Phosphorylation Modulates Catalytic Function and Regulation in the cAMP-Dependent Protein Kinase†

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ABSTRACT: Site-directed mutagenesis was used to remove a critical phosphorylation site, Thr-197, near the active site of the catalytic subunit of cAMP-dependent protein kinase. This residue is present in a number of protein kinases, and its phosphorylation largely influences catalytic activity. We changed Thr-197 to aspartic acid and alanine and measured the effects of these substitutions on the kinetic mechanism and inhibitor affinities. The mutants were expressed as the free catalytic subunit and as soluble fusion proteins of glutathione-*S*-transferase. The values for K_{ATP} and $K_{peptide}$ for all three mutants are raised by approximately 2 orders of magnitude relative to the wild-type enzyme. Viscosometric measurements indicate that elevations in $K_{peptide}$ are the result of reduced rates for phosphoryl transfer and not reduced substrate affinities. This implies that the loop that contains the phosphothreonine, the activation loop, does not reduce access to the substrate site as proposed for the inactive forms of cdk2 kinase [DeBont, H. L., et al. (1993) *Nature 363*, 595−602] and MAP kinase [Zhang, F., et al. (1994) *Nature 367*, 704−711]. The mutants associate slowly with the wild-type regulatory subunit, although the cAMP-free wild-type regulatory subunit inhibits the mutants stoichiometrically. A mutant regulatory subunit that binds cAMP poorly and rapidly inhibits the wild-type catalytic subunit does not inhibit the mutant proteins. These data suggest that the phosphothreonine region serves as a docking surface for the regulatory subunit in the holoenzyme complex. Under physiological conditions, the catalytic efficiency of the mutants is lowered by as much as 500-fold, implying that phosphorylation of this and other protein kinases in the activation loop provides a molecular switch for enzymic function.

Phosphorylation mediates the function of many proteins and enzymes in the cell. The catalysts responsible for this reversible modification are the protein kinases and protein phosphatases. In the last decades the discovery of new protein kinases has expanded geometrically (Hanks et al., 1988). They are the communication links between the plasma membrane and the internal structures of the cell. From the simplest metabolic processes to the elaborate orchestration of cell division, protein kinases play a key role in cell function and maintenance. Since their activity greatly alters the fate of the cell and the whole organism, their catalytic power is kept in check by a number of mechanisms. Ironically, the same chemical modifications that are used by protein kinases to alter the function of other proteins are used also to control the protein kinases. In this way phosphorylation cages the catalytic machinery of the eukaryotic cell so that it can be meted out at necessary moments.

The study of the catalytic properties of protein kinases is hampered by the inherent structural complexity of the protein kinase and its solubility in bacterial hosts. These problems are abrogated for the catalytic subunit of cAMP-dependent protein kinase (cAPK).[1] This enzyme is a tetramer of a regulatory dimer (R_2) and two catalytic subunits (C) that is easily dissociated by cAMP. The C-subunit is stable, small (40 800 kDa), and expressed abundantly in a soluble form in *Escherichia coli* (Slice & Taylor, 1989). The C-subunit phosphorylates serine and threonine residues in the consensus sequence, R-R-X-S/T-Hyd, where X is variable and Hyd is any hydrophobic amino acid (Kemp et al., 1977; Zetterqvist et al., 1990). The phosphoryl donor in the reaction is ATP, which requires a divalent metal for binding and catalysis (Armstrong et al., 1979). The γ phosphate is transferred to the hydroxyl group through direct, in-line displacement based on stereochemical precedence (Ho et al., 1988). Although a fixed metaphosphate intermediate is plausible, the arrangement of active site residues supports nucleophilic attack of the hydroxylic oxygen on the terminal phosphate of ATP (Zheng et al., 1995). The kinetic mechanism is steady-state random, although the primary association of ATP is favored over the heptapeptide substrate, LRRASLG, on thermodynamic grounds (Cook, 1982; Kong & Cook, 1988). The phosphorylation rate constant is fast so that the rate-determining step in k_{cat} is product release (Adams & Taylor, 1992).

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[1] Abbreviations: cAPK, cAMP-dependent protein kinase; C-subunit, catalytic subunit; Caps, 3-(cyclohexylamino)-1-propanesulfonic acid; GST, glutathione-*S*-transferase; GST-C(T197A), glutathione-*S*-transferase linked to C-subunit mutant with threonine-197 to alanine substitution; GST-C(T197D), glutathione-*S*-transferase linked to C-subunit mutant with threonine-197 to aspartate substitution; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; MTCN, 50 mM Mes, 25 mM Tris, 25 mM Caps, and 50 mM NaCl; PKI, protein kinase inhibitor for cAPK; R_I, type I regulatory subunit; R_I(R209K), type I regulatory subunit mutant with arginine-209 to lysine substitution; C(T197D), C-subunit mutant with threonine-197 to aspartate substitution; Tris, tris(hydroxymethyl)aminomethane.

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The recombinant C-subunit is phosphorylated on four residues (F. W. Herberg et al., manuscript in preparation). This posttranslational modification is autocatalytic in bacterial expression systems (Steinberg et al., 1993) but may be catalyzed by another, unidentified protein kinase in eukaryotes. Of the known phosphorylation sites only Thr-197 and Ser-338 are stable against phosphatase treatment (Chiu & Tao, 1978; Shoji et al., 1979). Removal of the other two phosphoryl groups does not hamper catalytic activity (F. W. Herberg et al., manuscript in preparation), and replacement of Ser-338, while it decreases stability, does not have a major effect on catalysis (Yonemoto et al., 1995). The X-ray crystal structure illustrates that the stability of Thr-197 is due to several polar and electrostatic contacts close to the enzyme's active site (Knighton et al., 1991a,b). In fact, some of these contacts provide the only bridge between the small and large domains of the C-subunit, implicating the phosphothreonine as a direct link between the lobes of the protein. The phosphoryl group of Thr-197 makes direct contacts with the catalytic loop at the substrate site (R_{165}DLKPEN) and is close in primary sequence to the metal-binding loop (D_{184}-FG). Both of these contacts are made through the ligands for the phosphothreonine and could impact both catalysis and substrate and nucleotide binding. Since the tertiary structures of the catalytic cores of protein kinases are likely to be similar, we suspect that regulation by phosphorylation within this conserved core may share similarities. We have used site-directed mutagenesis to change Thr-197 to aspartic acid and alanine to probe the significance of this critical phosphorylation site with respect to substrate processing and inhibitor binding. The results from this study establish Thr-197 as a key residue for ATP and regulatory subunit binding and phosphoryl transfer but not for the binding of substrate peptides. This paper outlines a detailed mechanistic appraisal of protein phosphorylation and its effects on catalysis and regulation for an archetypal protein kinase.

