Examination of an active-site electrostatic node in the cAMP-dependent protein kinase catalytic subunit

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Abstract

The active site of the cAMP-dependent protein kinase catalytic subunit harbors a cluster of acidic residues—Asp 127, Glu 170, Glu 203, Glu 230, and Asp 241—that are not conserved throughout the protein kinase family. Based on crystal structures of the catalytic subunit, these amino acids are removed from the site of phosphoryl transfer and are implicated in substrate recognition. Glu 230, the most buried of these acidic residues, was mutated to Ala (rC[E230A]) and Gln (rC[E230Q]) and overexpressed in *Escherichia coli*. In contrast to the mostly insoluble and destabilized rC[E230A], rC[E230Q] is largely soluble, purifies like wild-type enzyme, and displays wild-type-like thermal stability. The mutation in rC[E230Q] causes an order of magnitude decrease in the affinity for a heptapeptide substrate, Kemptide. In addition, two independent kinetic techniques were used to dissect phosphoryl transfer and product release steps in the reaction pathway. Viscosometric and pre-steady-state quench-flow analyses revealed that the phosphoryl transfer rate constant decreases by an order of magnitude, whereas the product release rate constant remains unperturbed. Electrostatic alterations in the rC[E230Q] active site were assessed using modeling techniques that provide molecular interpretations for the substrate affinity and phosphoryl transfer rate decreases observed experimentally. These observations indicate that subsite recognition elements in the catalytic subunit make electrostatic contributions that are important not only for peptide affinity, but also for catalysis. Protein kinases may, therefore, discriminate substrates by not only binding them tightly, but also by only turning over ones that complement the electrostatic character of the active site.

Keywords: cAMP-dependent protein kinase; catalytic subunit; electrostatic interaction; pre-steady-state kinetics; substrate specificity

The catalytic subunit of cAMP-dependent protein kinase is the simplest member of the protein kinase family because it is one of the smallest and its activity is regulated by a separate subunit (R-subunit). The holoenzyme form of the enzyme is an inactive complex composed of an R-subunit homodimer and two C-subunits (Taylor et al., 1989). Active monomeric C-subunits are released after cAMP binds with high affinity to the regulatory subunits (Gill & Garren, 1969) and phosphorylate a broad range of protein substrates involved in metabolic (Krebs, 1985), transcriptional (Yamamoto et al., 1988), and signal transduction events (Cook & McCormick, 1993). Active C-subunit phosphor-

ylates protein substrates on Ser and Thr residing in a common consensus sequence, Arg-Arg-X-Ser/Thr-Y, where X denotes any amino acid and Y is a hydrophobic residue (Zetterqvist et al., 1990). The P-3 and P-2 arginines³ confer 4–6 kcal/mol of binding energy (Walsh et al., 1990) to a 20-amino acid C-subunit inhibitor (IP20). C-subunit amino acids that complement these arginines were first visualized in a crystal structure of the C-subunit complexed with IP20 (Knighton et al., 1991). Comparison of this C-subunit crystal structure with structures of both Ser-specific (De Bondt et al., 1993; Hu et al., 1994; Zhang et al., 1994) and Tyr-specific (Hubbard et al., 1994) protein kinases are guiding speculation into the molecular mechanisms behind substrate specificity and catalysis.

Although the available protein kinase crystal structures depict some distinguishing features, they share common attributes. They are composed of two subdomains—a small N-terminal

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Abbreviations: cAPK, cAMP-dependent protein kinase; C-subunit, catalytic subunit; LRRASLG, Kemptide; LRRALG, Kemptide inhibitor analogue; LRRAS(P)LG, phospho-Kemptide; ATP, adenosine deoxynucleotide triphosphate; MOPS, 3-(N-morpholino) propane sulfonic acid; NADH, nicotinamide adenine dinucleotide, reduced; IPTG, isopropyl- β -D-thiogalactopyranoside.

³ The nomenclature for a peptide phosphorylated by the C-subunit designates the phosphorylated residue as the P site and residues N-terminal and C-terminal of the P position as P - n and P + n, respectively.

