corresponded to nt 1186−2002 (sense) and nt 1962−1941 (antisense) of the wild-type cDNA.[3] Inside primers for the T516A mutation were 5′→3′GAAAACATGG GCTTTTTGTGGGAC (sense) and 5′→3′GTCCCACAA-AAAGCCCATGTTTTC (antisense). Inside primers for the T516E mutation were 5′→3′AAACATGGGAATTTT-GTGGGACTCCAG (sense) and 5′→3′CCCACAAAA-TTCCCATGTTTTCTTTCC (antisense). The codons substituted for the wild-type threonine codon (ACT) are underlined. A 0.5 kb *Avr*II/*Afl*II fragment of each mutated PCR product was ligated into the *Avr*II/*Afl*II sites of a pUC/cGMP kinase vector to yield pUC/cgkA and pUC/cgkE, respectively. The inserted regions were entirely sequenced to ensure that only the desired mutations had been introduced.

To express the cGMP kinase mutants T516A and T516E in insect cells, baculovirus transfer vectors were constructed as follows. A 2.1 kb *Eco*RI/*Eco*RI cDNA fragment (nt −6 to 2119) containing the coding region of the cGMP kinase mutant (nt 1−2013) was excised from the corresponding pUC construct and subcloned into the *Eco*RI site of pVL1393, yielding the baculovirus transfer vectors pVL1393/cgkA and pVL1393/cgkE. The correct orientation of the inserted cDNA with respect to the polyhedrin promoter and the presence of the desired mutation were confirmed by restriction analysis and sequencing, respectively. Recombinant baculoviruses were generated as described previously (Feil et al., 1993a). Both mutant proteins were purified from Sf9 cell extracts by the procedure outlined above for the wild-type enzyme.

To express the T516E mutant in *E. coli*, a 1.6 kb *Xma*I/*Pac*I fragment was isolated from pUC/cgkE and ligated into the *Xma*I/*Pac*I sites of pET3a/cgk (Feil et al., 1993b). The resulting expression vector pET3a/cgkE was sequenced to confirm the integrity of the desired mutation. The mutant protein was expressed at 37 °C in *E. coli* BL21(DE3) as described for wild-type cGMP kinase (Feil et al., 1993b).

Enzyme Assays. Binding assays were performed according to Landgraf and Hofmann (1989). Equilibrium binding of [^{3}H]cGMP by cGMP kinase was conducted at 4 °C with 0.4 μg of purified enzyme per tube (200 μL reaction volume). The amount of cGMP bound to each site and the apparent K_d value for each site were calculated by the LIGAND computer program (Munson, 1983). The dissociation of [^{3}H]-cGMP from cGMP kinase was measured at 4 °C with 0.4 μg of purified enzyme per tube (200 μL reaction volume). The dissociation was initiated by the addition of 1 mM unlabeled cGMP. The cGMP-binding capacity of cell extracts (2 μg per tube) and the purified enzyme (0.2 μg per tube) was determined at 4 °C in a reaction volume of 100 μL in the presence of 0.7 μM [^{3}H]cGMP and 0.1 μM cAMP. Phosphotransferase activity of the enzyme was measured at 30 °C in the presence of 2 μM protein kinase inhibitor peptide (amino acids 5−24), 40 μM substrate peptide (GRTGRRNSI-amide), and various concentrations of cGMP (Ruth et al., 1991). The assay mixture (100 μL) contained 100 ng of cell extract, 10 ng of purified enzyme, or 20 μg of *E. coli* cell extract.

[3] Nucleotide numbering corresponds to the cDNA of bovine cGMP kinase Iα (Wernet et al., 1989).

Table 1: Affinity Purification of Recombinant cGMP Kinase from Insect Cells with 8-AEA-cAMP−Agarose

preparation	protein (mg)	cGMP binding capacity (nmol/mg)	kinase activity[a] μmol/(min mg)	purification factor (x-fold)[b]	yield[b] (%)
cell extract	673	0.42	0.40	1	100
flow through	507	0.05	0.04	–	8
cGMP eluate	11	8.1	10.9	23	38
gel filtration[c]	–	8.9	10.5	24	–
native enzyme	–	9.0	11.6	–	–

[a] Kinase activity was measured in the presence of 10 μM cGMP. [b] Each value was estimated from the specific cGMP-binding and kinase activity. Both calculation methods yielded similar results, and mean values are presented in the table. [c] Only a small aliquot of the cGMP eluate was subjected to gel filtration.