MATERIALS AND METHODS

Materials. Adenosine 5′-triphosphate (ATP), phosphoenolpyruvate, magnesium chloride, reduced nicotinamide adenine dinucleotide (NADH), 2-(*N*-morpholino)ethanesulfonic acid (Mes), tris(hydroxymethyl)aminomethane (Tris), 3-(cyclohexylamino)-1-propanesulfonic acid (Caps), pyruvate kinase (rabbit muscle), and lactate dehydrogenase (bovine heart) were purchased from Sigma. Sucrose was purchased from Mallinchrodt. Sodium chloride and glycerol were purchased from Fisher Scientific. Restriction endonucleases, T4 ligase, T7 polymerase, and RNase A were purchased from United States Biochemical or GIBCO/BRL. Radioactive nucleotides were purchased from Amersham or NEN Dupont. Media supplies were purchased from Difco. Various reagents and resins used for chromatography were purchased from Pharmacia, Sigma, Whatman, and Boeringer Mannheim. Custom oligonucleotides were synthesized with an Applied Biosystem DNA synthesizer, Model 380B. The following bacterial strains were used: *E. coli* RZ1032-(ATCC), *E. coli* JM101(ATCC), and *E. coli* BL21-(DE3) (gift from William Studier of Brookhaven National Laboratories, Upton, NY). The following vectors were used: phagemid pUC119 (ATCC), pLWS-3 (Slice & Taylor, 1989), pUC4K vectors(ATCC), and pGEX-KG (gift from Jack Dixon of Purdue University, West Layayette, IN).

Construction of Mutant Enzymes. Mutagenesis of Thr-197 was carried out as previously described (Yonemoto et al., 1991) using single-stranded Kunkel template. The mutant proteins are referred to as C(T197D) and C(T197A). These mutants were also subcloned into a modified GST fusion system. An *Nde*I site was created and an *Nco*I site was eliminated using a synthetic linker in the GST fusion vector, pGEX-KG (Guan & Dixon, 1991). The *kan* gene generated from pUC4K (Viera & Messing, 1982) was subcloned into the *Pst*I site of pGEX-KG. This rendered the resistant marker for ampicillin inactive and gave the plasmid kanamycin resistance. Insertion and orientation of the *kan* gene was confirmed by sequencing transformants which conveyed kanamycin resistance. The C(T197A,D) mutants were then subcloned into this modified pGEX-KG expression vector via *Nde*I and *Sst*I sites. Inserts were checked for orientation by sequencing, and protein expression checks were done as previously described. The fusion proteins are referred to as GST-C(T197D) and GST-C(T197A).

Peptide and Protein Purification. All peptides were synthesized at the Peptide and Oligonucleotide Facility at the University of California, San Diego. Peptides were purified by reverse-phase preparative HPLC chromatography. The concentration of some peptides was determined by turnover with the C-subunit under conditions of limiting peptide. The wild-type (R_I) and the mutant regulatory subunits R_I(R209K) were purified by previously published procedures using anion-exchange chromatography (León et al., 1991). The R_I(R209K) mutant was engineered as described previously and is defective in binding cAMP to site A (Bubis et al., 1988). In this form, the wild-type R_I subunit and R_I(R209K) have 2 and 1 equiv of cAMP bound, respectively. cAMP was removed from the wild-type regulatory subunit by urea-induced unfolding as previously described (Buechler et al., 1993). The recombinant wild-type C-subunit was expressed and purified from *E. coli* according to previously published procedures (Yonemoto et al., 1991). The concentration of proteins is measured by A_{280} ($A_{0.1\%} = 1.2$ for the C-subunit and 1.0 for the R_I-subunit). The concentration of GST-fused C-subunits was measured by titration with PKI. C(T197D) was purified by a modified version of the above procedure. After the mutant was bound to the P11 resin, the column was washed with 200 mL of 30 mM MES (pH 6.5), 1 mM EDTA, and 5 mM β-mercaptoethanol and 400 mL of 125 mM potassium phosphate (pH 6.5) and 5 mM β-mercaptoethanol. A 600-mL linear gradient of 125 to 500 mM potassium phosphate (pH 6.5) was run, and 10-mL fractions were collected. The active fractions were pooled and used directly for kinetic analysis.

GST-C(T197A) and GST-C(T197D) were purified by binding to a glutathione−agarose resin. Cells harvested from a 6-L growth culture were frozen at −80 °C overnight. The frozen cells were resuspended in 90 mL of 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 5 mM dithiothreitol. This suspension was then passed through a cold (4 °C) French pressure cell two times at 1000 psi. An equal volume (90 mL) of 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 1 mM dithiothreitol was added, and the lysate was clarified by centrifugation (4 °C) for 45 min at 15 000 rpm. The supernatant was batch bound to 7 mL of swelled glutathione cross-linked 4% beaded agarose for 4 h. The resin was poured into a

short column and then washed with 10 column volumes of 50 mM Tris, pH 7.5, 500 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 5 mM dithiothreitol. The resin was then mixed in the column with 10 mL of 10 mM glutathione in 50 mM Tris (pH 7.5), 500 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 5 mM dithiothreitol for 2 h. The resin was allowed to settle, and the flow-through was then collected. The major protein that eluted was the fusion enzyme. Protein impurities were removed by passing the fractions over an FPLC Mono-Q column pre-equilibrated with 20 mM Tris, pH 8.0, 150 mM NaCl, 0.5 mM dithiothreitol. Active C-subunit mutants flowed through the column.