ATP-binding subdomain and a larger C-terminal substrate-binding subdomain. The active site is a cleft formed at the junction of the two lobes and contains a number of conserved amino acids that appear important for substrate affinity and catalysis. Several conserved amino acids converge at the site of phosphoryl transfer to: (1) stabilize the triphosphates of ATP (Gly 50, Gly 52, Lys 72, Lys 168); (2) ligand the primary (Asp 184) and secondary (Asn 171) divalent metals; and (3) possibly stabilize the transition state (Asp 166) by general base catalysis. Although most of these conserved amino acids are within 4 Å of the phosphoryl transfer site, other C-subunit active-site amino acids further removed from the site of phosphoryl transfer encircle the substrate arginines. Amino acids in this acidic cluster (Asp 127, Glu 170, Glu 203, Glu 230, Asp 241) are specific to the cAPK C-subunit because they are not conserved throughout the protein kinase family (Hanks et al., 1988). Results from a cursory charged-to-alanine scan (Gibbs & Zoller, 1991) postulated that amino acids in this acidic cluster are important only for substrate-binding affinity because mutations increased the K_m for a heptapeptide substrate by two orders of magnitude while perturbing k_{cat} insignificantly. Substrate affinity and catalytic events were dissected here using steady-state and pre-steady-state kinetic techniques to better understand the individual contributions of these acidic residues that are specific to the C-subunit.

The role of Glu 230 was addressed specifically because it is positioned in the center of the acidic cluster/substrate Arg interface. Figure 1 depicts the environment around Glu 230. Glu 230 is: (1) within hydrogen bond distance to Arg 133 and Tyr 204; (2) close enough to the P – 2 Arg for an electrostatic contact; and (3) approximately 15 Å removed from the catalytic loop, magnesiums, ATP phosphates, and the site of phosphoryl transfer. Glu 230 was mutated to Ala (rC[E230A]) and Gln (rC[E230Q]), overexpressed in *Escherichia coli*, and purified to near-homo-

Fig. 1. Active site environment around Glu 230. Glu 230 can potentially form hydrogen bonds with Arg 133 and Tyr 204 and make an electrostatic contact with the P – 2 substrate Arg. The site of phosphoryl transfer, the catalytic loop, the ATP phosphates, and magnesium atoms are approximately 15 Å removed from Glu 230. Distances are in Å.

geneity. Comparing rC[E230Q] kinetic parameters to those for wild-type C-subunit elucidated the contribution of this subsite recognition element to ternary complex formation, catalysis, and product release. A model for the C-subunit kinetic reaction pathway derives from previous studies of the well-characterized wild-type enzyme. Substrate addition experiments determined that ATP and substrate bind in a random fashion to the enzyme (Cook et al., 1982; Whitehouse et al., 1983) with a preference for ATP binding first (Adams & Taylor, 1992). Stereochemical (Ho et al., 1988) and isotope partitioning (Kong & Cook, 1988) analyses of the reaction products indicated that phosphoryl transfer proceeds through a one-step, essentially irreversible process. Pre-steady-state quench-flow (Grant & Adams, 1996) kinetic studies measured phosphoryl transfer and product release rate constants of 500 and 20 s⁻¹, respectively. Quench-flow kinetic analysis also equated the directly measured K_d for the substrate peptide Kempptide (LRRASLG), with the K_i for an inhibitor analogue (LRRALG). The above pieces of information are culminated into the following reaction pathway (Pathway 1) for wild-type C-subunit:

where (S) is Kempptide. In this study, the wild-type C-subunit kinetic reaction pathway is compared with kinetic reaction pathway for rC[E230Q].

Results

Protein expression and purification

Glu 230 was mutated to Ala (rC[E230A]) and Gln (rC[E230Q]). rC[E230A] formed insoluble inclusion bodies when expressed in *E. coli*, whereas a significant portion of rC[E230Q] was soluble. rC[E230A] was rendered soluble when expressed from pGEX-KG (Guan & Dixon, 1991) as a fusion with glutathione S-transferase; however, its catalytic activity was lost completely at 4 °C in 7 days. This loss of rC[E230A] activity was not due to degradation as inactive protein still ran as one band following SDS-PAGE. In contrast to the Ala mutant, rC[E230Q] was typically stable for months at 4 °C. rC[E230Q] was purified to near homogeneity, based on Coomassie-stained SDS-PAGE, using the same purification scheme developed for wild-type C-subunit. Similar yields of homogenous enzyme, typically 5 mg/L of *E. coli* culture, were obtained for both wild-type and rC[E230Q] enzymes. The structural stability of rC[E230Q] was assessed by thermal denaturation of catalytic activity and rC[E230Q] exhibited an identical T_m (44 °C) as wild-type enzyme.