Protein Derivatization, Proteolytic Digestion, and Peptide Purification. The cysteines of cGMP kinase (50 μg) were reduced with DTT and incubated with 5 μL of vinylpyridine (Friedman et al., 1990). After the protein was desalted via ultrafiltration with an Amicon Centricon unit (10 kDa cutoff), cGMP kinase was incubated with 2 μg of endoproteinase LysC in 50 μL of 0.1 M Tris-HCl for 6 h at 37 °C. The peptide mixture was separated by reversed phase HPLC on a supersphere 60 RP select B column (Merck) at a flow rate of 0.3 mL/min. Solvent A was 0.1% trifluoroacetic acid, and solvent B was 0.1% trifluoroacetic acid in acetonitrile. The gradient was run from 0 to 70% in 70 min. The fractions were monitored at 206 nm and at 254 nm for detection of the pyridylethylated derivatives, which were isolated.

Characterization of the Peptides by Amino-Terminal Sequencing and Mass Spectroscopy. Peptides, which were expected to be phosphorylated, were amino-terminally sequenced by Edman degradation on a Porton LF 3600 gas phase sequencer. The phenylthiohydantion derivatives were identified by reversed phase HPLC. To measure the molecular mass (Covey et al., 1988), the peptides were infused into an atmospheric pressure ionization source fitted to a tandem quadrupole instrument API III (Sciex, Perkin-Elmer).

Miscellaneous Methods. The cAMP affinity material was synthesized by the aqueous coupling of 100 μmol of 8-AEA-cAMP (Biolog) with 10 mL of Affi-Gel 10 (Biorad), a cross-linked agarose support activated with a *N*-hydroxysuccinimide ester on a 10-carbon arm. The protein concentration was measured by the method of Bradford (1976) with bovine serum albumin as a standard. [^{3}H]cGMP was repurified on a poly(ethylenimine)−cellulose column. The peptide substrate GRTGRRNSI-amide was synthesized by solid phase peptide chemistry and HPLC-purified. Purified bovine lung cGMP kinase was obtained according to Landgraf and Hofmann (1989). SDS-PAGE was carried out according to Laemmli (1970) using 9% acrylamide gels. Native PAGE was performed on 6% separating gels in the absence of SDS and without boiling the protein sample prior to loading. Gels were stained with Coomassie Blue R-250. Gel filtration of purified proteins was carried out at room temperature in buffer B using a TSK-G3000SW column (LKB) at a flow rate of 0.8 mL/min. The column was calibrated with protein standards purchased from Boehringer. Ion exchange chromatography was performed on a 1 mL Resource Q column (Pharmacia) at room temperature at a flow rate of 1.0 mL/min. The purified enzyme was loaded onto the column in buffer B and eluted with a linear NaCl gradient from 0 to 550 mM in buffer B in 20 min.

RESULTS

Purification of Recombinant cGMP Kinase from Insect Cells and E. coli. It has been shown that large quantities of soluble and biologically active cGMP kinase Iα can be expressed in insect cells using a recombinant baculovirus (Feil et al., 1993a). The recombinant cGMP kinase was purified from insect cells in a single step to near homogeneity using a cAMP affinity column. cGMP kinase was eluted by low concentrations of free cGMP, which were reduced further to concentrations that did not interfere with the subsequent enzymological characterization of the purified enzyme (see Experimental Procedures). The results of the purification are presented in Table 1 and Figure 1A. cGMP kinase was purified 23-fold with a yield of 38% by the 8-AEA-cAMP−agarose column (Table 1). The purification factor indicated that the cell extract contained about 5% cGMP kinase. The enzyme was apparently homogeneous as demonstrated by SDS-PAGE (Figure 1A). In addition to the cGMP kinase protein band (75 kDa), some faint protein bands with apparent molecular masses below 75 kDa were

