Kinetic Analysis of cAMP-Dependent Protein Kinase: Mutations at Histidine 87 Affect Peptide Binding and pH Dependence†

Sarah Cox and Susan S. Taylor*

Department of Chemistry and Biochemistry, University of California, San Diego, Gilman Drive, La Jolla, California 92093-0654

Received June 8, 1995; Revised Manuscript Received October 2, 1995⊗

ABSTRACT: Mutation of His87 in the catalytic (C-) subunit of the cAMP-dependent protein kinase (cAPK) led to changes in the kinetic properties of this enzyme. The C-subunit is a bilobal structure, with catalysis occurring in the cleft between the two lobes. His87 lies at the edge of the cleft, making an interaction with phosphothreonine, 197. This is the only direct electrostatic or hydrogen-bonding interaction between the small and large lobes. Solvent viscosity studies of the His87Ala mutant of the C-subunit (rC[H87A]) revealed that binding of two peptides, LRRASLG and LRRASLG-NH_2, was impaired relative to that of the wild-type C-subunit. Consistent with this, the K_i's for two inhibitor peptides, LRRAALG and LRRAALG-NH_2, were 4 and 1.4 mM, respectively, 5- and 7-fold higher than the K_i's of the respective peptides for wild-type protein. Kinetic constants for three octapeptide substrates that differed only at the P+2 position suggested a direct interaction of His87 with residues at this site. The k_{cat} for rC[H87A] was 2−3-fold higher than k_{cat} for the wild-type enzyme, indicating an effect of the mutation on the rate-limiting step, product release. The pH dependence of kinetic parameters for rC[H87A] was also measured. A single pK_a of 6.5 was observed in $k_{cat}/K_{peptide}$ as compared to the two pK_a's of 6.5 and 8.5 for the wild-type enzyme. These changes suggest a role for His87 in substrate recognition and in stabilization of the catalytically competent conformation of the enzyme.

The catalytic (C-) subunit of cAMP-dependent protein kinase (cAPK)[1] catalyzes the transfer of phosphate from ATP to a serine or threonine residue in protein substrates. This phosphorylation typically causes a change in the equilibrium between active and inactive conformations of the substrate protein. The large number of protein kinases and their importance in controlling a vast number of cellular processes have led to a wide-ranging study of these proteins; however, cAPK has been particularly well-studied (Bramson et al., 1984; Taylor et al., 1990). The enzyme exists as an inactive tetramer which, in response to cAMP, dissociates into a regulatory (R) subunit dimer and two free active C-subunits. The C-subunit provides a simple model for this family of protein kinases, each of which contains a conserved catalyic core (Hanks & Quinn, 1991; Taylor et al., 1992). The development of an efficient expression system of C in *Escherichia coli* (Slice & Taylor, 1989) makes mutational analysis of the structure and mechanism of this enzyme feasible.

Several crystal structures of the recombinant mouse (Knighton et al., 1991, 1993; Zheng et al., 1993a,c) and mammalian porcine C-subunits (Bossemeyer et al., 1993; Karlsson et al., 1993; Zheng et al., 1993b) have been solved. The enzyme is comprised of two lobes that consitute the catalytic core. This core is flanked by an N-terminal helix and a C-terminal tail that wraps over the core. The catalytic site lies in a deep cleft between the two lobes, with ATP binding at the base of the cleft and largely associated with the smaller N-terminal lobe. Peptide inhibitor or substrate binding is at the edge of the cleft and associated largely with the larger C-terminal lobe.

Steady state kinetics (Cook, 1982; Whitehouse et al., 1983) and isotope-partitioning studies (Kong & Cook, 1988) indicated that the C-subunit binds ATP and peptide randomly although initial binding of the nucleotide is preferred. Viscosity studies indicated that phosphoryl transfer is rapid and that the rate-limiting step is release of ADP (Adams & Taylor, 1992). In general, the K_m for ATP corresponds well with the K_d for ATP binding (Whitehouse et al., 1983). The K_m's for short peptides, however, are typically lower than the K_d's for peptides due to the relative values for the microscopic rate constants which contribute significantly to the $K_{peptide}$ term (Adams & Taylor, 1992; Whitehouse et al., 1983). The proposed reaction mechanism for the C-subunit involves direct in-line attack of the serine hydroxyl on the γ phosphate of ATP (Ho et al., 1988), and the involvement of a catalytic base has been suggested (Yoon & Cook, 1987). From the crystal structures of the C-subunit complexed with an inhibitor peptide, PKI(5−24), and with ATP and PKI-(5−24), the most likely candidate for the catalytic base is Asp166 (Zheng et al., 1993a,c).

The pH dependence of catalytic efficiency on the C-subunit with peptide substrates is bell-shaped, and intrinsic pK_a's of 6.5 and 8.5 are observed (Adams & Taylor, 1993; Yoon & Cook, 1987). The lower pK_a has been proposed to

† This work was supported by grants to S.S.T. from the National Institutes of Health.

* To whom all correspondence should be addressed: Dr. Susan S. Taylor, Department of Chemistry, 0654, University of California, San Diego, Gilman Drive, La Jolla, CA 92093-0654. Phone: 619-534-3677. Fax: 619-534-8193. E-mail: staylor@ucsd.edu.

⊗ Abstract published in *Advance ACS Abstracts,* November 15, 1995.

[1] Abbreviations: cAPK, cAMP-dependent protein kinase; C-subunit, catalytic subunit of cAPK; rC-subunit, recombinant catalytic subunit of the murine cAMP-dependent protein kinase; R, regulatory subunit of cAPK; rC[H87A], His87Ala mutant of the C-subunit; CAPS, 3-(cyclohexylamino)propanesulfonic acid; MES, 2-(*N*-morpholino)-ethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid.

0006-2960/95/0434-16203$09.00/0

be due to the ionization of a catalytic base (Yoon & Cook, 1987) or to a residue involved in binding the peptide, in particular Glu230, that interacts with the P-2 Arg in peptide substrates (Adams & Taylor, 1993). No candidate for the upper pK_a has been proposed; however, it is most likely due to the ionization of a basic residue, Arg, Lys, His, or possibly Cys. A constant level of reduced activity is observed at high pH which implies that the fully ionized ternary complex is still capable of supporting phosphoryl transfer (Adams & Taylor, 1993).

