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Biochemistry

Probing the peptide binding site of the cAMP-dependent protein kinase by using a peptide-based photoaffinity label

(*p*-benzoylphenylalanine/catalytic subunit/peptide sequencing)

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ABSTRACT **A peptide-based photoaffinity label for the catalytic subunit of the cAMP-dependent protein kinase was prepared from the amino acid *p*-benzoyl-L-phenylalanine [L-Phe(*p*Bz)]. By using solid-phase peptide synthesis methodology, DL-Phe(*p*Bz) was incorporated into the cAMP-dependent protein kinase substrate Leu-Arg-Arg-Ala-Ser-Leu-Gly in place of the phosphorylatable serine. The diastereomeric peptides were separated by reverse-phase HPLC. The peptide substrate analog containing L-Phe(*p*Bz) had a K_i of approximately 110 μM at pH 7.5. When photolyzed at 350 nm in the presence of the enzyme, this peptide caused time- and concentration-dependent inactivation. Radioactive acetylated L-Phe(*p*Bz) peptide was used to establish the binding stoichiometry of peptide to enzyme; these results, together with protection experiments, showed the photoaffinity labeling to be specific (≈1:1). To identify the residues that were modified on the catalytic subunit, the photoinactivated enzyme was cleaved with CNBr and V8 protease (*Staphylococcus aureus*). The resulting peptide fragments were purified by HPLC and were sequenced; these experiments identified the modified residues as Gly-125 and Met-127. This region of the cAMP-dependent protein kinase catalytic subunit contains many residues that are conserved in serine- and tyrosine-protein kinases.**

The cAMP-dependent protein kinase is a central enzyme in eukaryotic metabolism and is believed to be responsible for all of the physiological effects of cAMP (1). The inactive holoenzyme contains two regulatory and two catalytic subunits. Upon binding of cAMP, the holoenzyme dissociates to give two catalytic subunits and a regulatory dimer (2). The catalytic subunit catalyzes the transfer of the γ-phosphoryl group of ATP to appropriate protein or peptide substrates.

The primary sequence of the catalytic subunit has been determined (3), but the structure of the enzyme active site remains unclear. The conformation of a peptide substrate bound to the enzyme has been deduced from NMR measurements (4, 5). Several investigators have used affinity labels to study the regulatory and catalytic subunits of the enzyme. The catalytic subunits from bovine heart (6) and porcine skeletal muscle (7) have been modified with the reagent 5′-(*p*-fluorosulfonylbenzoyl)adenosine; this label was shown to modify Lys-72 specifically (8). Bramson *et al.* (9) modified Cys-199 with the peptide Leu-Arg-Arg-Ala-Cys(3-nitro-2-pyridinesulfenyl)-Leu-Gly and concluded that this residue was in the enzyme active site. This experiment demonstrated the feasibility of using analogs of kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly), a good substrate of the enzyme (10), to modify the peptide binding site. Peptides containing N^{δ}-bromoacetylornithine have been used to alkylate Cys-199 and Thr-197 (ref. 11; M. Doughty and E.T.K., unpublished results).

This paper describes the synthesis of a photoaffinity label designed to modify the peptide binding site of the catalytic subunit. The amino acid *p*-benzoyl-L-phenylalanine [L-Phe(*p*Bz)] was chosen as the photoactive probe because of (*i*) the stability of benzophenones to peptide synthesis conditions, (*ii*) the high reactivity of the α,β-unsaturated ketones in the presence of light with wavelength >300 nm (12), and (*iii*) the low reactivity of these species to water (13). The development of this photoactive amino acid has been reported in a preliminary communication by us (14) and by other investigators (15).

MATERIALS AND METHODS

General. The chemicals used in the synthesis of DL-Phe(*p*Bz) were purchased from Aldrich. [1-^{14}C]Acetic anhydride and [1-^{3}H]acetic anhydride were from Amersham. ATP, cAMP, and adenosine 5′-[β,γ-methylene]triphosphate were purchased from Sigma. The chloromethylstyrene resin used in the peptide synthesis was purchased from Pierce, and the *tert*-butyloxycarbonyl (Boc) derivatives of amino acids were from Peninsula Laboratories (San Carlos, CA). TLC plates (silica support) were from Merck.