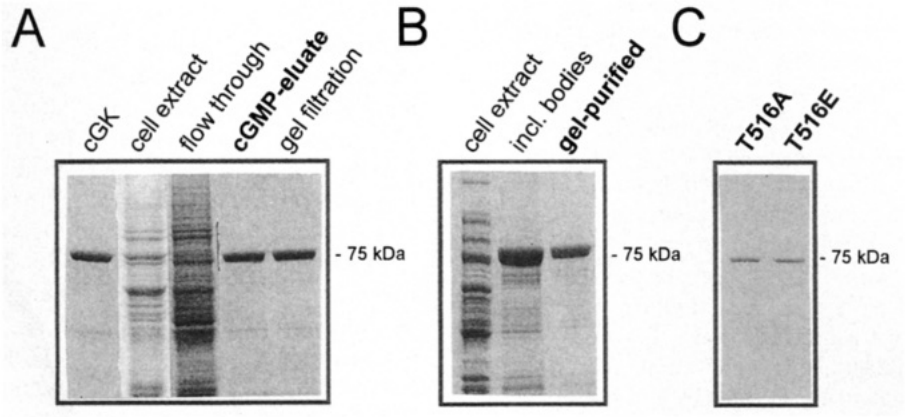


FIGURE 1: Purification of recombinant cGMP kinase. (A) Purification of the wild-type enzyme from insect cell extract by cAMP affinity chromatography, (B) purification of the wild-type enzyme from *E. coli* inclusion bodies by preparative gel electrophoresis, and (C) purification of the T516A and T516E mutants from insect cell extracts by cAMP affinity chromatography. Samples of the last purification step are indicated by bold letters. The amount of protein loaded onto the Coomassie-stained SDS gels was (A) 1.0 μg of pure bovine lung cGMP kinase (cGK), 20 μg of Sf9 cell extract, 20 μg of the flow through of the affinity column, and 1.0 μg of the cGMP eluate before and after gel filtration; (B) 10 μg of *E. coli* cell extract, the same fraction by volume of the insoluble cell fraction (incl. bodies), and 2.0 μg of the gel-purified preparation; and (C) 0.5 μg of the cGMP eluate.

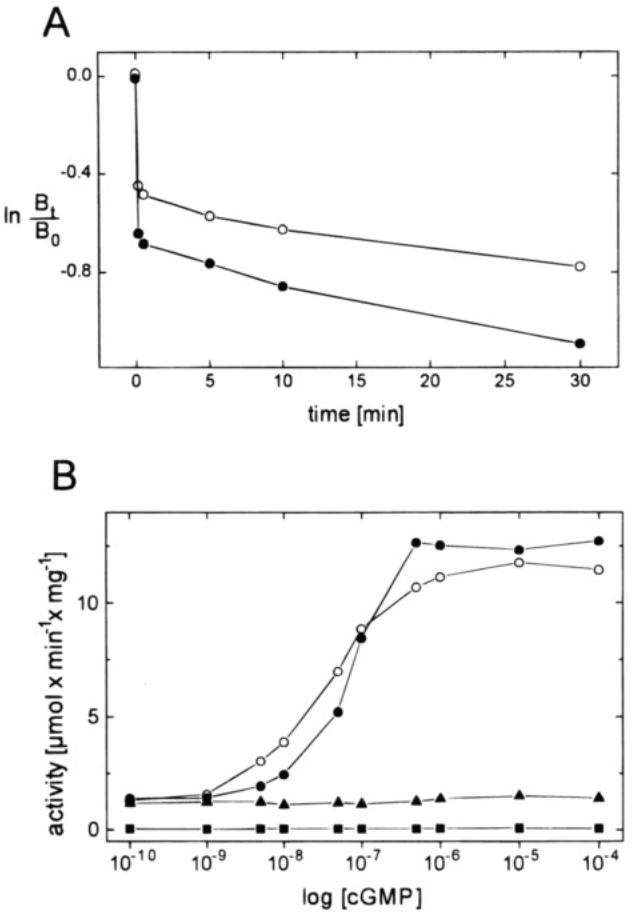


FIGURE 2: Enzymological characterization of the recombinant cGMP kinase purified from insect cells. (A) cGMP dissociation kinetics and (B) cGMP-dependent phosphotransferase activity of the purified native enzyme (○), the recombinant wild-type enzyme (●), the T516A mutant (■), and the T516E mutant (▲).