Steady-state Michaelis-Menten and inhibition parameters

All steady-state kinetic assays were conducted in 50 mM MOPS pH 7.0 and 10 mM free Mg²⁺. The steady-state Michaelis-Menten parameters reported in Table 1 were determined by initial velocity versus substrate concentration hyperbolic plots. Both K_m (Kempptide) and K_m (ATP) were measured under saturating ATP conditions (5 mM) and saturating Kempptide condi-

Table 1. Steady-state and inhibition parameters for wild-type and E230Q C-subunits

Parameter	Wild-type	rC[E230Q]	Ratio (WT/E230Q)
k_{cat} (s^{-1})	21.5 ± 1.5	10.0 ± 0.4	2
K_m (ATP) (μM)	20.4 ± 1.4	9.8 ± 0.9	2
K_m (Kemptide) (μM)	6.9 ± 1.7	$1,400 \pm 200$	1/200
k_{cat}/K_m ($\mu M^{-1} s^{-1}$) ^a	3.2 ± 0.8	0.007 ± 0.002	450
K_i (LRRALG) (μM) ^b	190 ± 20	$4,600 \pm 700$	1/25

^a The apparent second-order rate constant for Kemptide binding, k_{cat}/K_m , is reported.

^b LRRALG is a competitive inhibitor with respect to Kemptide for both wild-type and rC[E230Q] C-subunits.

tions (10 mM), respectively. Product formation was linear over the time range of these assays, indicating negligible product inhibition. As illustrated in Table 1, k_{cat} and K_m (ATP) were perturbed only twofold, whereas K_m (Kemptide) increased 200-fold in rC[E230Q]. The significant K_m (Kemptide) increase dominated the two-orders of magnitude reduction in the apparent second-order binding constant for Kemptide, k_{cat}/K_m in rC[E230Q].

An inhibitor analogue of Kemptide, LRRALG, was used to quantitate Kemptide binding affinity. This peptide analogue is a competitive inhibitor with respect to Kemptide (Yoon & Cook, 1987) for wild-type C-subunit, and K_i (LRRALG) equals the K_d (Kemptide) (Grant & Adams, 1996). A characteristic y-axis intersection point in a double-reciprocal initial velocity versus [Kemptide] plot (with inhibitor concentration as a fixed variable) established that LRRALG is a competitive inhibitor with respect to Kemptide in rC[E230Q] (data not shown). Linear extrapolation of Dixon plots yielded apparent K_i determinations that were converted into true K_i values (Segel, 1975) and reported in Table 1. The mutation in rC[E230Q] decreased substrate affinity by one order of magnitude.

Microviscosity effects on steady-state parameters

Solvent viscosity effects on k_{cat} and k_{cat}/K_m were determined by extrapolation from hyperbolic initial velocity versus [Kemptide] plots in the presence of 0, 16, 22, and 29% sucrose. Relative k_{cat} and k_{cat}/K_m values are ratios of a parameter measured in the absence and presence of sucrose. A relative k_{cat} and k_{cat}/K_m versus relative solvent viscosity plot is shown in Figure 2, and slope values from this plot are reported in Table 2. The rate-limiting step in the reaction catalyzed by wild-type C-subunit is diffusion-controlled because the slope on k_{cat} is equal to 1. A slope of 0.50 ± 0.05 for the rC[E230Q] k_{cat} indicated the rate-limiting step is partially diffusion-controlled in the mutant enzyme. There was no detectable solvent microviscosity perturbation on the apparent second-order binding constant k_{cat}/K_m for either wild-type enzyme or rC[E230Q]. k_{cat}/K_m was measured from initial velocity versus [Kemptide] plots and by initial velocities determined with Kemptide concentrations at least 10 times lower than the K_m .

Phosphoryl transfer and product release microscopic rate constants reported in Table 2 are calculated from the linear regres-

Fig. 2. Microviscosity effects on k_{cat} and k_{cat}/K_m for wild-type and rC[E230Q] C-subunits. Relative parameters are the ratios of the parameter measured in the presence and absence of sucrose. Relative k_{cat} values are depicted for wild-type (open circles) and rC[E230Q] (closed circles) C-subunits. Relative k_{cat}/K_m values are also shown for wild-type (open triangles) and rC[E230Q] (closed triangles) C-subunits. Relative viscosity measurements are outlined in Materials and methods.

sions in Figure 2. The following equations were derived from net rate constant expressions of steady-state parameters using the Stokes-Einstein relation (Adams & Taylor, 1992):

$$(k_{cat})^n = \frac{k_3}{k_3 + k_4},$$

$$(k_{cat}/K_m)^n = \frac{k_3}{k_3 + k_{-2}},$$

where $(k_{cat})^n$ and $(k_{cat}/K_m)^n$ are slopes from Figure 2. The lack of a solvent viscosity effect on k_{cat}/K_m for wild-type enzyme and rC[E230Q] indicates Kemptide is in rapid exchange with both of these enzymes ($k_{-2} \gg k_3$).