Kinetic Assay. The enzymatic activity of the C-subunit was measured spectrophotometrically (Cook, 1982). This assay couples the production of ADP with the oxidation of NADH by pyruvate kinase and lactate dehydrogenase. Typically, 2−20 mM ATP was preequilibrated with C-subunit in a buffer containing 1 mM phosphoenolpyruvate, 0.3 mM NADH, lactate dehydrogenase (12 units), and pyruvate kinase (4 units) in a final volume of 1 mL. Reactions were initiated by adding varying amounts of Kemptide (LRRASLG). Inhibition studies were done by preequilibrating ATP, C-subunit, and inhibitor before adding Kemptide. All reactions were done in a buffer containing 100 mM Tris (pH 8.0), 100 mM Mops (pH 7.0), or 50 mM Mes, 25 mM Tris, 25 mM Caps, and 50 mM NaCl (MTCN buffer) either in the presence or in the absence of glycerol or sucrose. The relative viscosity (η^{rel}) of buffers containing glycerol or sucrose was measured relative to a 100 mM Tris buffer at pH 8.0, 24 °C, using an Ostwald viscometer (Shoemaker & Garland, 1962). The pH of the MTCN buffers was adjusted by adding small volumes of concentrated HCl or NaOH. The MTCN buffer is a multicomponent buffer system that maintains constant ionic strength over a wide pH range. All kinetic measurements were performed at 24.0 °C, pH 6−10.8, and 10 mM free Mg^{2+}. The C-subunit was sufficiently stable at both low and high pH extremes within the time frame of ligand preequilibration and assay (data not shown).

Data Analysis. The observed values of k_{cat} and $K_{peptide}$ were determined from double-recriprocal plots of initial velocity vs substrate concentration at fixed, variable concentrations of ATP. All of these plots intersected at a common point on the x axis (1/[substrate] axis). The slopes and intercepts of these plots were then replotted to obtain the true values of k_{cat}, K_{ATP}, and $K_{peptide}$. The pH dependence in $k_{cat}/K_{peptide}$ was fit to eq 1,

$$y = \frac{C}{1 + 10^{(pK_1 - pH)} + 10^{(pH - pK_2)}} \quad (1)$$

where y is the observed $k_{cat}/K_{peptide}$ at a given pH, C is the maximum value of $k_{cat}/K_{peptide}$, and pK_1 and pK_2 are the lower and higher acid dissociation constants, respectively.

RESULTS

Protein Expression and Purification. The wild-type C-subunit of cAPK was expressed at high levels in *E. coli* in both a soluble and an active form. The purification of the enzyme from the crude lysate was accomplished readily by ion-exchange chromatography using phosphocellulose

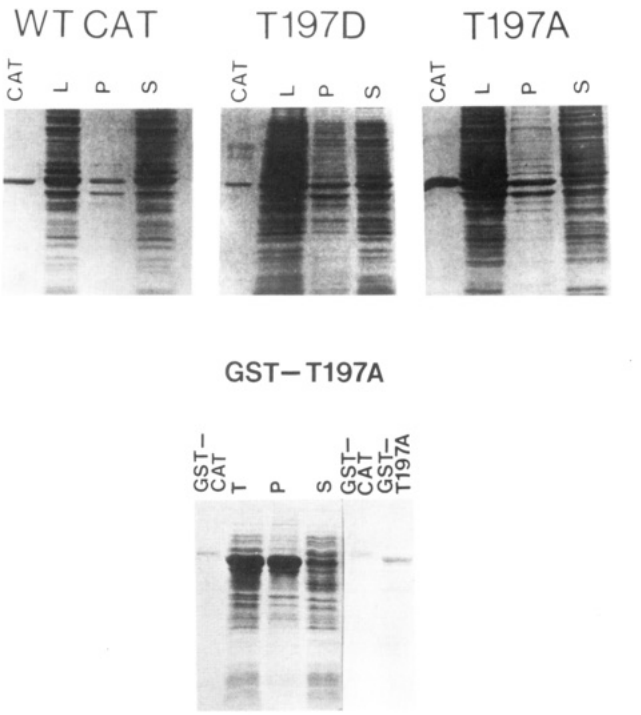


FIGURE 1: Expression and purification of mutant and wild-type C-subunits. Proteins were separated on a 12% SDS−polyacrylamide gel. (A, top) Equal volumes of the total cell lysate (L), pellet (P), and supernatant fractions (S) of wild-type C-subunit, C(T197D), and C(T197A) expressed in *E. coli*. (B, bottom) Equal volumes of the total cell lysate (L), pellet (P), and supernatant fractions (S) of GST-C(T197A) expressed in *E. coli*. The right-hand lane shows GST-C(T197A) after purification by glutathione agarose.

resin. C(T197D) was also expressed at high levels, although approximately one-half of the total protein was insoluble. Figure 1A shows an SDS−polyacrylamide gel of the wild-type and mutant C-subunits after cell lysis. C(T197D) and wild-type C-subunit are expressed as soluble enzymes, although C(T197A) was strictly expressed in an insoluble form. Approximately 100% of the wild-type C-subunit is expressed solubly in the supernatant fraction, while 50% of C(T197D) is expressed in the pellet fraction. Approximately 100% of C(T197A) was expressed in the pellet fraction with none observed in the supernatant. Coupling the N-terminus of C(T197A) to GST helped to solubilize some of this mutant. Figure 1B shows the expression and purification of GST-C(T197A) from crude lysate. Fusion with GST lead to an increase in the quantity of soluble enzyme which could be purified easily by glutathione agarose.

GST-C(T197A) migrated slightly below (i.e., lower molecular weight) the wild-type control. We suspect that the apparent band shift is due not to a proteolytic fragment of GST-C(T197A) but rather to a difference in overall charge caused by the removal of the phosphothreonine. This is supported by several observations. First, the fused mutants have similar K_m's for both ATP and substrate compared to C(T197D) (Table 1). Second, C-subunit that is isolated from bacteria shortly after induction is a mixture of two proteins—one that migrates with the normal C-subunit and has a phosphoryl group on Thr-197 and one that migrates below this that does not have a phosphoryl group on Thr-197 as judged by phosphoamino acid analysis (Steinberg et al., 1993). After further induction times the fast-running band disappears. Third, several GST fusion mutants of the C-subunit (e.g., GST-E230A & GST-D166A) migrate with

Table 1: Steady-State Kinetic Parameters for Wild-Type and Mutant Proteins[a]

protein	k_{cat} (s^{-1})	$K_{peptide}$ (mM)	K_{ATP} (mM)
wild type[b]	20	0.03	0.010
C(T197D)	18 ± 1.4	0.66 ± 0.15	0.65 ± 0.084
GST-C(T197D)	7.9 ± 0.36	0.80 ± 0.08	1.0 ± 0.16
GST-C(T197A)	1.4 ± 0.08	1.1 ± 0.03	1.4 ± 0.20

[a] Measured in pH 7.0 MTCN, 10 mM Mg^{2+}, 24 °C. [b] Taken from Adams and Taylor (1993b).

a lower apparent molecular weight than that of the wild-type control. Upon co-expression with wild-type C-subunit, the mutants migrate with the wild-type protein indicating that Thr-197 is phosphorylated (Slice & Taylor, 1989). Fourth, truncation of the C-subunit by a C-terminal-specific protease creates a mutant form with a molecular weight reduced by 15% that has no catalytic activity (Alhanaty et al., 1979, 1981).

