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Identification of Electrostatic Interactions That Determine the Phosphorylation Site Specificity of the cAMP-Dependent Protein Kinase†

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ABSTRACT: "Charged-to-alanine" scanning mutagenesis of the catalytic subunit of the *Saccharomyces cerevisiae* cAMP-dependent protein kinase (C_1) identified three glutamate residues, E171, E214, and E274, that are involved in the recognition of a peptide substrate, kemptide ($Leu^1Arg^2Arg^3Ala^4Ser^5Leu^6Gly^7$). These glutamate residues are conserved or conservatively substituted with asparate in the serine/threonine protein kinases that have a requirement for basic residues on the N-terminal side of their phosphorylation sites. Alanine replacement mutants in C_1 were subjected to kinetic analysis using alanine-substituted peptides as substrates. The additivity or nonadditivity of the effects of the alanine substitutions on the catalytic efficiency (k_{cat}/K_m) was analyzed. This allowed the identification of electrostatic interactions between the three glutamate residues in the enzyme and the two arginine residues present in the peptide substrate. The data suggest that E171 interacts with Arg^2 in the substrate and that E214 and E274 both interact with Arg^3. This may be a general method for identifying simple intermolecular interactions involving proteins when there is no three-dimensional structure available of the complex of interacting species. The identification of these interactions provides the potential for rational protein engineering of enzymes with alternative specificities.

The cAMP-dependent protein kinase phosphorylates specific serine and threonine residues in selected protein substrates in response to extracellular regulatory signals mediated by the second messenger cAMP. In this study we investigate the interactions that determine the phosphorylation site specificity of the yeast cAMP-dependent protein kinase (C_1).

The primary determinants of phosphorylation site specificity have been well defined for the mammalian enzyme by the extensive analysis of modified peptide substrates and inhibitors based on the sequences flanking the phosphorylation sites in protein substrates. A dibasic consensus sequence, RRX(S/T)Hy (Hy = hydrophobic) has been identified that is recognized by the cAMP-dependent protein kinase in protein substrates and that is essential in good peptide substrates and inhibitors [reviewed in Krebs and Beavo (1979) and Zetterqvist et al. (1990)]. When either of the arginine residues in the model substrate kemptide ($Leu^1Arg^2Arg^3Ala^4Ser^5Leu^6Gly^7$) was substituted with alanine, a large effect on specificity for the mammalian cAMP-dependent protein kinase was observed. The catalytic efficiency (k_{cat}/K_m) was decreased by 700- and 1500-fold for kemptide(Ala^2) and kemptide(Ala^3), respectively (Kemp et al., 1977). Recent studies comparing the yeast cAMP-dependent protein kinase (C_1) and the mammalian enzyme suggested that the two enzymes have similar determinants of substrate specificity (C. Denis, M. Zoller, and B. Kemp, personal communication).

The analysis of the crystal structures of several enzymes bound to their substrates has allowed the identification of interactions between the enzymes and their substrates. The manipulation of these interactions by site-directed mutagenesis and the use of alternative substrates has allowed the contributions of these interactions towards substrate specificity to be evaluated and has allowed the engineering of new substrate specificities (Fersht et al., 1985; Craik et al., 1985; Mayer et al., 1986; Wells et al., 1987; Russell & Fersht, 1987; Scrutton et al., 1990). Presently there is no three-dimensional structure of the cAMP-dependent protein kinase available, although the determination of the structure of the mammalian enzyme is

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at an advanced stage (S. Taylor, personal communication).

In the absence of a crystal structure of the enzyme, we used "charged-to-alanine" scanning mutagenesis of yeast C_1 to identify four mutants that appeared to be defective in the recognition of the peptide substrate (kemptide), while the recognition of the other substrate (MgATP) was unaffected (Gibbs & Zoller, 1991). The observed effects on the catalytic efficiency were also large, ranging between 100- and 1200-fold. Collectively, the mutants involved the substitution of a total of seven acidic amino acids with alanine. We suggested that some of these acidic side chains may participate in electrostatic interactions with the arginine side chains in the peptide substrate to contribute to substrate specificity.