detected in the SDS gel. These "contaminants" were present not only in the enzyme preparation from insect cells but also in the purified bovine lung enzyme. Apparently these bands represented fragments of cGMP kinase since they were also detected by an anti-cGMP kinase antibody (not shown). The fragments could not be removed by gel filtration of the purified cGMP kinase (Figure 1A). The specific activity of the enzyme was not increased after the gel filtration step (Table 1). Furthermore, the enzyme activity and the band pattern of the purified cGMP kinase remained unchanged during storage, indicating that the protein was not degraded. Most likely, these fragments of cGMP kinase arose during boiling of the sample for SDS-PAGE (North, 1990).

cGMP kinase is expressed in an insoluble and catalytically inactive form in *E. coli* (Feil et al., 1993b). Figure 1B shows that the cGMP kinase is stored mainly in inclusion bodies, which are a good source for isolating the recombinant protein. The cGMP kinase protein could be purified by preparative SDS-PAGE to homogeneity after solubilization of the isolated inclusion bodies (Figure 1B). A single electrophoresis run yielded 15 mg of purified protein from 1.5 L of culture.

Biochemical Characterization of the Recombinant cGMP Kinase Purified from Insect Cells. To ascertain that the Sf9 cells produced an authentic cGMP kinase, the properties of the purified kinase were characterized and compared with those of the pure bovine lung enzyme. The dissociation kinetics of cGMP from the enzyme (Figure 2A) clearly

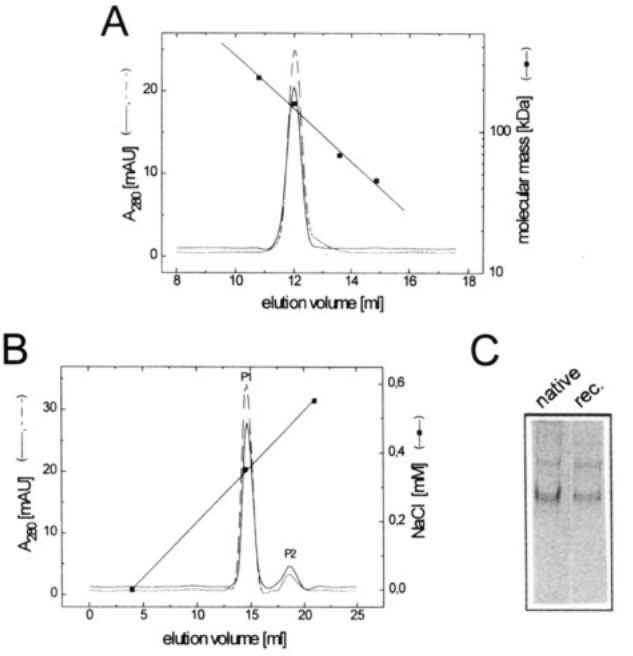
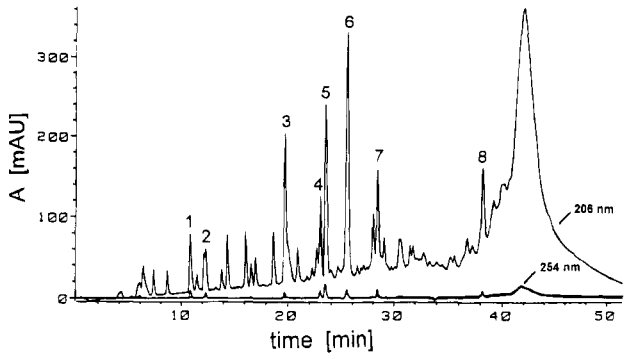


FIGURE 3: Physicochemical analysis of the recombinant cGMP kinase purified from insect cells. (A) Gel filtration, (B) ion exchange chromatography, and (C) native PAGE of the enzyme purified from bovine lung cells (—, native) and from insect cells (- - -, recombinant). The amount of protein subjected to chromatography and electrophoresis was 100 and 3 μg, respectively.