Steady-State Kinetic Analysis of Mutant Proteins. The steady-state kinetic parameters of C(T197D), GST-C(T197D), and GST-C(T197A) were measured in pH 7 MTCN buffer using LRRASLG and ATP as substrates. The production of ADP was coupled to NADH oxidation via pyruvate kinase and lactate dehydrogenase. All reaction rates were independent of the coupling enzyme concentrations and varied linearly with the C-subunit concentration (data not shown). All initial velocity data were collected using variable, fixed amounts of ATP (0.5−10 mM) and variable amounts of LRRASLG (0.15−4.1 mM) under conditions of 10 mM free Mg^{2+}. The data were fit to an initial velocity equation for a random, sequential bisubstrate reaction. All double-reciprocal plots of velocity vs substrate concentration were linear and intercepted at the substrate axis (data not shown). Table 1 summarizes all the steady-state kinetic parameters for all three mutants and the wild-type C-subunit.

pH Dependencies of Kinetic Parameters. The steady-state kinetic parameters for C(T197D) were also measured as a function of pH in MTCN buffer. Initial velocity data were collected under conditions of saturating ATP (8 mM), 10 mM free Mg^{2+}, and variable LRRASLG (0.25−2.0 mM). The values of k_{cat} were independent of pH between 6.6 and 9.6 and gave an average value of 18 ± 1 s^{-1}. The data for $k_{cat}/K_{peptide}$ are bell-shaped and gave $k_{cat}/K_{peptide}^{max}$ of 34 ± 3 mM^{-1} s^{-1}, pK_1 of 6.7 ± 0.12, and pK_2 of 9.4 ± 0.12. This compares with the wild-type values of $k_{cat}/K_{peptide}^{max}$ of 880 mM^{-1} s^{-1}, pK_1 of 6.5 , and pK_2 of 9.3 (Adams & Taylor, 1993b). Increasing the concentration of ATP by 20% at different pH values at a constant concentration of free Mg^{2+} did not change the observed initial velocities, indicating that the nucleotide concentration was saturating (data not shown).

Viscosity Effects on Mutant Enzyme Catalysis. The steady-state kinetic parameters k_{cat} and $k_{cat}/K_{peptide}$ for C(T197D) and GST-C(T197A) were measured as a function of the relative solvent viscosity at saturating ATP concentration and 10 mM free Mg^{2+} in pH 8 MTCN. The observed steady-state kinetic parameters were plotted as the ratio of the parameter in the absence and presence of added viscosogen (glycerol or sucrose) as a function of the relative viscosity of the buffer. All plots were linear with the slope values falling within the theoretical limits of 0 and 1. Table 2 summarizes the slope values for mutant and wild-type C-subunits. All the observed reaction velocities were dependent linearly on C-subunit concentration, indicating that added viscosogen did not affect the ability of the coupling agents to monitor ADP production (data not shown).

Table 2: Viscosity Effects on the Wild-Type and Mutant Protein Kinetics[a]

protein	$(k_{cat})^{\eta}$	$(k_{cat}/K_{peptide})^{\eta}$	k_3 (s^{-1})[e]	k_4 (s^{-1})[e]
wild type[b]	1.0	1.0	≥220	22
C(T197D)[c]	0.34 ± .050	~0	27 ± 3	53 ± 9
GST-C(T197A)[d]	0.17 ± .028	~0	3.6 ± 0.27	18 ± 3.2

[a] Measured in pH 8.0 MTCN in the absence and presence of glycerol or sucrose (see Materials and Methods), 10 mM Mg^{2+}, and varied LRRASLG, 24 °C. [b] Taken from Adams and Taylor (1993b). [c] Measured using 8 mM ATP, 18 mM $MgCl_2$, and 0.24−2.0 mM LRRASLG. [d] Measured using 10 mM ATP, 20 mM $MgCl_2$, and 0.47−2.8 mM LRRASLG. [e] $k_3 = k_{cat}/[1 - (k_{cat})^{\eta}]$; $k_4 = k_{cat}/(k_{cat})^{\eta}$ (Adams & Taylor, 1992).

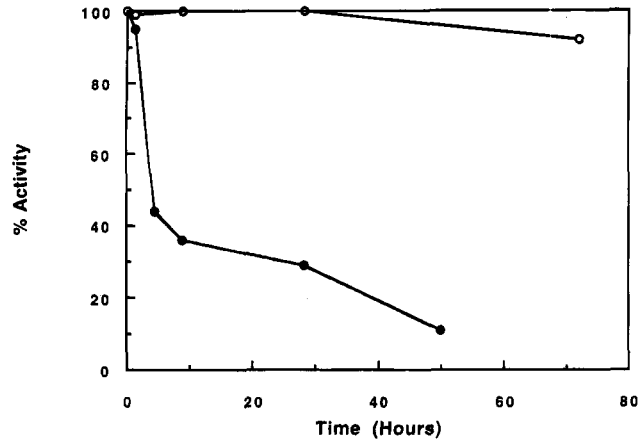


FIGURE 2: Percent change in the activity of wild-type C-subunit (●) and C(T197D) (○) as a function of time in the presence of R_I-subunit (see Results).