In this study, we undertake further site-directed mutagenesis of yeast C_1 to identify the single acidic amino acids involved in determining substrate specificity. Furthermore, the steady-state kinetic analysis of C_1 mutants with alanine-substituted peptide substrates is applied to determine whether the acidic amino acid side chains identified interact with the dibasic recognition sequence and, if so, which acidic residue in the enzyme interacts with which arginine residue in the substrate.

Experimental Procedures

Yeast Strains and Plasmids. Yeast strain LL8 [*MATa tpk1::URA3 tpk2::HIS3 tpk3::TRP1 BCY1 leu2* + YEp-(*ADE8*)*TPK1*] contains chromosomal disruptions in all three *TPK* genes encoding catalytic subunits of the yeast cAMP-dependent protein kinase, and viability is maintained by the presence of *TPK1* on a 2μ-based multicopy vector (Levin & Zoller, 1990). All the strains discussed in this study were derived by the transformation of LL8.

YEp(*LEU2*)*TPK1* is a multicopy yeast expression vector consisting of pUC119 containing the 2μ origin of replication, the *LEU2* gene, and the *TPK1* gene encoding C_1 (Levin & Zoller, 1990). YEp(*LEU2*)*TPK1* served as the template for all site-directed mutagenesis experiments.

YEp(*ADE8*)*ADH-BCY1*(T144N,S145A) is a multicopy yeast expression vector consisting of pUC119 containing the 2μ origin of replication, the *ADE8* gene, and the *BCY1* gene encoding the regulatory subunit of the cAMP-dependent protein kinase under the control of the *ADH* promoter. The coding region of the *BCY1* gene contains two mutations in the pseudosubstrate domain, T144N and S145A, that result in a regulatory subunit that binds more tightly to the catalytic subunit (Levin & Zoller, 1990). Yeast media and culture conditions were according to Sherman et al. (1983). Yeast strains were transformed by the lithium acetate procedure (Ito et al., 1983).

Site-Directed Mutagenesis and Construction of Mutant Yeast Strains. Alanine substitutions were introduced into the *TPK1* gene encoding yeast C_1 by oligonucleotide-directed mutagenesis on a single-stranded, uracil-containing template of YEp(*LEU2*)*TPK1* as described previously (Gibbs & Zoller, 1991). Yeast strain LL8 was transformed with the mutated plasmids, transformed strains were grown without selection, and strains that had lost the wild-type plasmid were selected by using the auxotrophic markers *LEU2* and *ADE8* (Zoller et al., 1991). Yeast strains containing mutant *TPK1* genes were transformed with YEp(*ADE8*)*ADH-BCY1*(T144N,S145A) to overexpress the tight-binding mutant regulatory subunit to amplify the expression of the mutant *TPK1* gene (Zoller et al., 1988) and to aid the purification of mutant catalytic subunits that have reduced affinity for the regulatory subunit (Levin & Zoller, 1990). Mutant plasmids were rescued from mutant yeast strains, and the presence of the correct mutation was confirmed by DNA sequencing.

Determination of Kinase Activity in Crude Cell Extracts. To screen mutant enzymes for defects in substrate recognition, crude cell extracts were made from 10-mL yeast cultures and phosphotransferase activity was measured at various substrate concentrations (Roskoski, 1983) as described previously (Gibbs & Zoller, 1991).