Two control experiments were performed to ensure that viscosogen perturbed k_{cat} only by altering solvent viscosity and not the structure of the mutant C-subunit. First, rC[E230Q] initial velocities remained constant when incubated for 30 min at 23 °C in the presence of 30% sucrose and diluted in the assay buffer. Second, apparent ATP hydrolysis rate constants of $1.38 \pm 0.10 \text{ min}^{-1}$ and $0.84 \pm 0.02 \text{ min}^{-1}$ were measured for wild-type and rC[E230Q] C-subunits, respectively. Thirty per-

Table 2. Microviscosometric effects on steady-state parameters and microscopic rate constants

Parameter	Wild-type	rC[E230Q]	Ratio (WT/E230Q)
$(k_{cat})^n$	1.1 ± 0.1	0.50 ± 0.05	2
$(k_{cat}/K_m)^n$	~ 0	~ 0	1
k_3 (s^{-1})	500 ± 60^a	20.0 ± 2.4	25
k_4 (s^{-1})	21.5 ± 1.5	20.0 ± 2.4	1

^a The tabulated value for the wild-type C-subunit k_3 of 500 s^{-1} was determined from quench-flow experiments under the same buffer conditions (Grant & Adams, 1996).

cent sucrose had no effect on the apparent $rC[E230Q]$ ATP hydrolysis rate constant within the 5% systematic error of the measurement.

Pre-steady-state kinetic analysis of $rC[E230Q]$

A quench-flow rapid mixing instrument followed product formation in the pre-steady-state time frame. Figure 3 compares pre-steady-state product formation profiles for wild-type and $rC[E230Q]$ C-subunits. The inset in Figure 3 is an expanded view of the first 200 ms. Buffer conditions, the 4:1 K_D to Kemptide concentration ratio, and the molar enzyme concentration were constant for wild-type and mutant C-subunit. Reaction conditions for $rC[E230Q]$ were 50 mM MOPS, pH 7.0, 1 mM ATP, 11 mM $MgCl_2$, 1 mM Kemptide, and 2 μM enzyme, whereas slightly lower concentrations of ATP and Kemptide (0.2 mM and 0.1 mM, respectively) were used for wild-type C-subunit. Control experiments, in which the reaction was quenched immediately with a 30% acetic acid-Kemptide mixture, determined both background activity (5%) as well as quenching efficiency (95%). Wild-type C-subunit data fit Equation 2 and yielded a "burst" rate constant of 250 s^{-1} and a linear rate constant of 20 s^{-1} (Grant & Adams, 1996). Because the $rC[E230Q]$ data fit both Equation 2 as well as a linear function, a burst rate constant is not reported. These fits both yielded a well-defined linear rate constant of $6.0 \pm 0.1\text{ s}^{-1}$, which was corrected to 15 s^{-1} (1.5-fold perturbed with respect to wild-type C-subunit) based on the wild-type C-subunit linear rate constant's hyperbolic dependence on [Kemptide] (Grant & Adams, 1996). The nearly linear plot for $rC[E230Q]$ clearly indicates that the mutation decreases the phosphoryl transfer rate constant, causing it to become partially rate limiting.

Electrostatic modeling

A model describing the electrostatic surface of the C-subunit was generated using the program DelPhi (Biosym Technologies, Inc., San Diego, California). Figure 4 depicts electrostatic potential

Fig. 3. Pre-steady-state quench-flow product formation time courses for wild-type and $rC[E230Q]$ C-subunits. Buffer conditions, [Kemptide] to K_D ratio, and molar enzyme concentration are constant for each enzyme.

slices through Glu 230 (Fig. 4A, without peptide; Fig. 4B, with peptide) and through Gln 230 (Fig. 4C, without peptide; Fig. 4D, with peptide). The many acidic residues clustered in the C-subunit active site form an intensely negative electrostatic field in the wild-type enzyme's active site (Fig. 4A) and addition of positively charged Kemptide partially neutralizes the negative charge (Fig. 4B). Mutating Glu 230's carboxyl to an amide significantly reduces the negative electrostatic potential in this region (Fig. 4C,D). Table 3 reports quantitative electrostatic calculations measured for specific mutations around Glu 230. The strength of individual interactions were estimated by assessing the effect of neutral mutations of Glu 230 and the substrate arginines on Kemptide binding energy ($\Delta\Delta G$). Although the P-3 Arg neutral mutation did not compensate unfavorable lone charge in $rC[E230Q]$ significantly, the neutrally mutated P-2 Arg compensated $rC[E230Q]$. These theoretical studies suggest that Glu 230 interacts most strongly with the P-2 substrate arginine.