^{1}H NMR data were recorded on a Nicolet 360-MHz Fourier transform spectrometer. Peptide synthesis was carried out on a Beckman model 990 peptide synthesizer. All mass spectra were obtained at the Rockefeller University Biotechnology Mass Spectrometric Research Resource. Amino acid analyses were carried out on a Dionex D300 amino acid analyzer after hydrolysis of the peptide in 6 N constant-boiling HCl (Pierce) at 110°C for 24 hr. The catalytic subunit of the cAMP-dependent protein kinase was purified from bovine heart (9).

Synthesis of DL-Phe(*p*Bz). *p*-Aminobenzophenone (0.03 mol) was dissolved in acetone (300 ml). The solution was cooled to 4°C in an ice bath, and concentrated HBr (16 ml of 48%) was added with stirring. The amine was diazotized with 60 ml of 5 M sodium nitrite. Acrylic acid (100 ml) and CuBr (300 mg) were added immediately, and the reaction mixture was allowed to warm to 25°C. After 90 min, N_2 evolution ceased, and the reaction was concentrated *in vacuo* to remove acetone and most of the acrylic acid and water. The residue was dissolved in 0.5 M $NaHCO_3$ (200 ml) and was washed twice with chloroform. The aqueous layer was acidified with concentrated HBr and washed several times with ether.

The α-bromohydrocinnamic acid was precipitated as the dicyclohexylamine salt by addition of one equivalent of dicyclohexylamine to the combined ether extracts. Crystals [mp 192–194°C (dec)] formed after 24 hr at −20°C and were washed with cold ether and treated with 5% citric acid to regenerate the acid. The racemic mixture of α-bromohydro-

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Abbreviations: Phe(*p*Bz), *p*-benzoylphenylalanine; peptide 1, Leu-Arg-Arg-Ala-L-Phe(*p*Bz)-Leu-Gly; peptide 2, Leu-Arg-Arg-Ala-D-Phe(*p*Bz)-Leu-Gly; Boc, *tert*-butyloxycarbonyl.

cinnamic acids was obtained as a yellow oil (70% yield): R_f 0.45 [$CHCl_3/CH_3OH/CH_3COOH$, 85:10:5 (vol/vol)]; ^1H NMR (C^2HCl_3) δ 3.50 (dd, 2H), 4.50 (t, 1H), 7.35 (d, 2H), 7.50 (t, 2H), 7.60 (t, 1H), 7.80 (d, 4H).

The racemic α-bromohydrocinnamic acid was dissolved in 400 ml of NH_4OH and left for 24 hr at 25°C. The solution was concentrated to dryness *in vacuo*, and 200 ml of 10% acetic acid was added to the residue. The resulting solution was washed four times with 100 ml of chloroform to remove remaining cinnamic acid. The aqueous layer was lyophilized to give DL-Phe(*p*Bz) in 50% yield. mp = 168–174° (dec); TLC R_f 0.65 (*t*-butyl alcohol/methyl ethyl ketone/formic acid/H_2O, 40:30:15:15); ^1H NMR (C^2H_3OD) δ 3.30 (dd, 2H), 4.40 (t, 1H), 7.38 (d, 2H), 7.44 (t, 2H), 7.60 (t, 1H), 7.66 (d, 4H); high-resolution mass spectrum, *m/z* 270.113 [$(M + H)^+$].

Synthesis of Peptides Containing Phe(*p*Bz). The racemic mixture of DL-Phe(*p*Bz) was treated with di-*t*-butyl dicarbonate to give the Boc-protected amino acid (16). This was utilized to incorporate DL-Phe(*p*Bz) into the peptide sequence Leu-Arg-Arg-Ala-DL-Phe(*p*Bz)-Leu-Gly by stepwise solid-phase synthesis on Merrifield resin. The Merrifield resin ester of N-protected glycine was prepared as described by Horiki *et al.* (17). The Boc-protected amino acids were coupled as symmetric anhydrides (18), except for Boc-DL-Phe(*p*Bz), which was incorporated by using hydroxybenzotriazole/dicyclohexylcarbodiimide coupling (19). The fully protected peptides were cleaved from the resin and simultaneously deprotected by treatment with HF (20).