Expression and Purification of Mutant Enzymes. One-liter cultures of mutant yeast strains were grown and harvested. Wild-type yeast C_1 and mutant enzymes were purified by ammonium sulfate fractionation and affinity chromatography using an immobilized anti-regulatory subunit monoclonal antibody (Zoller et al., 1988). Anti-regulatory subunit monoclonal antibody Mab BCY427 was purified from ascites fluid by precipitation with 30% saturated ammonium sulfate (Harlow & Lane, 1988) and was linked to cyanogen bromide activated Sepharose CL-4B (Pharmacia). Enzyme preparations were applied to 1-mL immunoaffinity columns in phosphate-buffered saline (PBS) (136.89 mM NaCl, 2.68 mM KCl, 7.96 mM Na_2HPO_4, and 1.47 mM KH_2PO_4, pH 7.2) at 4 °C and eluted batchwise with 1-mL aliquots of PBS containing 0.8 mM cAMP at 25 °C. Purified catalytic subunits were dialyzed against final sample buffer (PBS containing 5 mM β-mercaptoethanol, 0.02% Triton X-100, and 5% glycerol). Enzyme preparations were judged to be at least 95% pure as judged from SDS–polyacrylamide gels stained with Coomassie Blue. Enzyme concentration was determined from quantitative Western slot blots by using multiple estimates of two enzyme concentrations as described previously (Gibbs & Zoller, 1991).

Synthetic Peptide Substrates. Three peptide substrates—kemptide (NH_2-Leu^{1}Arg^{2}Arg^{3}Ala^{4}Ser^{5}Leu^{6}Gly^7-COOH), kemptide(Ala^2) (NH_2-Leu^{1}Ala^{2}Arg^{3}Ala^{4}Ser^{5}Leu^{6}Gly^7-COOH), and kemptide(Ala^3) (NH_2-Leu^{1}Arg^{2}Ala^{3}Ala^{4}Ser^{5}Leu^{6}Gly^7-COOH)—were synthesized manually by using Boc chemistry and purified by HPLC. The correct amino acid content was confirmed and the purity and concentration of the peptides was determined by amino acid analysis following hydrolysis. Concentrated 100 mM stock solutions of kemptide and kemptide(Ala^2) were made in aqueous solution and neutralized by titration with 1 M NaOH. Kemptide(Ala^3) was insoluble at 100 mM in aqueous solution at neutral pH and was dissolved in 10% dimethylformamide prior to neutralization. Concentrated stock solutions and diluted stock solutions were added directly to enzyme reaction mixes. The presence of up to 2% dimethylformamide in final reaction mixes was shown to have no effect on the measured enzyme activity.

Steady-State Kinetic Analysis. All combinations of mutant enzymes and substituted peptides were analyzed by steady-state kinetics. Aliquots (10 μL) containing between 30 and 200 ng of the purified mutant enzymes were used to start the reactions. The reaction mixtures contained 50 mM MOPS (pH 7.0), 0.25 mg·mL^{-1} BSA, 10 mM $MgCl_2$, 10 μM cAMP, 200 μM ATP, and 1000 cpm·pmol^{-1} [γ-^{32}P]ATP in a final volume of 50 μL. The concentration of the peptide substrates was varied at ten points ranging between 2× and 0.2× K_m. Reactions were incubated at 30 °C for 15 min and terminated by the addition of 150 μL of 40% acetic acid. The incorporation of ^{32}P into the peptide substrates was determined after removing unincorporated [γ-^{32}P]ATP by anion-exchange chromatography (Glass, 1983). Aliquots (100 μL) of terminated reaction mixtures were applied to disposable columns containing 2 mL of AG 1-X8 anion-exchange resin (Bio-Rad) equilibrated with 30% acetic acid. Radioactively labeled

Table I: Kinetic Parameters for Single Alanine Substitution Mutants Derived from the Double Mutant (E168A,E171A)

mutant	mutation	k_{cat} (s^{-1})	K_m (MgATP) (mM)	K_m (kemptide) (mM)	k_{cat}/K_m (kemptide) (s^{-1}/mM)
27	E168A,E171A	0.27	0.066	9.76	0.028
27X	E168A	3.60	0.097	0.228	15.79
27Y	E171A	0.36	0.039	9.11	0.040
wild type		8.60	0.043	0.136	63.24

peptide substrates were eluted directly into scintillation vials with five 1-mL aliquots of 30% acetic acid and were quantitated directly by Cerenkov counting.