His87, located in the small lobe, lies at the edge of the catalytic cleft between the two lobes of the enzyme and appears to play a unique role in formation of a contact site between the lobes. His87 forms an ionic or hydrogen-bonding interaction with the essential activating phosphorylation site, Thr197, in the large lobe, and this is the only strong electrostatic or hydrogen-bonding interaction between the two lobes. This contact, furthermore, is observed in the closed, but not in the open, conformation of the enzyme. In the open conformation, His87 is 6 Å away from the phosphate (Karlsson et al., 1993; Zheng et al., 1993b), while the distance is less than 3 Å in the closed conformation. This suggests a particular role for His87 in cleft opening and closing.

His87 is not conserved throughout the family of protein kinases and therefore may be expected to function in a capacity which is specific to the C-subunit. In a previous study, we demonstrated that mutations of His87 interfered with binding to the type I regulatory subunit (Cox & Taylor, 1994) and showed that the mutants, rC[H87A] and rC-[H87D], were altered in their ability to phosphorylate some peptide substrates. In the present study, we analyze more specifically the effects of the His87Ala mutation on peptide binding and on the pH dependence of the phosphorylation reaction.

EXPERIMENTAL PROCEDURES

Materials. Adenosine 5′-triphosphate (ATP), phosphoenolpyruvate, magnesium chloride, nicotinamide adenine dinucleotide (NAD), Tris, MES, CAPS, pyruvate kinase (rabbit muscle), and lactate dehydrogenase (bovine heart) were purchased from Sigma. All other chemicals were from Fischer.

Peptides and Proteins. All peptides were synthesized at the Peptide and Oligonucleotide Facility at the University of California, San Diego. Peptides were purified by reverse phase preparative high-performance liquid chromatography (HPLC). The concentration of each substrate peptide was determined by turnover with the catalytic subunit under conditions of limiting peptide. Recombinant C-subunits were expressed in *E. coli* and purified as previously described (Cox & Taylor, 1994; Slice & Taylor, 1989; Yonemoto et al., 1993). The concentration of enzyme was measured by the absorbance at 280 nm using an extinction coefficient of 1.2 and by titration of the number of active sites with the heat stable protein kinase inhibitor (PKI) (Walsh et al., 1971).

Kinetic Assays. Enzymatic activity was measured spectrophotometrically using a coupled assay system (Cook, 1982). Enzyme was preincubated with ATP and an assay mix (1 mM phosphoenolpuruvate, 0.3 mM NADH, 12 units of lactate dehydrogenase, and 4 units of pyruvate kinase) in MTCN buffer [50 mM MES, 25 mM CAPS, 25 mM Tris, 50 mM NaCl, and 10 mM $MgCl_2$ (pH 8.0)] in all experiments except for the thermal stability measurements. The pH of MTCN buffer was adjusted using small volumes of concentrated HCl or NaOH. MTCN, used for previous kinetic analyses of the C-subunit (Adams & Taylor, 1993), buffers over a wide pH range. The buffer also contained 50 mM NaCl so that the change in ionic strength due to the addition of acid and base was negligible. All kinetic measurements were performed at 23 °C and between pH 5.8 and 10.5. Both C-subunits were tested for stability at extreme pH. At all pH's in MTCN buffer, the $t_{1/2}$ (the time at which 50% of activity was lost) was greater than 30 min for each protein. Assays were carried out in duplicate for 2 min.

Thermal Stability Measurements. Aliquots (50 μL) of C-subunit at 1 μM were incubated at different temperatures for 2 min in buffer containing 20 mM potassium phosphate, 5 mM β-mercaptoethanol, 100 mM KCl, and 1 mM ethylenediaminetetraacetic acid (EDTA) (pH 6.5), and then immediately assayed in buffer A by dilution into an assay mix to give a final concentration of C-subunit of 40 nM.

Solution Viscosity Measurements. The relative viscosity (η^{rel}) of buffers containing glycerol or sucrose was measured using an Ostwald viscometer (Shoemaker & Garland, 1962). Buffers containg 20 and 29% glycerol (w/w) were used to obtain relative viscosities of 2.0 and 2.8, respectively. Buffers containing 26, 32, and 40% sucrose (w/w) were used to obtain viscosities of 3.3, 4.4, and 8.1, respectively. It was shown previously for the wild-type enzyme that the viscogenic agents do not exert their effect through specific active site interactions or major structural perturbations. This was also true for the mutant. After dialysis into 29% glycerol, the enzyme retained 90% activity, and the ATPase activity was unaffected by 30% sucrose.

Data Analysis. Values of $K_{peptide}$, K_{ATP} and V_{max} were determined from plots of initial velocity vs substrate concentration according to eq 1.

$$v = \frac{V_{max}[S]}{K_m [S]} \tag{1}$$

V_{max} is the maximal velocity, v is the initial velocity, [S] is the substrate concentration of varied substrate, and K_m is the Michaelis constant. V_{max} (micromoles per minute per milligram) was converted to k_{cat} (s^{-1}) using a molecular weight for the C-subunit of 40 800, assuming the enzyme was pure. Purity was based on polyacrylamide gel electrophoresis and by correlation of the protein concentration determined by the absorbance at 280 nm with the concentration of active kinase determined by titration with PKI.