Peptide Purification. The peptides were extracted from the resin with 10% acetic acid and loaded on a 1.5- × 90-cm column of Sephadex G-15, which was developed in 5% acetic acid. The diastereomeric peptides were separated and purified on an IBM LC/9533 HPLC employing an Altex C_{18} reverse-phase column. The column was equilibrated in 200 mM $NaClO_4$/25 mM NaH_2PO_4, pH 2.5, and the peptides were eluted with a linear gradient of 0–50% CH_3CN in the perchlorate/phosphate buffer. Effluent was monitored by a Perkin–Elmer LC75 variable wavelength detector set at 260 nm.

The amino acid compositions of the peptides were confirmed by amino acid analysis, 360-MHz NMR, and fission fragment ionization mass spectroscopy. The diastereomers were identified by digestion with leucine aminopeptidase (Worthington). Enzyme and peptide (1:100) were incubated at 37°C in 0.2 M triethylammonium acetate (pH 8.3) for 4 hr. These enzymatic digests were analyzed by HPLC as described in *Results*. Amino acid analysis for Leu-Arg-Arg-Ala-L-Phe(*p*Bz)-Leu-Gly (peptide 1): $Leu_{2.0}Arg_{2.2}Ala_{1.1}Gly_{1.2}$. L-Phe(*p*Bz) did not elute from the analyzer column. TLC R_f 0.26 (*n*-butyl alcohol/acetic acid/H_2O, 4:1:1); ^1H NMR (^2H_2O) δ 0.70 (m), 1.15 (d), 1.40–1.45 (m), 2.95 (m), 3.10 (q), 3.55 (t), 4.10 (m), 7.25 (d), 7.45 (t), 7.55 (t), 7.65 (d); mass spectrum, *m/z* 937.9 [$(M + H)^+$].

Enzyme Assays. The catalytic subunit of cAMP-dependent protein kinase was assayed according to the method of Bramson *et al.* (21). This method takes advantage of the spectral change at 430 nm due to the phosphorylation of Leu-Arg-Arg-Tyr(*o*-nitro)-Ser-Leu-Gly catalyzed by the catalytic subunit. These assays were typically done at pH 7.5 and contained 50 mM Mops, 10 mM $MgCl_2$, 0.15 M KCl, 2.0 mM ATP, bovine serum albumin at 0.2 mg/ml, and 0.2 mM dithiothreitol. The spectral changes were monitored continuously on a Perkin–Elmer Lambda 5 spectrophotometer.

Photolysis Experiments. Irradiations were performed at 25°C in a Rayonet RMR-500 photochemical reactor fitted with four RMR 3500-Å lamps (Southern New England Ultraviolet, Hamden, CT). The samples were clamped 3 cm from the lamps in borosilicate glass tubes. Photoinactivation assays typically contained 50 mM Mops, 10 mM $MgCl_2$, and 0.15 M KCl as well as enzyme and peptide, unless otherwise indicated.

Determination of Binding Stoichiometry. Irradiated enzyme–substrate complexes (in approximately 250-μl aliquots) were precipitated as follows: 100 μl of bovine serum albumin at 10 mg/ml was added with 500 μl of the Mops buffer. The protein in this solution was precipitated by addition of 100 μl of 70% $HClO_4$. The precipitate was collected on a Millipore HA filter, washed extensively with 7% $HClO_4$, resuspended in water, and assayed in Hydrofluor in a Beckman LS/7000 liquid scintillation counter.

Identification of the Modified Site. A 1.0-mM solution of [^{3}H]acetyl-peptide 1 (specific activity of 100,000 dpm/nmol) in 50 mM Mops/150 mM KCl, pH 7.0, was mixed with 2 mg of catalytic subunit (in 1 ml of Mops buffer) and irradiated for 20 min at 350 nm. Less than 10% enzyme activity remained after this treatment. The mixture was filtered through a Centricon-30 (M_r cutoff of 30,000) (Amicon) driven by centrifugation at 4640 × *g*. The retentate was washed several times with Mops buffer to remove excess peptide. The modified protein was dissolved in 2.5 ml of 70% (vol/vol) formic acid and was treated with a 50-fold excess of CNBr (22). This reaction proceeded for 24 hr at room temperature.