Inhibitor Binding Studies. The affinities of wild-type and mutant C-subunits for wild-type and mutant forms of the regulatory subunit were measured by two techniques. For the first method, C(T197D) and wild-type C-subunit (3 μM) were combined with wild-type regulatory subunit (6 μM) in a final volume of 800 μL and dialyzed for 70 h with 0.5 L of 50 mM potassium phosphate, 1 mM dithiothreitol, 18 mM $MgCl_2$, 8 mM ATP, and 5% glycerol, pH 6.5. At various times 40-μL aliquots were removed and assayed by the coupled enzyme assay using 18 mM $MgCl_2$, 8 mM ATP, and 195 μM Kemptide in 100 mM MOPS, pH 7.0. The amount of free C-subunit remaining in the initial mixture was determined by the ratio of the activity in the presence and absence of 10 μM cAMP. The time-dependent change in free C-subunit is shown in Figure 2. No formation of the holoenzyme complex as judged by activity was observed for C(T197D) after 70 h. The wild-type enzyme readily formed holoenzyme with a $t_{1/2}$ of approximately 4 h.

For the second method, C(T197D), GST-C(T197D), and GST-C(T197A) were mixed with varying amounts of cAMP-free wild-type or mutant regulatory subunits R_I(R209K) or PKI in a 1-mL cuvette containing 5−10 mM ATP, 15−20 mM $MgCl_2$, pH 7.0, and MTCN buffer or 100 mM Tris (pH 8.0). After 3 min of incubation, 100−850 μM Kemptide was added to start the reaction. Figure 3 shows the percentage of observed activity for GST-C(T197A) as a function of inhibitor concentration. PKI and cAMP-free R_I-subunit inhibited stoichiometrically GST-C(T197A), while

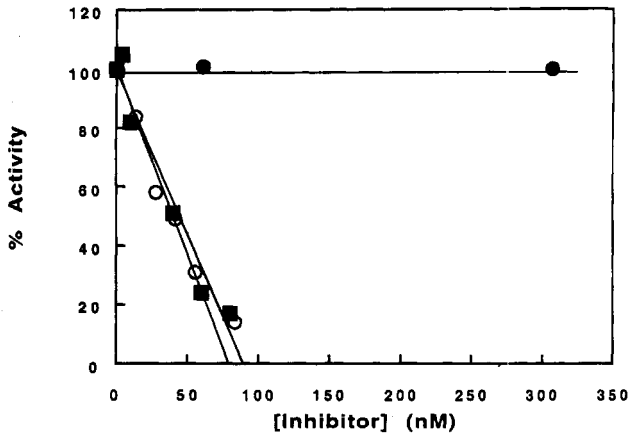


FIGURE 3: Percent change in the activity of GST-C(T197A) as a function of PKI (■), cAMP-free R_I-subunit (○), and R_I [R209K] (●). GST-C(T197A) was preequilibrated with 5 mM ATP, 15 mM $MgCl_2$, and varied inhibitor concentrations for 3 min before 1.4 mM LRRASLG was added to start the reaction (see Results).

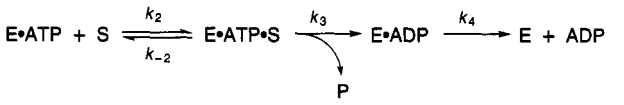
R_I(R209K) had no effect up to 4 equiv. Both C(T197D) and GST-C(T197D) were readily inhibited by equimolar concentrations of PKI; C(T197D) was inhibited stoichiometrically by cAMP-free R_I-subunit (data not shown). However, C(T197D) did not interact stoichiometrically with R_I(R209K). At 1.0 μM R_I(R209K) and 10 nM C(T197D), 70% of the activity remained (data not shown).

DISCUSSION

Although phosphoryl group-induced conformational changes in protein kinases are now widely accepted, the molecular details of these structural movements are not understood for any protein kinase. Insights can now be drawn from the X-ray structure solutions of three protein kinases—the C-subunit of cAPK (Knighton et al., 1991a), cdk2 kinase (DeBont et al., 1993), and MAP kinase (Zhang et al., 1994). These bilobal enzymes are structurally similar, although cdk2 and MAP kinase share only 24% sequence identity with the C-subunit. The large lobe is associated primarily with substrate binding, and the small lobe binds ATP. The catalytic residues, derived from both the large and small lobes, are situated in the cleft created by the two domains. All three enzymes also have an essential phosphorylation site that lies on the surface of the large lobe near the cleft interface (Taylor et al., 1993).

Despite a broad range of similarities between cAPK, MAP kinase, and cdk2 kinase, the crystal structures have important differences. The C-subunit of cAPK is phosphorylated at Thr-197, whereas the homologous residues in cdk2 kinase, Thr-160, and MAP kinase, Thr-183 and Tyr-185, are not phosphorylated. Thus, the structure of cAPK is that of an active enzyme, while those of cdk2 kinase and MAP kinase are inactive. One of the most significant differences in the three structures is in this "activation" loop. The loop that contains Thr-160 in cdk2 (residues 152−170) is significantly displaced relative to Thr-197. DeBondt et al. (1993) argue that this loop movement precludes substrate binding through steric constraints in the active site, although the high-temperature factors suggest that it may be rather distorted in solution. In this model phosphorylation at Thr-160 would then position the loop as in cAPK and permit the association of substrate. In yeast, this activation step for CDC2, a cyclin-dependent kinase related to cdk2, is mediated by another protein kinase and occurs only in the presence of a bound cyclin (Desai et al., 1992; Solomon et al., 1992). This provides a molecular switch for CDC2-linked processes. The dephosphorylated form of the activation loop in MAP kinase (residues 174−187) also shows movement relative to the loop in cAPK. This loop precedes the P+1 loop, which contains residues important for binding the substrate in both structures. The recombinant C-subunit of cAPK is phosphorylated at Thr-197, indicating that this enzyme, at least in *E. coli*, is autoactivated (Steinberg et al., 1993). Although it is not known whether eukaryotic cells recruit an auxiliary protein to process cAPK, the similarities of the three protein kinase structures suggest a common mechanism for activation. While the mechanism for phosphorylating this activation loop differs for each of the three enzymes, all require phosphorylation for full activity. With cAPK, we provide, for the first time, a kinetic means for understanding why this phosphorylation is important for catalysis.

Scheme 1



To test the functional importance of the phosphoryl group on Thr-197, two mutations were made. The first replacement, C(T197D), removes the phosphoryl group but maintains some of the negative charge. The second replacement, C(T197A), removes both the phosphoryl group and the charge. In the latter case, fusion of the N-terminus of the C-subunit with GST was required to increase the solubility of the mutation (Figure 1A,B).