Kinetic data were fitted to the Michaelis–Menten equation by the Marquardt–Levenberg nonlinear regression algorithm and used to calculate the kinetic parameters (k_{cat} and K_m) and their associated standard errors.

Results

Identification of Single Charged Residues in Yeast C_1 Involved in Interactions with a Peptide Substrate. In our previous study (Gibbs & Zoller, 1991), the charged amino acids (Asp, Glu, Arg, Lys, and His) in yeast C_1 were systematically mutated to alanine in groups of one, two, and three residues. Estimates of the kinetic paramters k_{cat}, K_m(kemptide), and K_m(MgATP) were determined for the mutant enzymes in crude cell extracts. Four "charged-to-alanine" mutants, 27 (E168A,E171A), 34Y (E214A), 43 (E274A), and 58 (E377A,E378A,D379A), were identified that appeared to be defective in interacting with the peptide substrate kemptide, displaying large increases in K_m for kemptide and small changes in k_{cat} and K_m for MgATP. The catalytic efficiency (k_{cat}/K_m) toward kemptide was decreased by 1200-, 350-, 680-, and 110-fold, respectively.

Two of the mutants, 27 (E168A,E171A) and 58 (E377A,E378A,D179A), involved the simultaneous substitution of multiple charged amino acids. In order to identify the single amino acid residues responsible for the kinetic defects, the alanine substitutions were constructed separately in C_1 and introduced into a yeast strain (LL8) that contains disruptions in all three chromosomal *TPK* genes. Kinetic analysis of the mutant enzymes in crude cell extracts was used to determine the kinetic parameters k_{cat}, K_m(kemptide), and K_m(MgATP). The values for k_{cat} determined in crude cell extracts have additional error derived from the estimation of the enzyme concentration by Western blotting (Gibbs & Zoller, 1991) that is not present in the values for k_{cat} determined for the purified enzymes in the latter portion of this study.

The single alanine substitution mutants derived from mutant 58 (E377A,E378A,D379A) displayed smaller defects in k_{cat}/K_m (2–8-fold) than previously observed for the multiple mutant (110-fold) and were eliminated from further analysis. The kinetic parameters determined for the single mutants 27X (E168A) and 27Y (E171A) derived from the double mutant 27 (E168A,E171A) are displayed in Table I. The effects of the double mutation on the recognition of kemptide are almost entirely attributable to the substitution of E171 in mutant 27Y, which displays a 67-fold increase in K_m for kemptide and a 24-fold decrease in k_{cat} that parallel the effects observed for the double mutant, 27. The other single mutant, 27X (E168A) has a phenotype almost like that of the wild-type enzyme, displaying only slight increases in K_m(kemptide) and K_m(MgATP) and a slight decrease in k_{cat}.

Strategy To Identify Interactions between the Enzyme and a Peptide Substrate. "Charged-to-alanine" scanning mutagenesis of yeast C_1, combined with the steady-state kinetic analysis of the mutant enzymes in crude cell extracts, has identified three glutamate residues, E171, E214, and E274, that appear to be involved in interactions with a peptide substrate (kemptide). The substitution of these residues with alanine caused large decreases (1200-, 350-, and 680-fold) in the catalytic efficiency (k_{cat}/K_m) toward kemptide.