The resultant solution was passed through a Centricon-30 to remove unreacted enzyme. The filtrate was then passed through a Centricon-10 (M_r cutoff of 10,000), and the retentate was washed repeatedly with water. The Centricon-10 filtrate contained ≈60% of the initial radioactivity, and the retentate contained the remainder. The Centricon-10 retentate was concentrated and dissolved in 50 mM NH_4HCO_3 (pH 7.8) and was incubated with 3.3 nmol (one-sixth equivalent) of V8 protease from *Staphylococcus aureus* (Cooper Biomedical, Malvern, PA). This reaction was allowed to proceed for 24 hr at 37°C. Afterwards, the reaction mixture was filtered through a Centricon-10 and washed several times with H_2O; the filtrate contained all of the radioactivity that had been present in the retentate.

Both of the Centricon-10 filtrate fractions were then analyzed by HPLC by using a Vydac C_{18} column (4.6 mm × 25 cm) (The Separations Group, Hesperia, CA). A 60-min gradient of 0–50% CH_3CN was employed in a solution containing 200 mM $NaClO_4$ and 25 mM NaH_2PO_4 (pH 2.5). Fractions corresponding to peaks of absorbance at 220 nm were collected, and an aliquot of each was counted in Hydrofluor. Fractions containing radioactivity were concentrated *in vacuo* and rechromatographed on the same column using a 60-min gradient of 0–50% CH_3CN in 0.1% trifluoroacetic acid. Active fractions from the second round of HPLC were concentrated *in vacuo* and subjected to the Edman degradation on an Applied Biosystems (Foster City, CA) gas-phase sequenator. The quantities of peptide sequenced in this way were 1–2 nmol per fragment.

RESULTS

Preparation of Photoaffinity Labels. Analogues of kemptide were synthesized in which the serine was replaced by the photolabile amino acids L-Phe(*p*Bz) and D-Phe(*p*Bz). The amino acid DL-Phe(*p*Bz) was prepared by means of the Meerwein reaction (23) from *p*-aminobenzophenone. Peptide 1 [Leu-Arg-Arg-Ala-L-Phe(*p*Bz)-Leu-Gly] and peptide 2 [Leu-Arg-Arg-Ala-D-Phe(*p*Bz)-Leu-Gly] were separated and purified on an Altex C_{18} reverse-phase column employing an IBM HPLC with a $NaClO_4/NaH_2PO_4/CH_3CN$ solvent system. The amino acid compositions of the peptides were confirmed by amino acid analysis, 360-MHz NMR, and fission fragment ionization mass spectrometry. The diastereomers were identified by digestion with leucine aminopeptidase (4 hr at 37°C). The digests were injected onto the same HPLC system described above and monitored at 262 nm. The digest of the first diastereomeric peptide yielded a new peak

that cochromatographed with Phe(*p*Bz). The digest of the second peptide yielded no peak at this position. Because leucine aminopeptidase is highly specific for L-amino acids (24), these results identify the first peptide as peptide 1 [containing L-Phe(*p*Bz)].

Photolysis of Peptide 1. Fig. 1 shows the effect of the length of time of irradiation on the absorption spectrum of peptide 1. The absorption at 262 nm decreases as a function of duration of photolysis, and the rate of photolysis follows apparent first-order kinetics (Fig. 1 *Inset*). The half-time is ≈70 sec under these conditions (50 mM Mops, pH 7.5). Incubation of peptide 1 with 400 μM Ser, 400 μM Cys, or 400 μM Ala had no effect on the photolysis. The products of photolysis under the nonenzymatic conditions have not been identified.

Reversible Binding of Peptides 1 and 2 by the Catalytic Subunit of cAMP-Dependent Protein Kinase. Peptides 1 and 2 were tested as competitive inhibitors of the catalytic subunit of the cAMP-dependent protein kinase. The spectrophotometric assay was used, as described in *Materials and Methods*. The enzyme concentration in the assays was 12.3 nM. Lineweaver–Burk plots were constructed, and the data were replotted as described by Dixon (25). From these plots the K_i values for peptides 1 and 2 were estimated to be 110 ± 15 μM and 650 ± 50 μM, respectively.