defined a fast-dissociating (low-affinity) site and a slow-dissociating (high-affinity) site. The corresponding k_{-1} values were 3.8 and 0.01 min^{-1} for the recombinant cGMP kinase and 2.7 and 0.01 min^{-1} for the native protein kinase. Equilibrium binding experiments (not shown) indicated that the recombinant enzyme bound 2 mol of cGMP per mole of subunit to a low- and high-affinity site. The apparent K_d values for the recombinant kinase were 52 and 11 nM and for the native enzyme 73 and 9 nM. The phosphotransferase activity (Figure 2B) of the insect cell enzyme was stimulated 10-fold by cGMP with apparent K_a and V_{max} values of 58 nM and 12.5 μmol/(min mg of protein), respectively. In the same experiment, the bovine lung kinase was stimulated 10-fold with apparent K_a and V_{max} values of 43 nM and 11.9 μmol/(min mg of protein), respectively. These data indicated that the cGMP-binding and catalytic properties of the recombinant cGMP kinase were indistinguishable from those of the native bovine lung cGMP kinase Iα.

The recombinant cGMP kinase had the same physicochemical properties as the native enzyme (Figure 3). Both proteins eluted as a single peak from a gel filtration column (Figure 3A). The molecular mass of 150 kDa was in agreement with the known dimeric structure of the native holoenzyme. Ion exchange chromatography of the recombinant and the native enzyme also resulted in an identical elution profile (Figure 3B). In each case, about 90% of the loaded protein eluted at 350 mM NaCl (P1) and 10% at 450 mM NaCl (P2). Both peaks, P1 and P2, contained kinase activity that was stimulated by cGMP and migrated as a single band of 75 kDa in a SDS gel (not shown). The relative amount of P1 and P2 was not changed when the protein kinase sample was preincubated with DTT or saturating concentrations of cGMP before application to the ion exchange column. P2 could not be converted to P1 by rechromatography. These findings suggested that the two peaks of cGMP kinase were not caused by oxidation of the

A



B

fraction no.	sequence
1	^{593}L C-pe R D N P S E.....
2	^{322}H L I G G L D D V D N K^{333}
3	^{42}C-pe Q S V L P V P S T.....
4	^{514}T W [T] F C-pe G T P E Y V A P E I I L N K^{532}
5	^{617}W F E G.....
6	^{629}G T L T P.....
7	^{105}N L E L S Q I.....
8	^{533}G H D I S.....

FIGURE 4: Isolation of the potentially phosphorylated peptide T^{514}–K^{532}. (A) HPLC elution profile of the reduced, pyridylethylated, and proteolyzed insect cell cGMP kinase monitored at 206 nm (upper profile) and 254 nm (lower profile). (B) Corresponding amino acid sequences of the numbered fractions, which were detected at 254 nm. The position of each identified peptide in the primary sequence of cGMP kinase is indicated. Peptides that were not completely sequenced are extended by a dotted line. Amino acids that could not be detected at the expected position are indicated by square brackets. The pyridylethylated derivative of cysteine is represented by C-pe.

protein or by a mixture of cGMP-free and cGMP-containing enzyme (Wolfe et al., 1987). The recombinant and the native cGMP kinase were also separated into several species during electrophoresis under nondenaturing conditions (Figure 3C). This behavior supports the idea that the recombinant cGMP kinase has the same properties as the native bovine lung enzyme. Most likely, the multiple enzyme species were caused by multiple phosphorylation of the enzyme in the amino-terminal region (Aitken et al., 1984).

Analysis of the Phosphorylation State of Active and Inactive cGMP Kinase. The phosphorylation state of the two potentially modified threonine residues Thr-514 and Thr-516 was analyzed by following a nonradioactive strategy. Thr-514 and Thr-516 are located in a peptide that is flanked by two lysine residues and contains a cysteine (Takio et al., 1984; Wernet et al., 1989). To identify the peptide T^{514}–K^{532}, the cysteines of cGMP kinase were labeled with vinylpyridine, allowing the selective detection of the relevant peptide at 254 nm. The labeled protein was digested with endoproteinase LysC, and the peptide mixture was separated by reversed phase HPLC (Figure 4A). The peptides of the fractions, which contained pyridylethylated cysteines, were amino-terminally sequenced (Figure 4B). Fraction 4 contained the sequence from Thr-514 to Lys-532 with the potentially phosphorylated threonine residues. Thr-514 was detected at the expected position. The expected amino acid at cycle three (Thr-516) could not be identified (Figure 4B), indicating that Thr-516 of the functional cGMP kinase was modified. The mass spectrum of peptide T^{514}–K^{532} derived from the active enzyme (Figure 5) yielded a molar mass of