For the determination of pK_{a1} and pK_{a2}, plots of $k_{cat}/K_{peptide}$, ascertained by eq 1, were fit to eq 2, where y is the observed $k_{cat}/K_{peptide}$ at a given pH and C is the maximum value of $k_{cat}/K_{peptide}$. pK_{a1} and pK_{a2} are the lower and upper dissociation constants, respectively.

$$y = \frac{C}{1 + 10^{pK_{a1}-pH} + 10^{pH-pK_{a2}}} \tag{2}$$

Competitive inhibition data were fit by a Dixon plot (Segel, 1975). The K_i values for peptides were determined from Dixon plots at a single substrate concentration. Assuming competitive inhibition, the X intercept is related to the K_i by

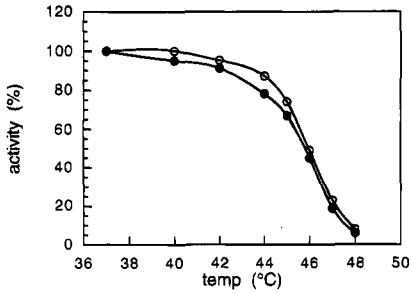


FIGURE 1: Thermal stability of the mutant rC[H87A]. Wild-type (●) or rC[H87A] (○) C-subunits were preincubated at the temperature indicated for 2 min before assay as described under Experimental Procedures.

Table 1: Kinetic Constants for the Wild-Type C-Subunit and rC[H87A][a]

	wild-type rC	rC[H87A]
Peptide Ia: LRRASLG		
$K_{peptide}$ (μM)	27.4 ± 1.9	213.0 ± 13.0
k_{cat} (s^{-1})	20.0 ± 1.0	60.0 ± 6.0
$k_{cat}/K_{peptide}$ (μM^{-1} s^{-1})	0.73 ± 0.03	0.28 ± 0.04
Peptide Ib: LRRASLG-NH_2		
$K_{peptide}$ (μM)	12.6 ± 2.0	11.2 ± 3.0
k_{cat} (s^{-1})	30.9 ± 2.3	51.0 ± 3.2
$k_{cat}/K_{peptide}$ (μM^{-1} s^{-1})	2.45 ± 0.5	4.55 ± 0.8
K_{ATP} (μM)	18.0 ± 3.0	21.0 ± 4.0
$k_{cat}/K_{peptide}$ (μM^{-1} s^{-1})	1.11 ± 0.5	2.85 ± 0.8

[a] Two heptapeptide substrates were used as indicated. The K_{ATP} was measured using peptide Ia, although similar values were obtained for peptide Ib.

eq 3:

$$X \text{ intercept} = K_i^{app} = K_i(1 + [S]/K_m) \quad (3)$$

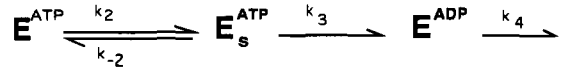
RESULTS

Thermal Stability and Steady State Kinetic Parameters for the Mutant rC[H87A]. To confirm that the overall stability of the protein was not altered by the mutation, the thermostability of rC[H87A] was compared to that of the wild-type rC-subunit. The inactivation curves shown in Figure 1 indicate that the stability of the protein was not compromised by the point mutation.

Steady State Kinetic Parameters. Two peptide substrates, shown in Table 1, were used for these steady state kinetic analyses. The two peptides differ only at the C-terminus. Peptide Ia had a free α-carboxylate, while in peptide Ib, the carboxyl group was amidated. The steady state kinetic parameters for the mutant C-subunit, rC[H87A], measured with the two heptapeptide substrates, Ia and Ib, are compared to those for the wild-type rC-subunit in Table 1. For Ia,the $K_{peptide}$ was elevated nearly 10-fold relative to that of the wild-type enzyme, while for Ib, the $K_{peptide}$'s of the wild-type and mutant proteins were equal. The $K_{peptide}$'s for Ib were significantly lower than the $K_{peptide}$'s for Ia for both wild-type rC and rC[H87A]. The difference in the $K_{peptide}$ for Ia vs Ib was nearly 20-fold compared with that of the mutant enzyme compared to a difference of 2-fold for the wild-type.

The k_{cat} for rC[H87A] was increased by approximately 2−3-fold relative to that of the wild-type C-subunits for both peptides. This is a significant increase. Some of the absolute values reported here differ slightly from those reported in our previous paper (Cox & Taylor, 1994) due to the use of different buffer systems.

Scheme 1



Effect of Viscosogenic Agents on the Steady State Parameters. The minimal kinetic requirements for the phosphorylation of peptide substrates by the C-subunit in the presence of saturating ATP are described in Scheme 1. The rate constants k_2, k_{-2}, and k_4 represent diffusion-controlled processes and are thus dependent on solvent viscosity, while the rate constant k_3, the chemical step in the reaction, is insensitive to solvent viscosity.

Using Cleland's method of net rate constants (Cleland, 1975), rate equations for k_{cat} and $k_{cat}/K_{peptide}$ can be derived. The rate constants in Scheme 1 are related to k_{cat} and $K_{peptide}$ by

$$k_{cat} = k_3k_4/(k_3 + k_4) \quad (4)$$

$$k_{cat}/K_{peptide} = k_2k_3/(k_{-2} + k_3) \quad (5)$$

If the ratios of k_{cat} and $k_{cat}/K_{peptide}$ are taken in the presence and absence of viscosogen (° denotes a parameter in the absence of viscosogen, $^\eta$ denotes the parameter in the presence of viscosogen), two linear equations (eqs 6 and 7) are derived. In these equations, k_{cat}^η and $(k_{cat}/K_{peptide})^\eta$ are the ratios of k_{cat} or $k_{cat}/K_{peptide}$ in the presence and absence of viscosogen (Adams & Taylor, 1992). The slopes of plots

$$k_{cat}^\eta = \frac{k_3}{k_3 + k_4^\circ} \quad (6)$$

$$(k_{cat}/K_{peptide})^\eta = \frac{k_3}{k_{-2}^\circ + k_3} \quad (7)$$

of these ratios, $k_{cat}^\circ/k_{cat}^\eta$ and $(k_{cat}/K_{peptide}^\circ)/(k_{cat}/K_{peptide})^\eta$, lie between theoretical limits of 0 and 1.

For the wild-type C-subunit, both k_{cat} and $k_{cat}/K_{peptide}$ are dependent on viscosity, with a slope ≈ 1 (Adams & Taylor, 1993). For the mutant C-subunit rC[H87A] assayed with either peptide Ia or Ib, the k_{cat}^η is also ≈ 1. Using eq 6, for both the wild-type and mutant C-subunits, the rate constant for phosphotransfer (k_3) must greatly exceed the rate constant for product dissociation (k_4). Therefore, for both enzymes, $k_{cat} \approx k_4$. Since the k_{cat} for rC[H87A] > 50 s^{-1} and the error in k_{cat}^η is on the order of 10%, a lower limit for phosphotransfer, k_3, of 500 s^{-1} can be assigned for rC[H87A].