Photoinactivation of the Catalytic Subunit by Photoaffinity Substrate Analogues. Catalytic subunit (100 nM) was incubated with various concentrations of peptide 1 in 50 mM Mops (pH 7.5) and photolyzed for up to 10 min. Aliquots were withdrawn and assayed for phosphotransferase activity by using the spectrophotometric assay described above. The enzyme was photoinactivated in a time- and concentration-dependent manner, as shown in Fig. 2. Here, 100% activity is taken to be the activity of the enzyme/peptide mixture before photolysis. No photoinactivation was observed when the enzyme was photolyzed alone for up to 20 min. Likewise,

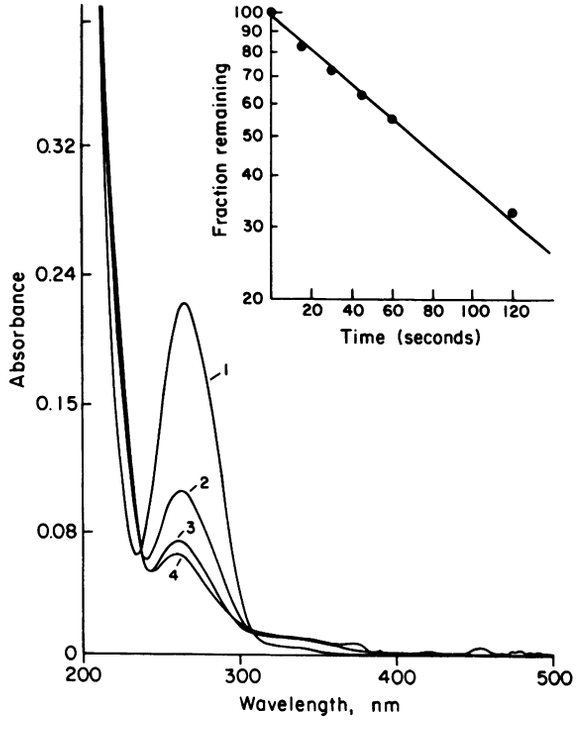

FIG. 1. Photolysis of peptide 1 in the absence of enzyme. Peptide 1 was photolyzed at 350 nm in 50 mM Mops (pH 7.5) at 25°C. Aliquots were removed at various time points and were analyzed. Spectra were recorded prior to photolysis (curve 1) and after 5 min (curve 2), 10 min (curve 3), and 20 min (curve 4) of photolysis. (*Inset*) Fraction of peptide 1 remaining as a function of duration of photolysis.

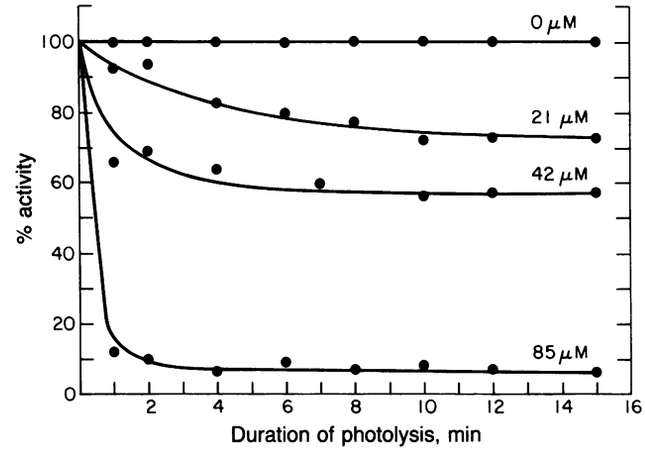

FIG. 2. Photoinactivation of the catalytic subunit by peptide 1. The concentrations of peptide 1 are given above the curves. The experiments were performed in 50 mM Mops (pH 7.5) at 25°C. The concentration of catalytic subunit was 100 nM. One hundred percent activity was taken to be the activity of the enzyme/peptide mixture before photolysis. Catalytic subunit was assayed by a spectrophotometric method (21).

the enzyme retained activity after photolysis in the presence of prephotolyzed peptide 1 or with 230 μM DL-Phe(*p*Bz) alone, arguing for the specific nature of the interaction.

When the analogous experiment was carried out with peptide 2, the enzyme was not significantly inactivated, even at concentrations approximately 3 times higher than those used for peptide 1 (data not shown).