Discussion

Protein kinase crystal structures are emerging at a rapid pace since the first structure of the cAPK C-subunit. The available structures suggest that most amino acids conserved among protein kinases reside close to the site of phosphoryl transfer. The C-subunit active site hosts a cluster of acidic amino acids not conserved throughout protein kinases—Asp 127, Glu 170, Glu 203, Glu 230, and Asp 241—that are removed from the site of phosphoryl transfer. Results from a C-subunit charged-to-alanine scan (Gibbs & Zoller, 1991) suggested that amino acids in this acidic cluster serve as simple binding determinants for substrate arginines. To more clearly assess the contribution of amino acids in this acidic cluster, Glu 230 was mutated to Gln, overexpressed in *E. coli*, purified to near-homogeneity, and analyzed using steady-state and pre-steady-state kinetic techniques. Comparing $rC[E230Q]$ kinetic parameters to those for wild-type C-subunit elucidated the contribution of a subsite recognition element to ternary complex formation, catalysis, and product release. Results from this analysis suggest that amino acids in the C-subunit acidic cluster contribute to catalysis as well as provide binding energy for substrate arginines.

Mutant stability

Glu 230 was mutated conservatively to Gln and nonconservatively to Ala. The carboxylate to amide replacement in $rC[E230Q]$ is

Table 3. Electrostatic free energy of Kemptide binding to wild-type and $rC[E230Q]$ C-subunits^a

Enzyme	Kemptide	$\Delta\Delta G$ (kcal/mol)
$rC[E230Q]$	Unaltered	23.0
$rC[E230Q]$	Neutral P-2 Arg	12.5
$rC[E230Q]$	Neutral P-3 Arg	19.6
Wild-type	Neutral P-2 Arg	3.4
Wild-type	Neutral P-3 Arg	9.9

^a Theoretical neutral mutations were made using the program Delphi, as described in Materials and Methods.

Fig. 4. Electrostatic potential slices through the C-subunit. The C-subunit backbone is in green and space-filling representations of Glu 230 and similarly positioned residues Asp 127, Glu 170, Glu 203, and Asp 241 are in sky blue. The slices are through Glu 230 without Kemptide (**A**) and with Kemptide depicted in cyan (**B**), and through Gln 230 without Kemptide (**C**) and with Kemptide (**D**). Dark blue represents positive electrostatic potential and red represents negative electrostatic potential. The Kemptide P – 2 Arg points toward Glu 230 in B and Gln 230 in D.

expected to retain hydrogen bonds to both Tyr 204 and Arg 133, which subsequently stabilize the structural framework of the C-subunit. In contrast, the carboxyl to methyl replacement in rC[E230A] will eliminate these hydrogen bonds. These hypotheses are consistent with the observed stability of the mutant enzymes. Although rC[E230Q] was stable for more than three months at 4 °C, rC[E230A] lost activity in seven days. Thermal

denaturation of rC[E230Q] catalytic activity yielded an identical T_m (44 °C) as wild-type enzyme. Although temperature stability studies may reflect gross structural perturbations only, correlation of rC[E230Q] catalytic parameters with those for peptide analogues (discussed below) indicates that the conservative carboxylate to amide mutation causes only subtle, if any, structural changes in rC[E230Q].