Unlike the wild-type C-subunit, the slope of the $(k_{cat}/K_{peptide})^\eta$ for rC[H87A] was independent of viscosity (≈0). Therefore, using eq 7, the rate of substrate dissociation (k_{-2}) for rC[H87A] must greatly exceed the rate of phosphotransfer (k_3). Cleland's method of net rate constants states that $K_m = k_4/k_2\ (k_{-2} + k_3/k_3 + k_4)$ (Cleland, 1975). Since $k_3 \gg k_4$ and $k_{-2} \gg k_3$, this reduces to $K_m = (k_{-2}/k_2)(k_4/k_3)$ and indicates that, for rC[H87A], the $K_{peptide}$'s for the peptides Ia and Ib were perturbed from the K_d by a factor of k_4/k_3.

Inhibition of the Mutant rC[H87A] by Peptides. The K_i values for two inhibitor peptides are given in Table 2. These inhibitor peptides, Ic and Id, had the same sequence as substrate peptides Ia and Ib except that the Ser at the site of phosphorylation was replaced with Ala. Peptide Ic, like

Table 2: Inhibition Constants K_i for the Wild-Type C-Subunit and rC[H87A]

	wild-type rC	rC[H87A]
	Peptide Ic: LRRAALG	
K_i (mM)	0.80 ± 0.2	4.0 ± 0.4
	Peptide Id: LRRAALG-NH_2	
K_i (mM)	0.16 ± 0.5	1.4 ± 0.2

petide Ia, had a free carboxylate at the C-terminus, and peptide Id, like Ib, was amidated. The K_i values for the mutant protein, rC[H87A], were 4.0 (Ic) and 1.4 (Id) mM, higher than the values for the wild-type of 0.8 (Ic) and 0.16 (Id).

For rC[H87A], the K_i for peptide Ic was 20-fold higher than the $K_{peptide}$ for substrate Ia. The K_i for peptide Id was >100-fold higher than the $K_{peptide}$ for the equivalent substrate peptide, Ib. Although these peptides are not ideal substrate mimics, the K_i for the peptide Id is close to the K_d for the substrate peptide measured with the wild-type protein (Whitehouse et al., 1983).

Interaction of His87 with the P+2 Residue in Peptide Substrates. Kinetic constants for phosphorylation of a series of peptides with differing residues at the P+2 position were also measured for rC[H87A] and the wild-type C-subunit (Table 3). Peptides IIa−c had the general consensus sequence and are similar to peptides Ia and Ib but are extended one residue at the C-terminus with a glycine residue. The penultimate residue, at the P+2 position, is Ala (IIa), Arg (IIb), or Asp (IIc). The k_{cat}'s for these peptides were similar for both the wild-type and rC[H87A], and for both proteins, the k_{cat}'s did not vary significantly.

For the wild-type C-subunit, substitution of a charged residue at the P+2 position had a modest effect on $K_{peptide}$. The best substrate was peptide IIa with Ala at P+2 which had a $k_{cat}/K_{peptide}$ of 3.0. The peptide IIc with Asp at P+2 was the worst, with a $k_{cat}/K_{peptide}$ of 0.7 due to a 6-fold increase in $K_{peptide}$. Peptide IIb, with Arg at P+2, was intermediate, with $k_{cat}/K_{peptide} = 0.9$. These differences were relatively small. Much larger differences were seen for the mutant rC[H87A]. Peptides IIa (Ala) and IIb (Arg) were phosphorylated with similar efficiency, with $k_{cat}/K_{peptide}$ values of 0.7 and 0.6, respectively. Peptide IIc (Asp), on the other hand, was a much worse substrate for rC[H87A], with a $k_{cat}/K_{peptide}$ of 0.03. The reduced catalytic efficiency was largely due to a 22-fold increase in $K_{peptide}$.

Effect of pH on the Steady State Kinetic Parameters for the Mutant rC[H87A]. For the wild-type C-subunit, the pH profile for $k_{cat}/K_{peptide}$ is bell-shaped, with two sharp pK_a's at 6.5 and 8.5. There is no pH dependence in k_{cat}. The k_{cat} and $k_{cat}/K_{peptide}$ for the mutant rC[H87A] with peptide Ia were measured as a function of pH and are compared to those of the wild-type rC-subunit in Figure 3. The mutant rC[H87A] showed a very different pH profile in $k_{cat}/K_{peptide}$. The pK_{a1} at 6.5 was identical to that of the wild-type C-subunit. However, the pK_{a2} at pH 8.5 was abolished. A small decrease in $k_{cat}/K_{peptide}$ at pH 10.5 indicated that, if there is a significant pK_{a2} at higher pH, it is greater than pH 11. As for the wild-type C-subunit, k_{cat} was constant over the range pH 5.8−10.5.

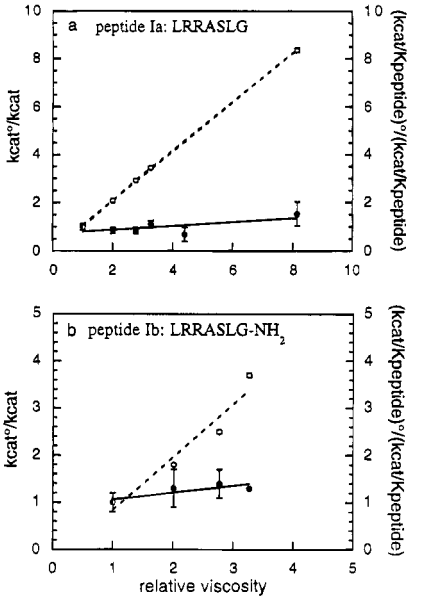


FIGURE 2: Effect of viscosogenic agents on the steady state parameters for rC[H87A]. Measurements were made at saturating ATP in buffer MTCN at pH 8.0 for peptide Ia (a) and peptide Ib (b). The change in k_{cat} (○) or $k_{cat}/K_{peptide}$ (●) at a given relative viscosity (η^{rel}) is expressed as a ratio of the observed parameter in the presence and absence of viscosogen. Linear dependencies were observed for both peptides.