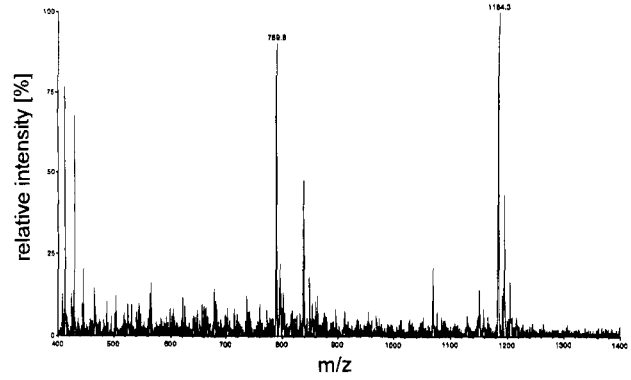


FIGURE 5: Ion spray mass spectrum of peptide T^{514}–K^{532} derived from the insect cell cGMP kinase. The peaks with a mass-to-charge ratio (*m/z*) of 789.8 and 1184.3 represent triple- and double-charged species of the ionized peptide, respectively. The molar mass (*M*) was calculated from the *m/z* peaks in the charge distribution file using the formula $M = n[(m/z) - 1]$ where n is the charge state of the peptide species (Covey et al., 1988).

Table 2: Molar Mass of Peptide T^{514}–K^{532} Derived from Insect Cells or *Escherichia coli*

	molar mass (g/mol)		
peptide from	sequence	MS	net difference
insect cells	2288	2366	78
E. coli	2288	2288	0

2366 g/mol (Table 2). The molar mass of this peptide deduced from its amino acid sequence is 2288 g/mol. The difference of 78 g/mol corresponds to an additional phosphate group. The combined results from sequence analysis and mass spectroscopy unambiguously demonstrated that the biologically active cGMP kinase purified from insect cells was phosphorylated at Thr-516.

The same procedure described above for the insect cell enzyme was used to isolate the peptide T^{514}–K^{532} from the inactive cGMP kinase expressed in *E. coli*. During amino acid sequencing of the peptide, Thr-514 and Thr-516 were detected at the expected positions (not shown). The molar mass of this peptide measured by mass spectroscopy was 2288 g/mol, identical with the molar mass calculated from the sequence (Table 2). These results indicated that the catalytically inactive cGMP kinase purified from *E. coli* inclusion bodies was not phosphorylated at Thr-514 or Thr-516.

Analysis of the Functional Importance of Phosphothreonine-516 by Site-Directed Mutagenesis. The functional significance of the identified phosphothreonine residue was addressed by substitution of Thr-516 by alanine and by glutamate to yield the cGMP kinase mutants T516A and T516E, respectively. Both mutant cGMP kinases were expressed in insect cells as soluble proteins and could be purified to homogeneity by cAMP affinity chromatography as described for the wild-type cGMP kinase (Figure 1C). The successful purification indicated that the cyclic nucleotide binding properties of either mutant were intact. This was confirmed by cGMP binding studies. Both mutant proteins bound cGMP to one slowly and one rapidly, exchanging site as the wild-type enzyme (not shown). However, the phosphotransferase activity of the purified mutant proteins was drastically altered when compared to that of wild-type cGMP kinase (Figure 2B). The T516A mutant showed no detectable kinase activity. Interestingly,

the T516E mutant had a basal phosphotransferase activity of 1.3 μmol/(min mg of protein) comparable to the wild-type enzyme. This activity was not stimulated by cGMP. The cGMP-independent activity was not due to degradation of the purified protein or due to a contamination of the sample with endogenous Sf9 proteins as indicated by SDS gel analysis (Figure 1C) and by the lack of phosphotransferase activity of the T516A mutant (Figure 2B). These findings demonstrate that phosphothreonine-516 is required for a functional catalytic core of cGMP kinase, whereas the glutamate side chain can only partially provide the functions of the phosphate group at Thr-516.