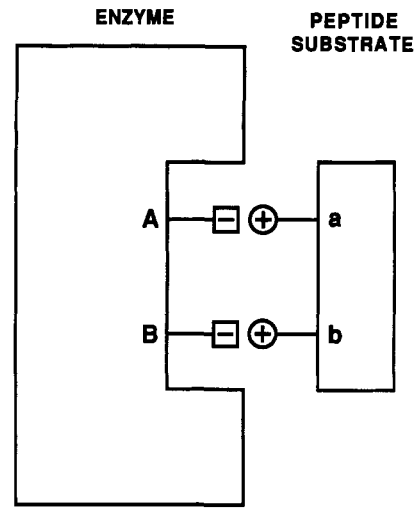

FIGURE 1: Identification of electrostatic interactions between the enzyme and a peptide substrate by combining site-directed mutagenesis with the use of substituted peptide substrates. In this model, negatively charged residues (A and B) on the enzyme participate in electrostatic interactions with positively charged residues on the peptide substrate (a and b). In the case where the residue mutated on the enzyme (A) is not interacting with the residue substituted in the substrate (b), the effects of substituting each residue are additive and two interactions are broken. In the other case, where the residue mutated on the enzyme (A) is interacting with the residue substituted in the substrate (a), the effects of each substitution are not additive and only one interaction is broken.

Previously, Kemp and co-workers demonstrated that when the two arginine residues adjacent to the phosphorylation site in the peptide substrate kemptide ($Leu^1Arg^2Arg^3Ala^4$-$Ser^5Leu^6Gly^7$) were individually substituted with alanine, the catalytic efficiency displayed by the mammalian cAMP-dependent protein kinase decreased markedly, by 700- and 1500-fold for kemptide(Ala^2) and kemptide(Ala^3), respectively (Kemp et al., 1977). This suggested that these two arginine residues participate in important interactions with the enzyme.

Since the glutamate residues identified in the enzyme and the arginine residues in the peptide substrate are of opposite charge and the effects of substituting either with alanine are similar, it is attractive to suggest that these residues may participate in electrostatic interactions with each other to contribute to substrate specificity. To test this hypothesis, the two methods of analysis were combined in a single experiment where the "charged-to-alanine" mutants of C_1 were subjected to kinetic analysis using the alanine-substituted peptides as substrates. In these experiments it would be expected that the effect of an alanine replacement mutation in the enzyme and the effect of an alanine substitution in the peptide substrate would be additive, except in the case where the two residues substituted interact with each other, as illustrated in Figure 1. In this way, the electrostatic interactions between the enzyme and the substrate may be identified in the absence of a crystal structure of the enzyme–substrate complex.

Kinetic Analysis of Mutant C_1 Enzymes in Combination with Substituted Peptide Substrates. The three C_1 mutants and the wild-type enzyme were purified by immunoaffinity chromatography and subjected to steady-state kinetic analysis with all combinations of kemptide and the two alanine-substituted peptides, kemptide(Ala^2) and kemptide(Ala^3). The

Table II: Kinetic Parameters for C_1 Mutants against Substituted Peptide Substrates

mutant	peptide substrate		k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1}/mM)	rel specificity[a]
wild type	kemptide	LRRASLG	4.18 ± 0.21	0.101 ± 0.01	41.4	
	kemptide(Ala^2)	LARASLG	1.88 ± 0.08	15.2 ± 0.72	0.124	334
	kemptide(Ala^3)	LRAASLG	1.81 ± 0.08	12.5 ± 0.45	0.145	286
E171A	kemptide	LRRASLG	1.72 ± 0.10	8.01 ± 0.89	0.215	
	kemptide(Ala^2)	LARASLG	3.70 ± 0.23	16.6 ± 1.70	0.223	0.96
	kemptide(Ala^3)	LRAASLG	0.21 ± 0.01	18.4 ± 1.62	0.011	19.5
E214A	kemptide	LRRAALG	1.70 ± 0.07	12.6 ± 0.83	0.135	
	kemptide(Ala^2)	LARASLG	0.19 ± 0.01	22.8 ± 2.18	0.008	16.9
	kemptide(Ala^3)	LRAASLG	1.16 ± 0.05	15.7 ± 1.21	0.074	1.82
E274A	kemptide	LRRASLG	1.91 ± 0.13	3.32 ± 0.27	0.575	
	kemptide(Ala^2)	LARASLG	0.36 ± 0.03	22.5 ± 2.03	0.016	35.9
	kemptide(Ala^3)	LRAASLG	4.39 ± 0.36	18.4 ± 2.67	0.239	2.41