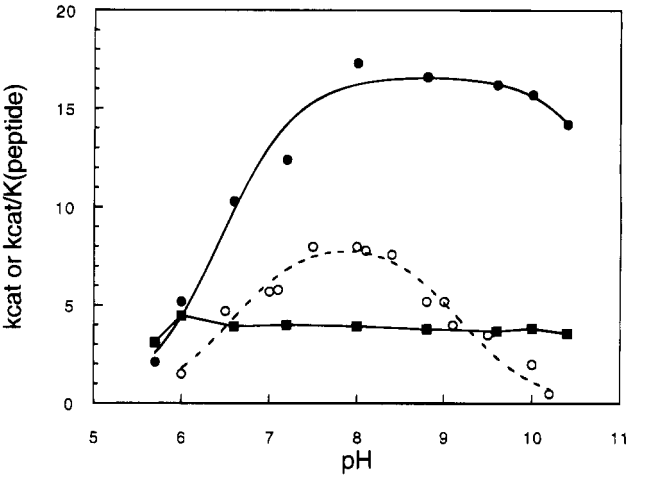


FIGURE 3: Effect of pH on steady state parameters for rC[H87A]. k_{cat} (■) and $k_{cat}/K_{peptide}$ (●) are plotted against pH. The $k_{cat}/K_{peptide}$ for wild-type (○) is also shown for reference (Adams & Taylor, 1993). Assays were carried out with peptide Ia in buffer MTCN.

Table 3: Kinetic Constants for Various Peptide Substrates[a]

	wild-type rC			rC[H87A]		
	$K_{peptide}$ (μM)	k_{cat} (s^{-1})	$k_{cat}/K_{peptide}$ (μM^{-1} s^{-1})	$K_{peptide}$ (μM)	k_{cat} (s^{-1})	$k_{cat}/K_{peptide}$ (μM^{-1} s^{-1})
peptide IIa: LRRASLAG	5.0 ± 2	15 ± 2	3	18 ± 5	13 ± 2	0.7
peptide IIb: LRRASLRG	18.0 ± 4	16 ± 2	0.9	44 ± 7	25 ± 3	0.6
peptide IIc: LRRASLDG	32.0 ± 6	22 ± 3	0.7	394 ± 61	12 ± 2	0.03

[a] Three octapeptide substrates were used as indicated.

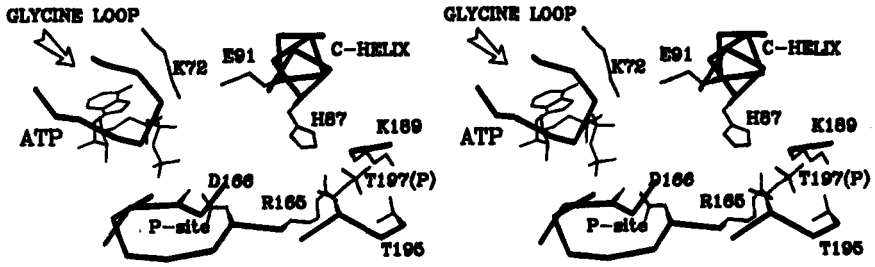


FIGURE 4: Stereoview of the interaction of His87 with Thr197(P). The C-α trace of the glycine rich loop, the C-helix, the essential phosphorylation site, and the catalytic loop are shown. Also shown are the P-site of the inhibitor peptide and ATP. Side chains of His87, Thr197, Arg165, Thr165, Asp166, Lys72, and Glu91 are shown and labeled. The link via Arg165 via the backbone to the catalytic base Asp166 can be seen.

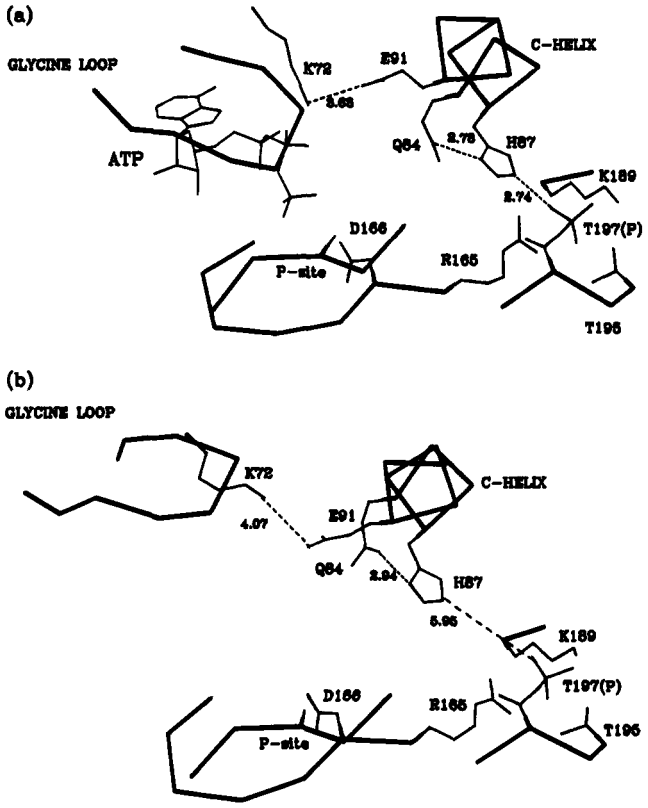


FIGURE 5: C-subunit of cAMP-dependent protein kinase showing the location and interactions of His87. Close-up of the region surrounding His87 in the C-subunit. (a) Closed and (b) Open conformations of the C-subunit. The distances between Glu91 and Lys72, His87 and Thr197(P), and His87 and Gln84 are indicated.