A possible reason for the expression of a biologically inactive cGMP kinase in *E. coli* was that the bacterially synthesized protein lacked the phosphate at Thr-516 (see above). This hypothesis was tested by expression of the T516E mutant in *E. coli*. The exchange of Thr-516 by glutamate did not lead to the expression of a catalytically active kinase in bacteria and did not improve the solubility of the recombinant protein (not shown). These results suggest that the absence of the phosphate at Thr-516 is not the cause of the incorrect folding and inactivity of the bacterially expressed cGMP kinase.

DISCUSSION

As noted previously (Feil et al., 1993a), a functional active cGMP kinase was expressed in insect cells. Milligram quantities of the enzyme have been purified from the cytosol to apparent homogeneity by a simple one-step procedure. The purified recombinant cGMP kinase was a dimer of the 75 kDa subunit and bound 2 mol of cGMP per mole of subunit to a low- and a high-affinity site with characteristics that were indistinguishable from that of the purified native bovine lung enzyme. Furthermore, the phosphotransferase activity of the recombinant enzyme was stimulated approximately 10-fold by cGMP with a K_a value that did not differ from that of the native kinase. The insect cell enzyme had the same specific activity as the purified lung enzyme. In contrast, the recombinant protein synthesized in *E. coli* was inactive, although its relative mobility on a SDS gel was identical with that of the native enzyme, indicating complete translation of the coding sequence. These results suggested that only the cGMP kinase expressed in insect cells was correctly folded and presumably post-translationally modified at the same positions as the enzyme isolated from mammalian tissues.

This hypothesis was further supported by the finding that Thr-516 was phosphorylated *in vivo* only in the insect cells but not in *E. coli*. Thr-516 was quantitatively phosphorylated in the Sf9 cells. An unmodified form of peptide T^{514}–K^{532} could not be detected by mass spectroscopy in the sample of fraction 4. The nonphosphorylated peptide could have been missed since it might have been eluted at a slightly different position from the reversed phase column than the phosphorylated peptide. However, this possibility is very unlikely since all fractions containing pyridylethylated cysteines were sequenced, but only fraction 4 yielded the sequence T^{514}–K^{532}. Therefore, the functional cGMP kinase Iα was quantitatively phosphorylated at Thr-516 in insect cells. This stoichiometry is in excellent agreement with the finding that cGMP kinase purified from bovine lung contained 1 mol of phosphate per mole of subunit in a metabolically stable linkage, which was not removed by phosphatase treatment or labeled by ^{32}P in an incubation with [^{32}P]γATP (Hofmann & Flockerzi, 1983).

Table 3: Activating Threonine/Serine Phosphorylation Sites (Boxed) of Several Protein Kinases in the Catalytic Core Region Corresponding to Thr-516 of cGMP Kinase[a]

cGPK	DFG	...	^{514}T	W	[T]	F	C	...	APE
cAPKα		...	^{195}T	W	[T]	L	C	...	
CDK2		...	^{159}Y	[T]	H	E	V	...	
MAPK		...	^{182}L	[T]	E	Y	V	...	
MAPKK		...	^{219}A	N	[S]	F	V	...	
PKCα		...	^{495}T	R	[T]	F	C	...	
CKII		...	^{186}Y	N	V	R	V	...	
CAMII		...	^{170}W	F	G	F	A	...	
MLCK		...	^{450}L	K	V	L	F	...	
PHK		...	^{180}L	R	E	V	C	...	

[a] The sequences were aligned according to Hanks and Quinn (1991).

The functional role of phosphothreonine-516 was investigated by site-directed mutagenesis of Thr-516 to alanine and to glutamate. In insect cells, both mutant proteins were expressed in a soluble form with functional cGMP binding domains, indicating that the amino acid substitution did not interfere with the general folding of each protein species. Exchange of Thr-516 by alanine renders the protein inactive in its phosphotransferase function, demonstrating that phosphotreonine-516 is an essential residue in the catalytic core region of cGMP kinase. Interestingly, when Thr-516 is substituted by glutamate, the basal catalytic activity but not the stimulatory effect of cGMP is retained. This finding suggests that the negative charge of the glutamate side chain can provide at least some of the interactions of phosphothreonine-516 in the wild-type enzyme.