Catalytic Properties of Wild-Type and Mutant C-Subunits. Viscosometric studies were used to define a complete kinetic mechanism for substrate phosphorylation that accounts for all initial velocity data (Adams & Taylor, 1992). This mechanism unifies the steady-state kinetic parameters and the individual steps in the mechanism. Several surprising results stem from this investigation. For the wild-type C-subunit, k_{cat} does not reflect the chemical step of the reaction (i.e., phosphoryl transfer) but rather represents the dissociation of the product, ADP (Adams & Taylor, 1992). Furthermore, $K_{peptide}$ does not reflect the affinity of the substrate but rather reflects a complex combination of rate constants for phosphoryl transfer, product release, and substrate association and dissociation. For wild-type C-subunit, the K_d for LRRASLG was placed at 500 μM (Whitehouse et al., 1983) which compares with the measured $K_{peptide}$ of 30 μM (Table 1). These complications make it difficult, if not impossible, to interpret the effects of point-site replacements on the individual steps within the reaction by steady-state kinetics solely.

Through viscosometric studies on the C-subunit we reduced the complexity of the steady-state kinetic parameters and interpreted them according to a simple reaction mechanism (Scheme 1) involving substrate binding (k_2 and k_{-2}), phosphoryl transfer (k_3), and ADP release (k_4). The dissociation rate constant for phosphorylated peptide is included in k_3 since it is presumed to be much larger than k_4 owing to the large difference in K_d values for both (Whitehouse et al., 1983). The Stokes−Einstein equation relates inversely the diffusion-controlled rate constant for a bimolecular reaction to the intrinsic viscosity of the solvent. The ratio

of this rate constant in the absence and presence of added viscosogen can then be equated with the relative viscosity of the solvent. If we presume that the chemical step is insensitive to solvent viscosity, we can write a rate law for Scheme 1 which includes a relative viscosity term (η^{rel}). We can then express the rate law as the ratio in the absence and presence of added viscosogen. Plots of the ratio of k_{cat} and $k_{cat}/K_{peptide}$ in the absence and presence of added viscosogen vs η^{rel} will be linear. The slopes of these plots are given by the expressions below:

$$(k_{cat})^{\eta} = \frac{k_3}{k_3 + k_4} \tag{2}$$

$$(k_{cat}/K_{peptide})^{\eta} = \frac{k_3}{k_{-2} + k_3} \tag{3}$$

where $(k_{cat})^{\eta}$ and $(k_{cat}/K_{peptide})^{\eta}$ are the slopes of those parameters in the absence and presence of added viscosogen vs η^{rel}. These slope values fall between the theoretical limits of 0 and 1. They can be combined with expressions for k_{cat} and $k_{cat}/K_{peptide}$ to determine or put limits on all the rate constants in Scheme 1. These new equations are represented in the footnotes of Table 2.

The results of this viscosometric analysis are summarized in Table 2. The steady-state kinetic parameters of both mutants show a reduced sensitivity to viscosogen. Although substitution of Thr-197 with aspartic acid has no large effect on k_{cat} (18 vs 7.9 s^{-1}), there are reductions approaching 10-fold in the rate constant for phosphoryl transfer (k_3). The relatively small effect on k_{cat} is due to a 2-fold increase in k_4 and more than a 10-fold reduction in k_3. For GST-C(T197A), the 10-fold reduction in k_{cat} is due to a greater than 60-fold reduction in k_3 with no effect on ADP release. We presume that the moderate effects of mutation on the ADP release rate compared to the K_m for ATP do not reflect differential affinities of these two nucleotides but rather reflect a reduced association rate constant for both. This latter presumption implies that the mutants favor a less productive binding form compared to the wild-type enzyme. Overall, all replacements at Thr-197 lead to marked decreases in their ability to transfer the γ phosphate of ATP to the peptide with little or no effect on the desorption of the products.

The insensitivity of $k_{cat}/K_{peptide}$ to added viscosogens [i.e., $(k_{cat}/K_{peptide})^{\eta} \approx 0$] indicates that substrate dissociates faster than the rate of phosphoryl transfer for the mutants compared to wild-type C-subunit (i.e., $k_{-2} > k_3$). It follows from the expression for $K_{peptide}$ that when substrate rapidly dissociates and k_3 no longer exceeds k_4, $K_{peptide}$ will closely represent the true affinity of the substrate for the binary enzyme form, E·ATP.[2] If we use the K_d of 500 μM for LRRASLG to the wild-type enzyme (Whitehouse et al., 1983), then $K_{peptide}$ for the mutants will either equal or slightly exceed this value. It is likely that, with the inherent error in measuring K_d in the active ternary complex, the true substrate affinity for the mutants is mostly unaffected. This is a clear example illustrating the value of knowing the individual rate constants in a kinetic mechanism. Without knowing the rate constants for each step in the phosphorylation mechanism, we would misinterpret the effects on $K_{peptide}$.

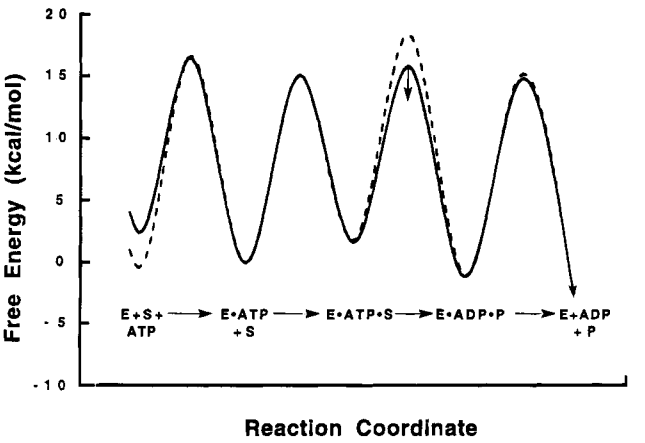


FIGURE 4: Free energy reaction coordinate diagram for wild-type C-subunit and GST-C(T197A). ATP and LRRASLG standard states were set at 1.0 mM and 50 μM. The K_m for the substrate and GST-C(T197A) was used for the K_d. For wild-type C-subunit the K_I of 800 μM for LRRAALG was used for the K_d of the substrate (unpublished results). The association rate constants for the substrate and wild-type C-subunit and GST-C(T197A) were assumed to be equivalent and were set at 0.9 μM^{-1} s^{-1} (Adams & Taylor, 1993b). The dissociation rate constants for ATP and the wild-type C-subunit (Adams & Taylor, 1992) and GST-C(T197A) were assumed to be equivalent since those for ADP were unaffected. The difference in the K_d of ATP for the wild-type and mutant proteins was set by the association rate constants. The internal equilibrium constant was assumed to be unaffected by mutation and was set at 100 (Adams & Taylor, 1993a).