DISCUSSION

His87 plays a unique role in the C-subunit of cAPK. Its functional importance physiologically was first suggested when a spontaneous mutant H87N/W196R yielded an unregulated phenotype in human JEG cells (Orellana & McKnight, 1992). In the crystal structure of C-subunit, His87, a critical residue at the cleft interface, is the only residue in the small lobe of the conserved catalytic core that makes a direct, either electrostatic or hydrogen-bonding, contact with the large lobe. This contact is with the phosphate on Thr197 [Thr197(P)], a post-translational modification that is essential for full activity. As seen in Figure 4, Thr197(P) also interacts with Arg165, Lys189, and Thr195 in the large lobe. Figure 5 compares this region in the closed and open conformations. In the open conformation, the cleft is more open due to a concerted rotation of the small lobe relative to the large lobe (Karlsson et al., 1993; Zheng et al., 1993b). As a result, the contact between His87 and Thr197(P) is broken. Three major consequences of mutation of His87 to Ala are reported here. (i) Recognition of residues at the P+2 position was altered. (ii) The upper pK_{a2} in the pH dependency of $k_{cat}/K_{peptide}$ seen in the wild-type C-subunit was lost. (iii) The rate-limiting step, release of ADP, was increased. The kinetic basis for each of these findings is given below, and each is discussed in terms of the crystal structures.

(i) Peptide Binding and Interaction with the P+2 Site. The kinetic mechanism for the wild-type C-subunit was given in Scheme 1. For the wild-type C-subunit, assayed in MTCN, both k_{cat} and $k_{cat}/K_{peptide}$ were dependent on viscosity, with slopes $\approx$ 1.0 (Adams & Taylor, 1993) . Thus, $k_3 \gg k_4$ and $k_3 \gg k_{-2}$. Since the rate of phosphotransfer (k_3) is much faster than the release of unphosphorylated peptide substrate (k_{-2}), the peptide can be said to be a "sticky" substrate. Once it is bound, it is committed to catalysis. The $K_{peptide}$ is perturbed from the binding affinity, K_d, such that $K_m = k_4/k_2$. The rate-limiting step is k_4, the release of product.

Mutation of His87 in the C-subunit reduced the affinity of peptide binding and also changed the relative rates of individual rate constants. The viscosity dependence was measured for two peptides Ia and Ib (Figure 2). These peptides had the same sequence LRRASLG; however, peptide Ia terminated with a free carboxylate, while in peptide Ib, the carboxylate was amidated so the C-terminus was no longer charged. On the basis of the viscosity studies for rC[H87A], $k_3 \gg k_4$ and $k_{-2} \gg k_3$. Since $k_{-2} \gg k_3$, in marked contrast to the wild-type C-subunit, neither peptide Ia nor peptide Ib were sticky substrates for rC[H87A], even though the $K_{peptide}$ for Ib for this mutant was very close to that of the wild-type enzyme. For the mutant enzyme, the $K_{peptide}$ was perturbed from the K_d by a factor of k_4/k_3. The k_{cat} was dependent on viscosity, with a slope = 1. Therefore, as in the case of the wild-type enzyme, the rate-limiting step is, most likely, release of product, k_4.

The effect of the His87Ala mutation on peptide affinity was corroborated using inhibitor peptides (Table 2). The two inhibitor peptides Ic and Id were equivalent to Ia and Ib, respectively, but had an Ala instead of a Ser at the phosphorylation site. The K_i values for peptides Ic and Id were 5-fold and 7-fold higher than they were for wild-type C-subunit. This reduced peptide affinity is sufficient to account for the change in kinetic mechanism, i.e., the switch from $k_3 \gg k_{-2}$ for the wild-type enzyme to $k_{-2} \gg k_3$ for the mutant.

A difference in k_3, the rate of phosphotransfer, for phosphorylation of peptides Ia and Ib by rC[H87A] was also

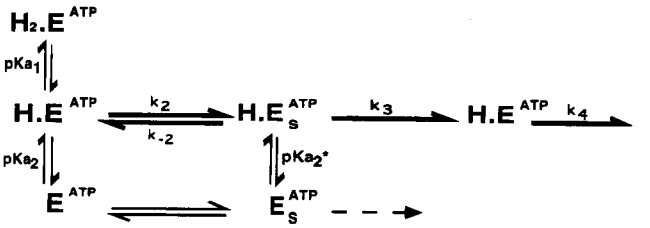
indicated. The amidated substrate, peptide Ib, was phosphorylated with a $K_{peptide}$ 20-fold lower than that for the substrate with the free carboxylate, peptide Ia, while the effect on the K_i for the inhibitor peptides, Id vs Ic, was only 3-fold. For rC[H87A], $K_{peptide} = (k_{-2}/k_2)(k_4/k_3)$, and K_i is dependent only on the k_2 and k_{-2} for the peptide. Therefore, there is an involvement of the k_4/k_3 term in the different $K_{peptide}$'s for these substrates. The k_{cat}'s (k_4) for phosphorylation of the peptides Ia and Ib are very similar. Thus, a difference in k_3 for phosphorylation of these two peptides on the order of 6-fold is indicated.

His87 was shown to be important for recognition of the P+2 site of peptide substrates. The consequence of introducing a negative charge close to the P+2 site was demonstrated initially by comparison of peptides Ia and Ib which differed only at their C-terminus. Peptide Ib was a much better substrate than peptide Ia for both the wild-type enzyme and rC[H87A]; however, the difference was much greater for the mutant enzyme (Table 1). To determine whether a negatively charged side chain at the P+2 site was also badly tolerated by the mutant enzyme, kinetic constants for three octapeptides were measured (Table 3). These peptides extended to P+3 with a Gly and differed only at the P+2 position. Peptide IIc, with Asp at the P+2 position, was the worst substrate for both enzymes. The effect of placing the negative charge at the P+2 position was much more dramatic for rC[H87A] than for the wild-type C-subunit. Neither a negative charge nor steric constraints were major factors for residues at, or close to, the P+2 position for the wild-type C-subunit. The mutant rC[H87A], however, was particularly sensitive to a negative charge in this region.