[a] Relative specificity = $[k_{cat}/K_m(\text{kemptide})]/[k_{cat}/K_m(\text{Ala peptide})]$.

kinetic parameters determined for each pairwise combination are displayed in Table II. The catalytic efficiency (k_{cat}/K_m) of each mutant toward each peptide was calculated as a measure of substrate specificity (Fersht, 1977). The *relative specificity* of each mutant toward each substituted peptide was calculated from the ratio of the catalytic efficiency toward kemptide to the catalytic efficiency toward the substituted peptide.

The substitution of either of the two arginine residues in kemptide with alanine caused decreases in the catalytic efficiency of wild-type C_1 of 334-fold for kemptide(Ala^2) and 286-fold for kemptide(Ala^3). The substitution of glutamate residues in C_1 with alanine caused decreases of a similar magnitude in the catalytic efficiency of the mutant enzymes toward kemptide. In some cases the pairwise combination of the mutant enzymes with the substituted peptides caused no further decrease in the catalytic efficiency, in which case it would be predicted that the residue mutated in the enzyme and the residue substituted in the peptide are interacting with each other. In other cases, the catalytic efficiency decreased further, in which case it would be predicted that the two residues substituted are involved in separate interactions between the enzyme and substrate.

The catalytic efficiency of the mutant E171A toward kemptide(Ala^2) is the same as that toward kemptide. However the catalytic efficiency toward kemptide(Ala^3) is further decreased by a factor of 20, suggesting that an additional interaction (or interactions) has been broken. Thus, mutant E171A has a preference for kemptide over kemptide(Ala^3) (relative specificity = 19.5) but no preference for kemptide over kemptide(Ala^2) (relative specificity = 1.0). This suggests that E171 in C_1 interacts with Arg^2 in kemptide.

Mutants E214A and E274A display the opposite pattern of substrate specificity. The substitution of Arg^3 in kemptide(Ala^3) had little effect on the catalytic efficiency of these mutants. However, the substitution of Arg^2 in kemptide(Ala^2) caused 17- and 36-fold decreases in the catalytic efficiency of these of these mutants, respectively. In other words, the mutants E214A and E274A display little preference for kemptide over kemptide(Ala^3) (relative specificity = 1.8 and 2.4) but display a preference for kemptide over kemptide(Ala^2) (relative specificity = 16.9 and 35.9). This suggests that E214 and E274 in C_1 interact with Arg^3 in kemptide.

Discussion

"Charged-to-alanine" scanning mutagenesis of the catalytic subunit of yeast cAMP-dependent protein kinase (C_1) has identified three glutamate residues, E171, E214, and E274, that appear to be involved in interactions with a peptide substrate, kemptide. The additivity of the effects observed in the kinetic analysis of pairwise combinations of C_1 mutants and substituted peptide substrates in this study (Table II) supports the suggestion that these residues contribute to substrate specificity by interacting with the two arginine residues of the substrate recognition sequence, RRX(S/T)Hy (Kemp et al., 1977; Krebs & Beavo, 1979; Zetterqvist et al., 1990).

The patterns of conservation of these glutamate residues among the protein kinases are consistent with the suggestion that they are involved in phosphorylation site specificity (Hanks et al., 1988). E171, E214, and E274 (E127, E170, and E230 in the mammalian enzyme) are conserved or conservatively substituted with aspartate in the cyclic nucleotide dependent, Ca^{2+}/calmodulin-dependent, protein kinase C, and CDC28 subfamilies of the serine/threonine protein kinases that recognize substrates with basic residues on the N-terminal side of the phosphorylation site (Kemp & Pearson, 1990). E214 and E274 are replaced by histidine and serine, respectively, in human, rat, *Drosophila*, and bovine casein kinase II (Meisener et al., 1989), which require an acidic recognition sequence (Kuenzel & Krebs, 1985; Pinna et al., 1990; Kemp & Pearson, 1990).