Michaelis ternary complex formation

Because buffer components were shown to influence the steady-state kinetic parameters for Kemptide phosphorylation (Yoon & Cook, 1987; Kong & Cook, 1988; Adams & Taylor, 1992), rC[E230Q] was compared with wild-type C-subunit using a single buffer condition. Although k_{cat} and the K_m (ATP) were not significantly perturbed, K_m (Kemptide) increased by two orders of magnitude on mutation of Glu 230. Because K_m (Kemptide) does not simply reflect peptide affinity, the increase in K_i (LRR AALG) by one order of magnitude more accurately measures the perturbation in substrate affinity caused by the mutation. Both k_{cat} and K_m parameters reported here are similar in magnitude to those measured in a charged-to-alanine scan for the *Saccharomyces cerevisiae* C-subunit (Gibbs et al., 1992), even though the mutation in the murine C-subunit is less invasive. Because both an amide and a carboxyl can form hydrogen bonds stabilizing enzyme structure, the salient difference between wild-type and rC[E230Q] is a change in the electrostatic potential around this region (Fig. 4). An acidic electrostatic potential in wild-type C-subunit shifts to one that is more basic in rC[E230Q]. The 23 kcal/mol free energy increase calculated for rC[E230Q] represents only the altered electrostatic potential in the mutant, and its value most likely decreases as a result of compensatory steric, hydrophobic, and hydrogen bonding effects in rC[E230Q]. The decrease in rC[E230Q] Kemptide affinity is compensated by the theoretical neutral mutation of the substrate P-2 Arg (Table 3), indicating that Glu 230 interacts most strongly with the P-2 Arg. The positively polarized electrostatic potential in rC[E230Q] decreases binding energy for the positively charged substrate P-2 Arg and thereby decreases ligand affinity. Steady-state kinetic data in this study suggests that perturbations in the Glu 230/P-2 Arg electrostatic node decrease substrate affinity by about 2 kcal/mol.

Phosphoryl transfer and product release

Mutation of subsite recognition elements to Ala caused only minor perturbations in k_{cat} (Gibbs et al., 1992) in a charged-to-alanine scan of the *S. cerevisiae* C-subunit. Although mutation of Glu 230 to Gln in the recombinant murine C-subunit caused a twofold reduction in k_{cat} , k_{cat} was further defined in this study by two independent kinetic techniques. Results from viscosometric and quench-flow analysis established that, although the product release rate constant is perturbed insignificantly, the phosphoryl transfer rate constant is reduced by an order of magnitude (Table 2). Agreement between the two methods further confirms phosphoryl transfer is nondiffusion controlled for the C-subunit. The decrease in phosphoryl transfer accompanying mutation of Glu 230 was not expected from previous studies. Disruption of the Glu 230/P-2 Arg electrostatic node by conservative mutation of Glu 230, in fact, decreases substrate binding and catalysis equivalently.

Disruption of the Glu 230/P-2 Arg electrostatic node by mutation of the enzyme or the peptide substrate causes the same perturbation in catalytic parameters. A partial viscosity effect on k_{cat} was observed previously for wild-type enzyme turnover of a substrate missing a P-2 Arg, LRANSI (Adams & Taylor, 1993a). Turnover of this peptide by wild-type C-subunit yielded identical k_3 and k_4 parameters as when Kemptide is turned over by rC[E230Q]. Because removal of the Glu 230/P-2 Arg elec-

trostatic node by mutation of the peptide could not alter the enzyme structure significantly, the conservative carboxylate to amide mutation in rC[E230Q] most likely causes only subtle structural perturbations, if any occur at all.

The decrease of the rC[E230Q] phosphoryl transfer rate constant by an order of magnitude suggests that the mutation destabilizes the phosphoryl transfer transition state. The decrease in negative charge density (Fig. 4) due to the carboxyl to amide mutation in rC[E230Q] may destabilize a positively polarized transition state. A positively polarized transition state is consistent with a phosphoryl transfer mechanism in which the phosphate acceptor hydroxyl proton leaves late in the transition state. Glu 230 can provide charge stabilization to the transition state from a distance of 15 Å as electrostatic field perturbations resulting from amino acid substitution were shown to effect pK_a s of amino acids 20 Å away (Loewenthal et al., 1993). Alternatively, transition-state destabilization could also be caused by small structural perturbations in the rC[E230Q] active site. Weaker substrate interactions could result in poor phosphoryl acceptor positioning. In addition, the P-2 Arg, which no longer forms a salt bridge with Glu 230 in rC[E230Q], may neutralize its charge by forming a stronger interaction with Glu 170; movement of Glu 170 could conceivably result in altered positions of catalytic loop residues necessary for transition-state stabilization. rC[E230Q] crystallization trials have begun and information from future structural studies as well as studies that characterize the transition state will delineate requirements for the protein kinase phosphoryl transfer.