Protection from Inactivation by Active Site-Directed Reagents. Addition of saturating amounts of ATP or the nonhydrolyzable ATP analog adenosine 5′-[β,γ-methylene]triphosphate to the photolysis mixture protected the enzyme against inactivation by peptide 1. Catalytic subunit (2.0–2.5 μM), when photolyzed in the presence of peptide 1 (70–105 μM) and saturating $MgCl_2$, was protected about 70% with 2.0 mM ATP or 2.0 mM adenosine 5′-[β,γ-methylene]triphosphate. When 270 μM kemptide was added to this reaction mixture, 95% protection was observed. These results are consistent with a specific interaction between peptide 1 and the active site of the catalytic subunit.

Determination of the Stoichiometry of Binding and Identification of the Site of Modification. Peptide 1 was acetylated on the N terminus with [1-^{14}C]acetic anhydride. Radioactive acetylated peptide 1 (2000 dpm/nmol) was used to determine the stoichiometry of binding of peptide to enzyme. The photolysis was carried out as before, with various concentrations of peptide and enzyme. Aliquots were removed at various time points and assayed for enzyme activity. Extra aliquots were also removed at these time points to assess the extent of label incorporation into the catalytic subunit. These results showed that the protein modification was covalent and that the stoichiometry was ≈1:1 (Fig. 3).

In a separate experiment, the catalytic subunit (2 mg) was irradiated together with [^{3}H]acetyl-peptide 1 (100,000 dpm/nmol) until >90% loss of activity resulted. The excess photoaffinity label was removed by filtration over a Centricon-30. The modified enzyme was then cleaved with CNBr and filtered successively over a Centricon-30 and a Centricon-10. Approximately 60% of the radioactivity flowed through the latter membrane, indicating that the label was present in peptide fragments from the CNBr cleavage reaction that were of a M_r <10,000. The fraction containing the remaining 40% of the radioactivity was recovered and digested with *S. aureus* V8 protease and filtered through a Centricon-10.

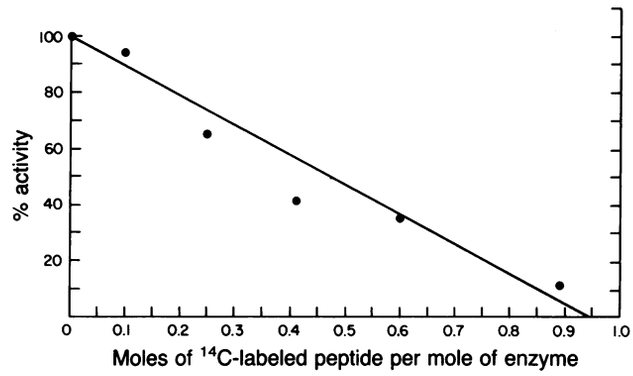

FIG. 3. Covalent incorporation of [1-^{14}C]acetyl-peptide 1 into catalytic subunit. Photolysis experiments were performed in 50 mM Mops (pH 7.5) at 25°C, with various concentrations of peptide and enzyme. Aliquots were removed for spectrophotometric enzyme assay. After photolysis, the modified enzyme was precipitated with 70% $HClO_4$, washed on a Millipore HA filter, and counted in a liquid scintillation counter.

Both batches of Centricon-10 filtrate were then analyzed by analytical HPLC using a reverse-phase C_{18} column. The radioactive fragments were purified initially using a linear gradient of CH_3CN and an aqueous solution containing $NaClO_4$ and NaH_2PO_4. The major peak of radioactivity from the small peptide produced by cleavage with CNBr eluted at 46 min (Fig. 4*B*). Major peaks of radioactivity from the V8 protease digest of the large fragment from CNBr cleavage eluted at 36 and 45 min (Fig. 4*C*). For both samples, fractions were collected, concentrated, and further purified by rechromatography by C_{18} HPLC using 0.1% trifluoroacetic acid. The elution times of the small CNBr fragment and the major product from the V8 protease digest of the large CNBr fragment were 45 and 34 min, respectively, in the trifluoroacetic acid buffer system. The radioactive fractions from the second round of HPLC were applied to a gas-phase sequenator and carried through the Edman degradation. The fragments obtained and the sites of modification are shown in Table 1. The two residues modified were Gly-125 and Met-127. These residues are contained in the CNBr peptide beginning at Met-119 and ending at Met-127; however, modification of the latter residue apparently blocked CNBr cleavage at that site, generating the large CNBr fragment. As a reference, native, unmodified catalytic subunit (1 mg) was carried through the CNBr cleavage, HPLC analysis, and sequencing. The unmodified CNBr peptide from Met-119 to Met-127 eluted at 38 min in the initial gradient (Fig. 4*A*).