Biochemical studies on the mammalian enzyme have also implicated these residues in interactions with peptide substrates. The residue equivalent to E171 in the mammalian enzyme (E127) is flanked by two residues, G126 and M128, that were labeled by a photoaffinity analogue of kemptide. Labeling was accompanied by inactivation of the enzyme but could be partially prevented by the presence of MgATP and completely prevented by MgATP in the presence of kemptide (Miller & Kaiser, 1988). The residue equivalent to E241 in the mammalian enzyme (E170) was protected from modification by a water-soluble carbodiimide by an inhibitor peptide in the presence of MgATP (Buechler & Taylor, 1990). Similarly, E230, the residue in the mammalian enzyme equivalent to E274, was modified by a hydrophobic carbodiimide. Modification of this residue was prevented by an inhibitor peptide and MgATP but not by MgATP alone (Buechler & Taylor, 1988).

In our study, the additivity or nonadditivity of the effects of pairwise combinations of disruptive alanine substitution mutants in C_1 and alanine substitutions in kemptide on the catalytic efficiency (k_{cat}/K_m) allowed the identification of electrostatic interactions between the enzyme and substrate. The kinetic data presented in Table II suggest that E171 in C_1 interacts with Arg^2 in the substrate and that E214 and E274 both interact with Arg^3, as illustrated in Figure 2. The hydrogen-bonding capacity of an arginine side chain is sufficient to accommodate interactions with carboxyl groups from two glutamate residues. The data base of highly resolved

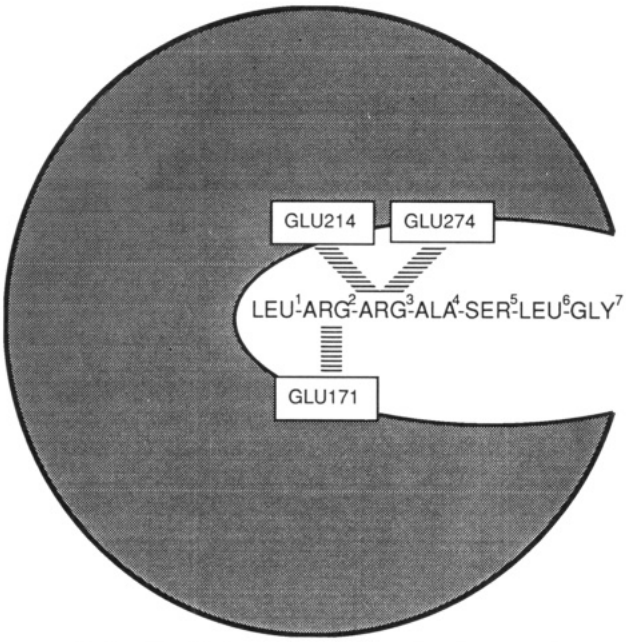

FIGURE 2: Predicted electrostatic interactions between the yeast cAMP-dependent protein kinase and the peptide substrate kemptide. Predicted electrostatic interactions between negatively charged residues in the enzyme and positively charged residues on the substrate are indicated by a broken line. The two arginine residues that constitute the recognition sequence found in natural substrates of the cAMP-dependent protein kinase and in the naturally occurring protein kinase inhibitor, PKI, are predicted to interact with the enzyme in a similar way.

protein structures was searched for examples of two carboxyl groups interacting with a single arginine side chain. The examples found included the interactions between D32, D82, and R35 in thermolysin (Holmes & Matthews, 1982), between E35, D125, and R129 in alcohol dehydrogenase (Eklund et al., 1976), and between E172, E190, and R195 in actinidin (Baker, 1980).