Protein kinase substrate specificity

A free energy diagram assembled for wild-type and rC[E230Q] (Fig. 5) summarizes perturbations in kinetic parameters caused by the mutation. Transition-state energies were calculated by operating the Eyring equation (Fersht, 1977) on rate constants parameterized in this and other studies (Adams & Taylor, 1992). Relative perturbations in ground-state free energies were calcu-

Fig. 5. Free energy diagrams for wild-type (solid) and rC[E230Q] (dashed) phosphoryl transfer reaction pathways. Ground-state free energies are calculated from equilibrium parameters and transition-state free energies of activation are calculated from rate constants for individual steps in the reaction pathway. The free energy of uncomplexed enzymes was arbitrarily set to zero and an internal equilibrium constant of 100 was used.

lated from equilibrium constants for steps along the reaction pathway. Assumptions included in the free energy diagrams are: (1) mutations do not perturb free enzyme ground-state free energy; (2) mutations do not perturb association microscopic rate constants (Adams & Taylor, 1993b); and (3) an internal equilibrium constant of 100 determines the relation between substrate and product ternary complex ground-state free energies for wild-type (Kong & Cook, 1988) and mutant enzymes. Figure 5 illustrates the insignificant effect on ATP binding, the order of magnitude decrease in substrate affinity, the order of magnitude decrease in the phosphoryl transfer rate constant, and the unperturbed rate of product release in rC[E230Q].

In contrast to cAPK, protein kinases that phosphorylate amino acids flanked by acidic residues substitute subsite recognition elements with basic and neutral amino acids. Casein kinase II substitutes Glu 230 with Ser and Glu 203, Glu 170, and Asp 241 with His. Tyrosine protein kinases typically substitute Glu 230 and Asp 241 with either Ile or Val, Glu 170 with either Ala or Arg, and Asp 127 and Glu 203 with Leu and Lys. In the case of the cAPK C-subunit, analysis of rC[E230Q] suggests that the Glu 230/P – 2 Arg electrostatic node contributes 2 kcal/mol to substrate binding affinity and 2 kcal/mol to transition-state stabilization. C-subunit crystal structures predict that Asp 127, Glu 170, Glu 203, and Asp 241 play similar roles as Glu 230 because they are similarly positioned with respect to the substrate arginines. This cAPK-specific acidic cluster dictates substrate specificity by: (1) providing binding energy for only substrates that are positively polarized by arginines; and (2) stabilizing the phosphoryl transfer transition state only when an optimal electrostatic field is attained in the Michaelis complex.

Materials and methods

Materials

Reagents were purchased as follows: ATP, MOPS, lactate dehydrogenase, pyruvate kinase, NADH, and phosphoenolpyruvate from Sigma Chemicals; IPTG from United States Biochemical; magnesium chloride, phosphoric acid, β -mercaptoethanol, and liquid scintillant from Fisher Scientific; phosphocellulose filter disks and resin from Whatman; mutagenesis buffers from Biorad; a Mono S Fast Performance Liquid Chromatography (FPLC) column from Pharmacia; [γ - 32 P] ATP and [α - 35 S] dATP from ICN Laboratories; peptides, LRRASLG (Kemptide), and LRR AALG were synthesized at the Peptide and Oligonucleotide Facility at the University of California, San Diego, and purified by HPLC.

Enzyme mutagenesis, expression, and purification

Active-site mutations were introduced into the mouse C-subunit gene (Uhler et al., 1986) by oligonucleotide mutagenesis (Zoller & Smith, 1987) and confirmed by [α - 35 S] dideoxy ATP DNA sequencing. *E. coli* strain BL21-DE3 was transformed with pRSET-B harboring the mutant C-subunit gene, and transformants were selected by ampicillin resistance. Transformed bacteria were cultured in YT media containing 200 μ g/mL ampicillin, and gene expression was induced at 25 °C with 1 mM IPTG once the culture's optical density was 0.6 AU at 600 nm. At the end of a 5-h induction, cells were harvested by centrifugation at 4,000 $\times$ g for 10 min. After lysis in a French pressure cell, C-subunit was

purified using phosphocellulose resin and MonoS FPLC as described previously (Herberg et al., 1993). C-subunit concentration was measured by its absorbance at 280 nm ($A_{0.1\%} = 1.2$).

Coupled enzyme assay

Enzymic activity was determined as described previously (Cook et al., 1982). Oxidation of NADH, monitored spectrophotometrically as an absorbance decrease at 340 nm, was coupled to the production of ADP by lactate dehydrogenase and pyruvate kinase. C-subunit (50–100 nM) was typically incubated in a 60- μ L volume containing 50 mM MOPS, pH 7.0, 5 mM ATP, 15 mM magnesium chloride, 1 mM phosphoenolpyruvate, 0.3 mM NADH, 12 units of lactate dehydrogenase, and 4 units of pyruvate kinase at 23 °C. Reactions were incubated in a quartz microcuvette, initiated with varying amounts of Kemptide, and monitored in a DU 640 Beckman spectrophotometer. Kemptide stock concentrations were determined by turnover with wild-type C-subunit under conditions of limiting peptide in the spectrophotometric assay. For inhibition studies, LRR AALG was pre-equilibrated with enzyme for 1 min before addition of Kemptide.