DISCUSSION

High-resolution structural data are not available for any of the protein kinases, although a preliminary study of crystals of the cAMP-dependent protein kinase has been reported (29). Extensive amino acid homology exists among the catalytic domains of the serine- and tyrosine-protein kinases (26). To begin to identify residues and domains important in binding and catalysis, we have chosen as our model system the catalytic subunit of the bovine heart cAMP-dependent protein kinase. This enzyme is readily purified in milligram quantities, and a synthetic peptide substrate (kemptide) is available, which is phosphorylated with favorable kinetic parameters (10). The conformation of this peptide bound to the active site of cAMP-dependent protein kinase has been elucidated (4, 5).

We report here the extension of our peptide-based affinity labeling studies to photoaffinity labeling. Because peptides in which the reactive serine of kemptide is replaced by 3-nitro-2-pyridinesulfenyl-cysteine also bind to the catalytic subunit (9), this site was chosen for the introduction of the photolabile

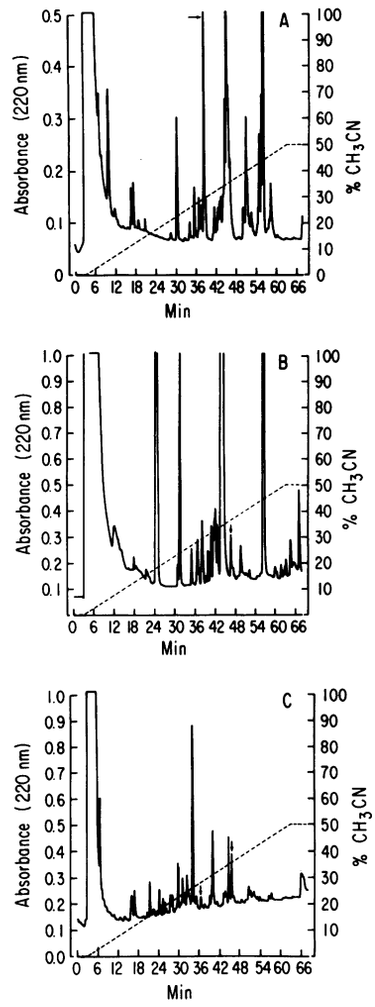

FIG. 4. HPLC purification of peptidic fragments. The solvent system is described in the text; the flow rate was 1.0 ml/min. (*A*) Elution profile of native catalytic subunit after proteolysis with CNBr. →, Peptide from Met-119 to Met-127. (*B*) Elution profile of the small (M_r < 10,000) peptides produced by cleavage with CNBr of the photoinactivated catalytic subunit. ↓, Peak of radioactivity. (*C*) Elution profile of the larger (M_r > 10,000) fragments from the CNBr reaction, after digestion with V8 protease. ↓, Peaks of radioactivity.

amino acid L-Phe(*p*Bz). Peptide 1 was synthesized and found to have a K_i of 110 μM when tested as a competitive inhibitor of the catalytic subunit. It was also a potent photoinactivator (Fig. 2); it left the enzyme ≈90% inactive after 1 min of photolysis at 25°C in 50 mM Mops (pH 7.5).

A potential problem with photoaffinity labeling experiments is the possibility of nonspecific interactions on the enzyme or receptor surface (27). To address this question, the catalytic subunit was photolyzed in the presence of (*i*) Phe(*p*Bz) itself, and (*ii*) peptide 2, the analog of kemptide containing D-Phe(*p*Bz) in place of serine. No photoinactivation was observed in either case, indicating that tight binding at the active site is required for the effect. To strengthen this

Table 1. Proteolytic fragments and sites of modification

Source	Sequenced fragment	Site of modification
Small CNBr fragment	Glu-Tyr-Val-Pro-Gly ...	Gly-125
Large CNBr fragment, V8 protease digest	Tyr-Val-Pro-Gly-Gly-Glu-X	Met-127

argument, protection experiments were carried out in which other active site-directed reagents protected the photoinactivation of the enzyme by peptide 1. Finally, radiolabeled peptide 1 was used to establish a linear relationship between photoinactivation and covalent attachment (Fig. 3). This experiment demonstrates that at 100% inactivation 1 mol of photoaffinity label would be incorporated per mol of enzyme.