The elevated $K_{peptide}$ for the substrates bearing a negative charge at P+2 for the wild-type enzyme demonstrated that the negative charge at the C-terminus near the P+2 position was an unfavorable determinant for phosphorylation. This is consistent with early studies of Kemp et al. (1976) which showed that peptides which were amidated at the C-terminus were better substrates for the wild-type C-subunit and with more recent studies of Songyang et al. (1994), who, using a random library of peptide substrates, found that the least favored residues at the P+2 position were Asp or Glu. However, the detrimental effect of having a negative charge at this position is modest for the wild-type C-subunit, and several good physiological substrates of cAPK, such as phosphofructokinase and muscle glycogen synthase (site 1b), have an Asp or Glu at P+2. In the absence of His87, there is a marked impairment of phosphorylation of the peptides with a negative charge at this position. The presence of this His could, therefore, be an important determinant for substrate specificity, but rather than increasing, it reduces selectivity, allowing cAPK to be a broad specificity kinase. The efficiency of phosphorylation of different substrates by this multisubstrate kinase will affect the physiological function of the kinase (Walsh & Patten, 1994), and it is important to understand all the determinants that affect substrate selection.

In structural terms, a direct effect of His87 on peptide phosphorylation by interaction with the residues at the P+2 position is plausible. The C-terminus of the peptide is disordered in all of the crystal structures; however, it is possible that the residues at P+2 or P+3 could make contact with His87 in solution. A protonated His may directly

Scheme 2



compensate for the negative charge on the Asp at P+2 in peptide IIc or the carboxylate end group in peptide Ia. Additionally, in the absence of the interaction of His87 with the Thr197(P), the charge on the Thr197(P) could be felt more by the Asp residue or the carboxylate, leading to electrostatic repulsion. The cluster of negative charge in proximity to the active site (Figure 4) could also influence the chemistry of the phosphotransfer reaction, leading to a reduced k_3.

(ii) pH Dependence. The peptide studies with the rC[H87A] mutant were carried out at pH 8.0 and suggest that His87 may be protonated at this pH, 1.5 pH units above the pK_a for free histidine. The wild-type C-subunit shows two pK_a's in $k_{cat}/K_{peptide}$. The lower pK_a is pH 6.5, and the upper pK_a depends on the conditions of the assay. Scheme 2 describes the kinetic requirements for an ionizable C-subunit. In the wild-type enzyme when $k_3 \gg k_{-2}$, the equilibrium, pK_{a2}, is perturbed to pK_{a2}*. When assayed with peptide Ia in MTCN, a value of 9.3 is obtained. This value is perturbed from the intrinsic pK_a of 8.5 since the substrate is sticky as described above (Adams & Taylor, 1993).

No upper pK_a was observed for rC[H87A]. In the rC[H87A] mutant enzyme where $k_{-2} \gg k_3$, the substrate is not sticky, so pK_{a2} is not perturbed. The simplest explanation for the loss of the pK_{a2} at pH 8.5 (or 9.3) in the rC[H87A] mutant is that the ionization of His87 is responsible for the upper pK_a observed in the wild-type enzyme. This would mean that the pK_a for this His is shifted from that of His in solution by 1.5 pH units.

The pH dependence in $k_{cat}/K_{peptide}$ and k_{cat} was independent of pH, indicating that the protonation state of the enzyme affected peptide binding. If His87 is indeed resposible for pK_{a2}, this is consistent with the observation that mutation of this residue affects peptide binding, and since rC[H87A] is an active protein, it is also consistent with the suggestion of Adams and Taylor (1993) that the fully deprotonated C-subunit can support catalysis. Furthermore, a shifted pK_a for His87 can be explained by the crystal structure. In the closed conformation, both NH groups of the immidazole ring of His87 are hydrogen-bonded, one to the Thr197(P), as described above, the other to the side chain of Gln84 (Figure 5).

It cannot be ruled out, however, that the removal of His87 alters the structure of the enzyme such that the pK_a of another residue is shifted from pH 8.5 to >11. One possibility is that mutation of this residue would affect the pK_a of the key residue Lys72. Figures 4 and 5 show the link between His87 and Lys72. His87 is located at the beginning of the C-helix. At the next turn of the helix is the conserved Glu91, which makes a buried salt bridge with Lys72. This essential Lys interacts with the β phosphate of the ATP in the ternary complex. Mutation of Lys72 in many kinases leads to a decrease of 3 orders of magnitude in the activity of the

enzyme. The possibility that mutation of His87 alters the pK_a of Lys72 is considered unlikely since the enzyme is active and there is no effect on ATP binding. Alternatively, the pK_a could be due to one of the phosphate oxygens of Thr197(P) which could be altered by removal of His87. Mutations at Thr197 have a dramatic effect on binding of both peptide and ATP and also on the rate of phosphotransfer, k_3 (Adams et al., 1995). This possibility is also considered less likely since removing the His would be expected to shift the pK_a of the phosphate to a lower pH.

(iii) Rate of Product Release and the Open and Closed Conformations. The rate limiting step, k_4, for rC[H87A] was increased by approximately 2−3-fold relative to that of wild-type C-subunit. The rate-limiting step in the reaction is release of ADP, so this increase may reflect weaker ADP binding to the mutant enzyme. However, the K_m for ATP was not altered. Another explanation for the increase in k_{cat} is that a conformational change from a closed conformation, required for catalysis, to an open conformation, required for ADP release, occurs more readily in the mutant protein.

In summary, His87 in the C-subunit of cAPK has a role in peptide binding, and it is likely that ionization of this residue is responsible for the upper pK_a observed in $k_{cat}/K_{peptide}$ for the wild-type enzyme. The His acts to neutralize the detrimental effects of negatively charged substituents at the P+2 position in substrates. Finally, the elevated rate of k_4 suggests that this residue and the interactions it makes may also influence the equilibrium between open and closed conformations of the kinase.