Solution viscosity measurements

The relative viscosity (η^{rel}) of buffers containing sucrose was measured relative to 50 mM MOPS, pH 7.0, at 23 °C using an Ostwald viscometer (Shoemaker & Garland, 1962). Relative solvent viscosities of 1.0, 1.6, 1.9, and 2.4 were obtained for buffers containing 0, 16, 22, and 29% (w/v) sucrose. The measurements were made in triplicate and did not deviate by more than 2%.

Rapid quench-flow measurements

Pre-steady-state kinetic measurements were made using a Kin-Tek Corporation Quench Flow Apparatus Model RGF-3. The apparatus contains three syringes driven by a stepping motor. Quench-flow experiments were executed as described previously (Grant & Adams, 1996).

Data analysis

Michaelis–Menten initial velocity versus substrate concentration data were fit to a simple hyperbolic function:

$$v_0 = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

Linear fits of Dixon plots determined K_i (LRR AALG) values. Data from quench-flow time courses were fitted to an empirical function containing a single exponential and a linear component:

$$y = \alpha[1 - \exp(-k_b \cdot t)] + L \cdot t, \quad (2)$$

where y is the concentration of phospho-Kemptide, α is the observed burst amplitude, k_b is the observed single-exponential burst rate constant, and L is the observed linear rate. Data were fitted using the Macintosh computer graphics program, Kaleidagraph (Synergy Software), which utilizes an iterative least-squares algorithm.

Molecular modeling

The electrostatic field and changes in the electrostatic energy of interaction between the C-subunit and IP20 were investi-

gated for a ternary complex (Zheng et al., 1993) containing the C-subunit, IP20, and MnATP. This structure represents the closed conformation of the C-subunit. When substrate was modeled instead of IP20, the positioning of the P-site hydroxyl was based on the crystal structure of the C-subunit with MgADP and a substrate analogue of IP20 (Madhusudan et al., 1994).

The electrostatic field was calculated by solving the linearized Poisson-Boltzmann equation on a 0.9-Å grid using the program DelPhi 2.5. The dielectric constant of the solvent was set to 80 and that of the protein interior was set to 4. The ionic strength was equivalent to 0.145 M sodium chloride. The ionizable residues Glu, Arg, Lys, and Asp were assigned a full charge as expected at pH 7, whereas His was assigned a 0.5 positive charge. In addition, the phosphate on Thr 197 was assigned a 1.5 negative charge. The AMBER partial charge distribution (Weiner et al., 1984) was used to place partial charges on all atoms in the system. The total electrostatic energy of the system comprising the C-subunit and peptide was calculated as a sum of the electrostatic solvent interaction term with Coulombic energy (Gilson & Honig, 1988).

The total electrostatic free energy of peptide binding, V° , was estimated using a three-step thermodynamic process. First, both molecules were fully solvated and infinitely separated from each other. Second, the solvent around these molecules was replaced by media with a dielectric constant of 4, which is the same dielectric constant used for the protein interior. Third, the work of the assembly was calculated using Coulomb's Law and the solvent term of the electrostatic free energy when the enzyme complex was solvated.

In determinations of paired amino acids, ionizable amino acids in the enzyme and the substrate were mutated to neutral moieties using two different methods. The substrate P-2 and P-3 arginines were neutralized by removing only the charge, whereas Glu 230 was neutralized by mutation to Gln using Insight II (Biosym Technologies, Inc., San Diego, California). The side-chain conformation of each modified residue was adjusted by energy minimization using Discover (Biosym Technologies, Inc., San Diego, California) for 1,000 iterations to relax strains introduced by modifications. The total electrostatic binding free energy for the mutated system, V^m , was calculated after this minimization. Single replacements were made in an effort to determine the extent to which each ionizable residue contributed to peptide binding. Double replacements, one in the enzyme and one in the inhibitor, were introduced to investigate possible synergistic compensations caused by the removal of unfavorable lone charges. Differences between wild-type V° and $rC[E230Q]$ V^m were expressed as $\Delta\Delta G$ values.

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