Site-directed mutagenesis and the crystal structure of several protein kinases have suggested that phosphorylation of a threonine in the highly conserved catalytic core region of protein kinases is important for their function (Taylor & Radzio-Andzelm, 1994; Wei et al., 1994). Table 3 shows that the phosphorylated Thr-516 of cGMP kinase corresponds to the stably phosphorylated Thr-197 of cAMP kinase (Shoji et al., 1979) and that many other protein kinases also contain serine/threonine phosphorylation sites in this general region between the nearly invariant sequence motivs "DFG" and "APE". Phosphorylation of cAMP kinase at Thr-197 drastically improved its enzymatic activity (Steinberg et al., 1993). The phosphate of Thr-197 apparently contributes to the correct folding of the catalytic center through multiple interactions with nearby side chains (Knighton et al., 1991). Cyclin-dependent protein kinase (Gu et al., 1992) and mitogen-activated protein kinase (Payne et al., 1991) are

phosphorylated in this region by separate protein kinases. In both cases, this modification is essential for the catalytic function. The crystal structure of both proteins indicated that phosphorylation of the respective threonine could activate the phoshotransferase activity of these enzymes by a mechanism similar to that of cAMP kinase (De Bondt et al., 1993; Zhang et al., 1994). Mitogen-activated protein kinase (Alessi et al., 1994) and protein kinase C (Cazaubon et al., 1994) contain an activating phosphorylation site at the corresponding position. Other serine/threonine protein kinases such as ribosomal S6 kinase (Sutherland et al., 1993) or β-adrenergic receptor kinase (Benovic et al., 1989) are phosphorylated in this general region or contain potential phosphate acceptor sites in this segment, respectively (not shown). Autophosphorylation of many protein tyrosine kinases, e.g. of the insulin receptor (Tornqvist & Avruch, 1988), takes place in an analogous region (not shown). Thus, the phosphorylation of a threonine, serine, or tyrosine in the catalytic core appears to be a common necessity for the phosphotransferase activity of distantly related members of the protein kinase superfamily.

On the other hand, there are protein kinases that do not contain a threonine or serine at positions analogous to Thr-516 of cGMP kinase (Table 3), and therefore, phosphorylation in this region is unnecessary for the activity of these enzymes. Interestingly, these proteins, i.e. casein kinase II (Grankowski et al., 1991), Ca^{2+}/calmodulin-dependent protein kinase II (Hagiwara et al., 1991), and the catalytic domains of myosin light chain kinase (Bagchi et al., 1989) and phosphorylase kinase (Cox & Johnson, 1992), were expressed in *E. coli* as active protein kinases. With one exception, protein kinases that need a phosphorylated residue in the catalytic core region are not expressed in an active form in *E. coli*. Only cAMP kinase could be expressed as an active kinase in *E. coli* (Slice & Taylor, 1989), apparently since Thr-197 can be phosphorylated in an autocatalytic reaction (Yonemoto et al., 1993; Steinberg et al., 1993). In contrast to the functional cGMP kinase from Sf9 cells, the catalytically inactive cGMP kinase expressed in *E. coli* was not phosphorylated at Thr-516. Provided that the absence of this modification caused the misfolding of recombinant cGMP kinase in bacteria, the T516E mutant, which was synthesized as a soluble, principally active enzyme in insect cells, should also be expressed successfully in *E. coli*. However, the glutamate mutant was produced in *E. coli* as a catalytically inactive protein, mainly in the form of inclusion bodies as was the case for the wild-type enzyme. Thus, factors other than the lack of the phosphate at Thr-516 interfere with the correct folding of cGMP kinase in *E. coli*.

In conclusion, the identification of the stable *in vivo* phosphorylation site of cGMP kinase Iα at Thr-516 and the assignment of its essential functional role described in this report are important steps for the understanding of the structure–function relationships of this particular enzyme. Furthermore, this finding extends the emerging general view that the activity of many protein kinases depends on the phosphorylation state of residues in the catalytic core.

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