This last point is of most significance for the whole family of protein kinases. Many other protein kinases contain an essential activating phosphorylation site analogous to Thr197. In these kinases, Arg165 and Lys189, the two basic residues that anchor the phosphate in the large lobe, are typically conserved. His87, or a basic residue at this position, is not, however, conserved. Each kinase will be unique in how this segment of the small lobe specifically interacts with the large lobe. The positioning of both the C-helix and the loop containing the activating phosphorylation site (activation loop) is critical for the activity of protein kinases. This is empasized clearly by the recently solved structures of cdk-2 in both an inactive conformation (De Bondt et al., 1993) and a partially activated complex with cyclin A (Jeffery et al., 1995). The C-helix, which includes the cyclin-binding PSTAIRE motif, in the inactive complex is twisted so that there is no interaction between Lys72 and Glu91. The activation loop containing Thr160, the equivalent of Thr197, is folded across the active site. Upon cyclin A binding, this helix and the activation loop are brought into close allignment to their positions in the C-subunit. Thr160 is not phosphorylated in this partially active structure; however, three basic residues form a cationic pocket ready to recieve the phosphate. One of these residues is the R of the PSTAIRE sequence. In the sequence allignment of these two kinases, the Thr in the PSTAIRE sequence alligns with His87. However, the Arg, one turn along the helix, fills the space occupied by His87 in the C-subunit and could have a similar function to that of His87 described here.

ACKNOWLEDGMENT

The authors thank all the members of the Taylor group for helpful discussions and in particular Dr. J. Adams (San Diego State University), who guided the kinetic analysis. Dr. W. Hemmer and B. Grant are thanked for critical reading of the manuscript, and S. Bell and S. Garrod are thanked for providing highly purified wild-type C-subunit and peptides, respectively.

REFERENCES

Adams, J. A., & Taylor, S. S. (1992) *Biochemistry 31*, 8516−8522.

Adams, J. A., & Taylor, S. S. (1993) *J. Biol. Chem. 268*, 7747−7752.

Adams, J. A., McGlone, M. L., Gibson, R., & Taylor, S. S. (1995) *Biochemistry 34*, 2447−2454.

Bossemeyer, D., Engh, R. A., Kinzel, V., Ponstingl, H., & Huber, R. (1993) *EMBO J. 12*, 849−859.

Bramson, H. N., Kaiser, E. T., & Mildvan, A. S. (1984) *Crit. Rev. Biochem. 15*, 93−124.

Cleland, W. W. (1975) *Biochemistry 14*, 3220−3224.

Cook, P. F. (1982) *Biochemistry 21*, 113−116.

Cox, S., & Taylor, S. S. (1994) *J. Biol. Chem. 269*, 22614−22622.

De Bondt, H. L., Rosenblatt, J., Jancarik, J., Jones, H. D., Morgan, D. O., & Kim, S. H. (1993) *Nature 363*, 595−602.

Hanks, S., & Quinn, A. M. (1991) in *Protein Phosphorylation (Part A)* (Hunter, T. & Sefton, B. M., Eds.) pp 38−62, Academic Press, Inc., San Diego.

Ho, M.-f., Bramson, H. N., Hansen, D. E., Knowles, J. R., & Kaiser, E. T. (1988) *J. Am. Chem. Soc. 110*, 2680−2681.

Jeffery, P. D., Russo, A. A., Polyak, K., Gibbs, E., Hurwitz, J., Massague, J., & Pavletich, N. P. (1995) *Nature 376*, 313−320.

Karlsson, R., Zheng, J., Xuong, N.-h., Taylor, S. S., & Sowadski, J. M. (1993) *Acta Crystallogr. D49*, 381−388.

Kemp, B. E., Benjamini, E., & Krebs, E. G. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73* (4), 1038−1042.

Knighton, D. R., Zheng, J., Ten Eyck, L. F., Ashford, V. A., Xuong, N.-h., Taylor, S. S., & Sowadski, J. M. (1991) *Science 253*, 407−414.

Knighton, D. R., Bell, S. M., Zheng, J., Ten Eyck, L. F., Xuong, N.-h., Taylor, S. S., & Sowadski, J. M. (1993) *Acta Crystallogr. D49*, 357−361.

Kong, C.-T., & Cook, P. F. (1988) *Biochemistry 27*, 4795−4799.

Orellana, S. A., & McKnight, G. S. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 4726−4730.

Slice, L. W., & Taylor, S. S. (1989) *J. Biol. Chem. 264*, 20940−20946.

Songyang, Z., Blechner, S., Hoagland, N., Hoekstra, M. F., Piwnica-Worms, H., & Cantley, L. C. (1994) *Curr. Biol. 4*, 973−982.

Taylor, S. S., Buechler, J. A., & Yonemoto, W. (1990) *Annu. Rev. Biochem. 59*, 971−1005.

Taylor, S. S., Knighton, D. R., Zheng, J., Ten Eyck, L. F., & Sowadski, J. M. (1992) *Annu. Rev. Cell Biol. 8*, 429−462.

Walsh, D. A., Ashby, C. D., Gonzalez, C., Calkins, D., Fischer, E. H., & Krebs, E. G. (1971) *J. Biol. Chem. 246*, 1977−1985.

Whitehouse, S., Feramisco, J. R., Casnellie, J. E., Krebs, E. G., & Walsh, D. A. (1983) *J. Biol. Chem. 258*, 3693−3701.

Yonemoto, W., Garrod, S. M., Bell, S. M., & Taylor, S. S. (1993) *J. Biol. Chem. 268*, 18626−18632.

Yoon, M.-Y., & Cook, P. F. (1987) *Biochemistry 26*, 4118−4125.

Zheng, J., Knighton, D. R., Ten Eyck, L. F., Karlsson, R., Xuong, N.-h., Taylor, S. S., & Sowadski, J. M. (1993a) *Biochemistry 32*, 2154−2161.

Zheng, J., Knighton, D. R., Xuong, N.-h., Taylor, S. S., Sowadski, J. M., & Ten Eyck, L. F. (1993b) *Protein Sci. 2*, 1559−1573.

Zheng, J., Trafny, E. A., Knighton, D. R., Xuong, N.-h., Taylor, S. S., Ten Eyck, L. F., & Sowadski, J. M. (1993c) *Acta Crystallogr. D49*, 362−365.

BI9512859