The substitution of charged groups on the enzyme and substrate with alanine had large effects on the catalytic efficiency (k_{cat}/K_m). The effects were distributed between k_{cat} and K_m (Table II), as has been observed in previous mutagenesis experiments on a variety of enzymes involving the substitution of residues that are interacting with substrates (Wilkinson et al., 1983; Craik et al., 1985; Fersht et al., 1985; Leatherbarrow et al., 1985; Wells et al., 1987). This is consistent with the suggestion that some of the binding energy between the enzyme and substrate may not be realized until the enzyme–transition state complex is formed where some of the binding energy may be diverted to stabilize the transition state (Fersht, 1977).

A comparison of the catalytic efficiencies observed for the mutant and wild-type enzymes against the substituted peptides reveals that in two cases a mutation has slightly improved the catalytic efficiency of the enzyme toward a substituted peptide (Table II). The catalytic efficiency of mutant E171A against kemptide(Ala^2) is greater than that of the wild-type enzyme by 1.8-fold, and the catalytic efficiency of E274A against kemptide(Ala^3) is 1.6-fold greater than that of wild-type. This suggests that leaving an unpaired charged group in the active site of the enzyme may be disadvantageous. The magnitude of these improvements is small, and although statistically significant, they may be irrelevant, considering that the catalytic efficiency of these mutants is very low. However, these improvements suggest that it may be possible to engineer alternative substrate specificities into the enzyme by manipulation of the interactions at these sites.

It should be appreciated that the interpretation of the patterns of additivity and nonadditivity observed for the combination of disruptive substitutions in the enzyme and substrate in terms of electrostatic interactions between individual residues of opposite charge is a simple interpretation of what may be a complex system. The interpretation of the kinetic data requires assumptions that the substitutions introduced into the enzyme and substrate have not radically altered the conformation of the enzyme or the configuration of the peptide substrate and that the binding mode of the substrate and the kinetic mechanism of the enzyme are unchanged. It is not trival to test the validity of any of these assumptions; however, the fact that all of the mutant enzymes support the growth of yeast cells suggests that they continue to recognize their natural substrates. No examples of substrate inhibition were observed, suggesting that the substituted peptide substrates do not bind to the enzymes in a nonproductive mode. The ultimate test of the accuracy of the conclusions drawn in this study is the solution of the three-dimensional structure of the enzyme–substrate complex, which is currently in progress.

The conclusions drawn from this study are equivalent to those that normally require the interpretation of a crystal structure of the enzyme–substrate complex, as indicated above. The combined analysis used in this study, where intermolecular interactions are identified by examining the patterns of additivity and nonadditivity between disruptive modifications made in the interacting species, may be a general method for identifying simple interactions between enzymes and substrates, between hormones and receptors, and in other protein–protein interactions. The identification of these interactions allows the potential for rational protein engineering of alternative specificities in systems where there is no crystal structure available of the interacting species. A similar method of analysis has been applied to identify an interaction important for the DNA binding specificity of the *lac* repressor (Ebright, 1986).

Added in Proof.

Subsequent to the submission of this manuscript, the three-dimensional structure of the mammalian cAMP-dependent protein kinase bound to a peptide derived from the heat stable protein kinase inhibitor (PKI) has been solved (S. S. Taylor, Department of Chemistry, University of California, San Diego, personal communication and submitted to *Science*). The conclusions presented in our study are in agreement with the data from the crystal srtucture. The residues in the mammalian enzyme equivalent to those identified by us (E127, E170, and E230) are positioned and oriented such that they can interact with the two arginine residues in the peptide in the manner predicted in our study.

Acknowledgments

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Registry No. Arg, 74-79-3; Glu, 56-86-0; MgATP, 1476-84-2; kemptide, 65189-71-1; kemptide(Ala^2), 133496-45-4; kemptide(Ala^3), 133496-46-5; protein kinase, 9026-43-1.

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