The kinetic data presented in Tables 1 and 2 can be illustrated in a free energy reaction coordinate diagram. Figure 4 compares the phosphorylation of the peptide substrate by wild-type C-subunit and GST-C(T197A) under the standard states of 1.0 mM ATP and 50 μM LRRASLG. This representation shows clearly the overall effects of phosphoryl group removal at Thr-197 on catalytic efficiency. It also pinpoints the real effects of the phosphoryl group on the kinetic mechanism. This free energy plot reveals that only two steps in the overall reaction scheme are altered—the binding of ATP and the rate of phosphoryl transfer. Both substrate binding and product release steps are unaffected. Phosphorylation of the activation loop bestows optimum catalytic flux by stabilizing the binary E·ATP complex relative to its unbound form and by lowering the transition-state energy of the chemical step. We will discuss later how the loop may accomplish this specific task.

The inability of mutations at Thr-197 to influence substrate binding is also reflected in the pH sensitivity of the enzyme reaction. The ionization of two residues is required for maximum activity of the wild-type C-subunit. Plots of $k_{cat}/K_{peptide}$ are bell-shaped with pK values of 6.4 and 9.4 (Adams & Taylor, 1993b). A maximum value for this parameter is achieved at pH $\approx$ 8. Since k_{cat} is insensitive over a wide pH range, Yoon and Cook (1987) concluded that three forms of the ATP-bound enzyme equilibrate in solution with substrate binding to a single form. The pH-dependent steady-state kinetics for C(T197D) conform to this mechanism. For this mutant, k_{cat} is insensitive to pH, and pK_1 and pK_2 are close in value to those of the wild-type enzyme. We conclude from these findings that mutation at Thr-197 does not affect greatly the environment of the ionizing residues

[2] For the mechanism in Scheme 1,

$$K_{peptide} = \frac{k_4}{k_2}\left(\frac{k_{-2} + k_3}{k_3 + k_4}\right)$$

when $k_{-2} \gg k_3$ and $k_3 < k_4$, $K_{peptide} \approx k_{-2}/k_2 \approx K_d$.

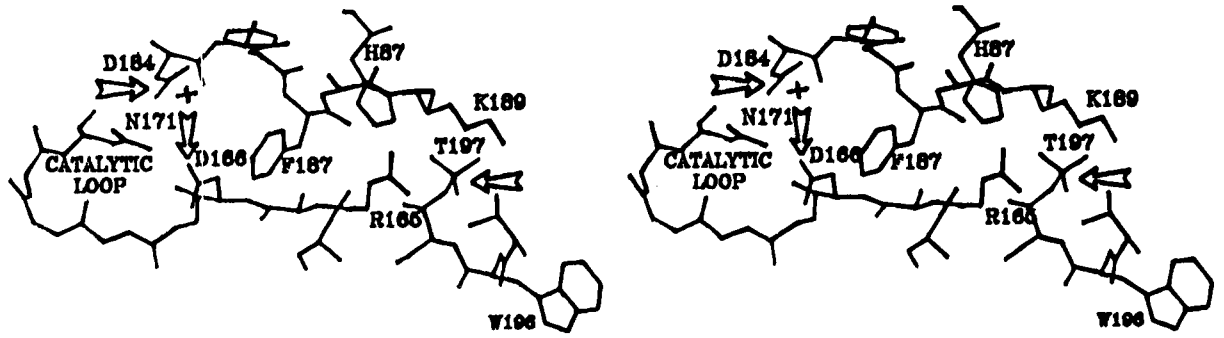


FIGURE 5: Stereoview of the environment around the phosphoryl group of Thr-197. The open arrow on the right indicates the phosphoryl group of Thr-197, while the dotted lines show contacts made with His-87, Arg-165, and Lys-189. The two open arrows on the left point out the metal chelator, Asp-184, and Asp-166 of the catalytic loop (Knighton et al., 1991a).

required for substrate binding and catalysis. It is still unclear what residues control the pH dependence of $k_{cat}/K_{peptide}$. The proposal that the ionization of a general base catalyst is responsible for the lower pK (Yoon & Cook, 1987) is challenged by substrate analog studies (Adams & Taylor, 1993b). Nonetheless, the similar shape of the pH rate profiles for wild-type C-subunit and C(T197D) argues against large structural changes in the mutant.

Inhibitor Binding. The mutations at Thr-197 not only have pronounced effects on ATP binding and phosphoryl transfer but also greatly affect the binding of physiological inhibitors. This effect on the binding of inhibitor proteins is, however, highly selective. Removal of the phosphothreonine severely impacts protein−protein contacts with the R_I-subunit but has little effect on PKI binding. Wild-type R_I-subunit which has 2 equiv of cAMP bound inhibits poorly the activity of C(T197D), implying that the phosphothreonine is an important feature of the interaction surface for the regulatory subunit. This observation is cAMP-dependent since GST-C(T197A) (Figure 3) and C(T197D) were inhibited readily by cAMP-free R_I-subunit. In addition, a mutant regulatory subunit, R_I(R209K), that is defective in binding cAMP to one of its two domains does not inhibit GST-C(T197A) or C(T197D). On the basis of modeling studies of the crystal structure of the catabolite gene activator protein and the R_I-subunit, Arg-209 is likely to interact with the phosphoryl moiety of cAMP (Weber et al., 1987). Taken together, these data imply that the phosphothreonine environment contacts the cAMP-binding domain of the regulatory subunit. It is likely that Arg-209 contributes either directly or indirectly to the binding of the phosphothreonine in the holoenzyme complex. The binding of cAMP would disrupt this contact, thereby weakening the interaction of R_I with the C-subunit. In this way, the phosphothreonine may shuttle between the catalytic and regulatory subunits. It is reasonable to suspect that the ligands to the phosphoryl group of Thr-197 are also part of the recognition surface for R_I. In the yeast C-subunit, mutation of the residue equivalents of Thr-197, Lys-189, Arg-165, and His-87 leads to reductions in the affinity of these mutants for the regulatory subunit (Gibbs et al., 1992).