To identify the residues modified, the catalytic subunit was photolyzed together with [^{3}H]acetyl-peptide 1 and was subsequently cleaved with CNBr and V8 protease. To separate different sized fragments quickly, M_r 10,000 and M_r 30,000 cutoff Centricon devices were used with centrifugation at 4640 × g, as reported previously (11). By this analysis ≈60% of the radioactivity was shown to be associated with a small (M_r < 10,000) CNBr peptide. This peptide was purified by two rounds of HPLC purification and subjected to N-terminal Edman degradation on a gas-phase sequenator. The sequence obtained corresponds to the amino acids between positions 120 and 124 of the catalytic subunit and did not include Glu-126 (Table 1). Since no proteolytic cleavage was expected between Gly-125 and Glu-126, these results indicate the site of attachment to be Gly-125.

The larger (M_r > 10,000) CNBr fragments that contained 40% of the original radioactivity were digested with V8 protease and were analyzed by the same methodology. In this case, the sequence corresponded to residues 121–126 of the enzyme, with sequence termination and observation of an unknown phenylthiohydantoin at the next round of sequencing. These results indicate modification at residue Met-127; this modification apparently blocks both the CNBr reaction at that position and V8 protease cleavage at Glu-126.

Alignment of the amino acid sequences in catalytic domains of serine- and tyrosine-protein kinases shows that a high degree of homology exists among these enzymes (26). When the sequences are arranged as described by Hunter and Cooper (26), the portion of the cAMP-dependent protein kinase modified by peptide 1 falls in a region that has homology with other protein kinases, with Gly-125 being a conserved residue (Table 2). In addition, the length of the peptide chain between Gly-125 and Lys-72, a residue important in ATP binding in the cAMP-dependent protein kinase (8), is approximately conserved in other serine- and tyrosine-protein kinases. Taken together, this argues that this protein of the active site in the cAMP-dependent protein kinase contains structural features that may be preserved in other protein kinases.

Table 2. Homology in region modified by peptide 1

Type of protein kinase	Sequence
Serine	
cAMP-dependent protein kinase	Y-V-P-G-**G**-E-M-F-S-H
cGMP-dependent protein kinase	A-C-L-G-**G**-E-L-W-T-I
Phosphorylase kinase γ subunit	L-M-K-K-**G**-E-L-F-D-Y
Tyrosine	
pp60$^{v\text{-}src}$	- - - K-**G**-S-L-L-D-F
P90$^{gag\text{-}yes}$	- - - K-**G**-S-L-L-D-F
P70$^{gag\text{-}fgr}$	- - - H-**G**-S-L-L-E-F
P120$^{gag\text{-}abl}$	- - - Y-**G**-N-L-L-D-Y
P140$^{gag\text{-}fps}$	L-V-Q-G-**G**-D-F-L-S-F
P85$^{gag\text{-}fes}$	L-V-Q-G-**G**-D-F-L-T-F
P68$^{gag\text{-}ros}$	L-M-E-G-**G**-D-L-L-S-Y
gp68$^{v\text{-}erb\text{-}B}$	L-M-P-Y-**G**-C-L-L-D-Y
EGF receptor kinase	L-M-P-F-**G**-C-L-L-D-Y
Insulin receptor	L-M-A-H-**G**-D-L-K-S-Y

Amino acids are identified by the one-letter code. EGF, epidermal growth factor. The boldface G marks the position of Gly-125 in the cAMP-dependent protein kinase.

The photochemistry of diaryl ketones is well understood from model systems (12, 28). Upon irradiation at 350 nm, the ketone carbonyl of Phe(pBz) is expected to undergo a n,π^* transition to give a triplet biradical. This species has been shown to react preferentially with C–H bonds as compared to water (13). The resulting tertiary alcohol is a stable adduct. In the present study, a glycine and a methionine residue have been modified. In the case of glycine, the target for the photochemical reaction is presumably the α-carbon of glycine. The nature of the adduct with methionine has not been elucidated. Photoaffinity labels containing L-Phe(pBz) should be complementary to other types of affinity labels, as the photogenerated free radicals are anticipated to modify covalently non-nucleophilic sites on proteins.

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