The Thr-197 mutants were inhibited stoichiometrically by PKI, while C(T197D) and GST-C(T197A) were inhibited stoichiometrically by cAMP-free R_I-subunit. This does not mean that either of these inhibitors shows normal affinity for the C-subunit mutants. This follows from the use of the high enzyme concentration needed to monitor inhibitor-dependent activity decreases. Only an upper limit can be placed on the K_d for the R_I-subunit and PKI that is equivalent to the enzyme concentration (46−117 nM). Since the K_d for PKI and R_I to the wild-type C-subunit is less than 0.1 nM in the presence of ATP (F. W. Herberg et al., manuscript in preparation), the affinity of the inhibitors may be raised by several orders of magnitude without any effects on the titration curves of Figure 3. In fact, Steinberg et al. (1993) showed that substitution of alanine at Thr-197 creates a mutant C-subunit defective in binding a PKI affinity resin. Since the binding of LRRASLG is unaffected by the mutation, we conclude that if there are affinity losses for any of the mutants and PKI or the R_I-subunit, then they are due to changes in the interaction of these inhibitors with regions outside the immediate consensus region of the substrate (i.e., R-R-X-S).

Model for Phosphoryl Group-Induced Activity Changes. The study of point-site mutations of cAPK indicates that the phosphoryl group on Thr-197 is critically important for modulating catalytic flux in the C-subunit. At arbitrarily chosen concentrations of 1 mM ATP and 50 μM LRRASLG, the wild-type C-subunit is 16-, 53-, and 490-fold more effective at catalyzing substrate turnover compared to C(T197D), GST-C(T197D), and GST-C(T197A), respectively. The reductions in catalytic efficiency were due to the specific effects of weakened ATP affinity and reduced rates of phosphoryl transfer (Figure 4). No effect on the binding of peptide substrates suggests that the overall conformation of the substrate-binding site in the large domain of the C-subunit is unaffected by phosphoryl group removal at Thr-197. Whether physiological protein substrates recognize the surface that lies beyond the consensus site is unknown.

The large rate reductions observed for phosphoryl transfer and the large decreases in ATP affinity in all mutants may result from semilocal effects in the active site. The environment around Thr-197 is shown in Figure 5. The phosphoryl group of Thr-197 makes a strong electrostatic contact with Arg-165, the first residue of the catalytic loop (R_{165}-DLKPEN). Loss of this contact may alter the orientation of the catalytic loop, which contains a number of invariant residues essential for phosphoryl transfer. For instance, Asp-166 hydrogen bonds to the hydroxyl proton of the substrate and may act as a general base catalyst. Lys-168 forms a strong salt bridge with the γ phosphate of ATP and may stabilize charge in the reaction transition state. Lys-168 also hydrogen bonds to the α carbonyl of the P-1 residue in substrate and inhibitor peptides (Madhusudan et al., 1994). Replacement of Asp-166 and Lys-168 with Ala in the yeast C-subunit leads to 330- and 50-fold reductions in k_{cat} (Gibbs

& Zoller, 1991), supporting these claims. The phosphoryl group of Thr-197 is also linked indirectly to Asp-184, an invariant residue required for chelation of the primary Mg^{2+} in the active site. Both of these residues lie in loop regions that are separated by β strand 9. Lys-189, in the middle of this strand, is another of the ligands to Thr-197. Substitution of Mg^{2+} for Mn^{2+} or Co^{2+} has profound influences on the rate of phosphoryl transfer, suggesting that mild change in the position or character of the divalent metal can strongly influence catalysis. For example, the chemical step in Scheme 1 (k_3) is lowered by more than 1 order of magnitude when Mg^{2+} is replaced by Mn^{2+} in the primary position (Adams & Taylor, 1993a). Furthermore, the active site metal strongly enhances ATP binding. In the presence of Mg^{2+}, lin-benzo-ADP binds to the C-subunit with a K_d of 10 μM but fails to associate in its absence (Hartl et al., 1983). In cdk2 kinase, Asp-145 (Asp-184 in cAPK) is repositioned and a twisted conformation for the phosphates of ATP is adopted that cannot support in-line transfer of the γ phosphate. These data support the hypothesis that phosphorylation of the activation loop could influence ATP binding and phosphoryl transfer by distorting the position of Asp-184 or the catalytic loop.

Although the three-dimensional structure of one of the Thr-197 mutants is not yet available, the role of phosphorylation in modulating catalytic function in cAPK may be inferred from the recent X-ray solutions of cdk2 kinase and MAP kinase. In cdk2 kinase, displacement of the activation loop prompted DeBondt et al. (1994) to suggest that the dephosphorylated loop acts as an autoinhibitor by obstructing access to the active site. In MAP kinase the activation loop takes on a conformation distinct from that of the active cAPK loop as well. These movements, although more subtle than in the case of cdk2 kinase, distort residues neighboring the phosphorylation sites of the enzyme, Thr-183 and Tyr-185. Arg-192, Ala-187, and Arg-189 (Leu-205, Gly-200, and Pro-202 in cAPK) converge at what would be the P+1 pocket in MAP kinase. Structural differences between the P+1 pockets of cAPK and MAP kinase suggest that substrate binding would be disfavored for the latter enzyme. This may account, in part, for the decreased activity of the dephosphorylated form of MAP kinase (Zhang et al., 1994).

Although the K_m's for the substrate and the mutants are raised relative to that of the wild-type enzyme, the kinetic data presented here show clearly that there are no effects on substrate affinity. We conclude that activation of cAPK through phosphorylation does not occur by the modulation of the substrate's accessibility as proposed for MAP and cdk2 kinase. These observations suggest that the mechanism for cAPK activation may be distinct, although clarification of the structural consequences of dephosphorylation on the activation loop in cAPK awaits the X-ray solution of one or more of the mutants at Thr-197.

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