Differential Labeling of the Catalytic Subunit of cAMP-Dependent Protein Kinase with a Water-Soluble Carbodiimide: Identification of Carboxyl Groups Protected by MgATP and Inhibitor Peptides†

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ABSTRACT: The catalytic subunit of cAMP-dependent protein kinase typically phosphorylates protein substrates containing basic amino acids preceding the phosphorylation site. To identify amino acids in the catalytic subunit that might interact with these basic residues in the protein substrate, the enzyme was treated with a water-soluble carbodiimide, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), in the presence of [^{14}C]glycine ethyl ester. Modification of the catalytic subunit in the absence of substrates led to the irreversible, first-order inhibition of activity. Neither MgATP nor a 6-residue inhibitor peptide alone was sufficient to protect the catalytic subunit against inactivation by the carbodiimide. However, the inhibitor peptide and MgATP together completely blocked the inhibitory effects of EDC. Several carboxyl groups in the free catalytic subunit were radiolabeled after the catalytic subunit was modified with EDC and [^{14}C]glycine ethyl ester. After purification and sequencing, these carboxyl groups were identified as Glu 107, Glu 170, Asp 241, Asp 328, Asp 329, Glu 331, Glu 332, and Glu 333. Three of these amino acids, Glu 331, Glu 107, and Asp 241, were labeled regardless of the presence of substrates, while Glu 333 and Asp 329 were modified to a slight extent only in the free catalytic subunit. Glu 170, Asp 328, and Glu 332 were all very reactive in the apoenzyme but fully protected from modification by EDC in the presence of MgATP and an inhibitor peptide. Glu 170 is conserved in other protein kinases that recognize basic residues in their protein substrates and is thus a good candidate for interacting with the basic residues preceding the phosphorylated amino acid in the protein substrates of the catalytic subunit. Asp 328 and/or Glu 332, as part of a cluster of six acidic groups near the carboxy terminus, may also play a role in peptide recognition. In any case, the results indicate a dramatic change in the accessibility of this region of the catalytic subunit in the presence and absence of substrates.

In the presence of cAMP, the catalytic subunit of cAMP-dependent protein kinase is a monomeric enzyme with a calculated M_r of 40900 (Shoji et al., 1983). Although many proteins serve as a substrate for this kinase, each has a common sequence surrounding the phosphorylation site. This sequence, typically consisting of two basic residues preceding the phosphorylated amino acid, was first recognized by comparing the phosphorylation sites in known protein substrates such as liver pyruvate kinase (Hjelmquist et al., 1974). A consensus sequence was confirmed by using synthetic peptides capable of serving as substrates for the enzyme. The optimal sequence recognized by the catalytic subunit of cAMP-dependent protein kinase is Arg-Arg-X-Ser/Thr (Feramisco et al., 1980; Kemp et al., 1977). The two basic residues preceding the phosphorylation site are important determinants for substrate recognition.

A number of approaches have been used to identify functional groups at the active site of the catalytic subunit. Affinity labeling with an analogue of ATP localized Lys 72 near the γ-phosphate of ATP (Zoller et al., 1981; Bhatnagar et al., 1984). Differential labeling of lysine residues with acetic anhydride confirmed that the region flanking Lys 72 is sensitive to ATP binding (Buechler et al., 1989). A hydrophobic carbodiimide, dicyclohexylcarbodiimide (DCCD), was used to identify a carboxyl group, Asp 184, that is close to the ATP binding site (Buechler & Taylor, 1988). In the absence of MgATP, DCCD mediates the cross-linking of Asp 184 and Lys 72, thus localizing both of these residues in close proximity at the active site (Buechler & Taylor, 1989).

These approaches described above provide some insights into the amino acids constituting the active site of the enzyme. Various approaches also were used to map the peptide binding site. For example, Bramson et al. (1982) used a peptide substrate analogue to demonstrate that Cys 199 was close to the phosphorylated amino acid in the protein substrate. More recently, a peptide analogue containing a photoreactive group in place of the phosphorylatable serine labeled Gly 125 and Met 127 (Miller & Kaiser, 1988). Two different peptides, each containing N^{δ}-(bromoacetyl)ornithine in place of one of the arginine residues in the peptide substrate, were shown to modify Cys 199, Thr 197, and Glu 346 (Mobashery & Kaiser, 1988). Finally, Granot et al. (1981) concluded from their study that the substrate peptide assumed an extended coil conformation when it was bound to the catalytic subunit. Thus, the peptide recognition site could extend over a considerable distance on the surface of the enzyme. Although the above approaches have provided some insights into the features that constitute the peptide binding site, they nevertheless have not yet provided definitive information about the specific residues in the catalytic subunit that are important for recognition of the two arginines that are crucial determinants for protein substrate recognition by this kinase.

To identify specific carboxyl groups that may play a role in recognizing the protein substrate, the catalytic subunit was treated with the water-soluble carbodiimide 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC).

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The rationale here was that the groups important for peptide recognition should be on the surface of the enzyme and thus accessible to modification by a water-soluble carbodiimide. A preliminary paper also suggested that the activity of the catalytic subunit was sensitive to EDC (Matsuo et al., 1980). EDC was capable of inhibiting the catalytic subunit, and MgATP or inhibitor peptide alone was not sufficient to protect the catalytic subunit against inactivation. After it was established that MgATP and an inhibitor peptide together protected completely against inactivation, specific carboxyl groups were identified that were modified by EDC in the free catalytic subunit and protected in the presence of both substrates. The potential participation in substrate recognition for the carboxyl groups that are protected from modification by EDC in the presence of MgATP and inhibitor peptide is discussed.

Experimental Procedures

Materials. Reagents were obtained from the following sources: [^{14}C]glycine ethyl ester (50 mCi/mmol), New England Nuclear; 2-(*N*-morpholino)ethanesulfonic acid (MES) and glycine ethyl ester, United States Biochemical Corp.; 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC), adenosine 5′-triphosphate (ATP), trypsin treated with L-1-(tosylamino)-2-phenylethyl chloromethyl ketone (TPCK), thermolysin (type X), and α-chymotrypsin, Sigma; trifluoroacetic acid (TFA, HPLC grade), Pierce; Cytoscint, ICN Radiochemicals.

Source of Proteins and Peptides. The catalytic subunit was prepared from porcine heart as described previously (Nelson & Taylor, 1981). The inhibitor peptide, T-T-Y-A-D-F-I-A-S-G-R-T-G-R-R-N-A-I-H-D, which represents a fragment of the heat-stable inhibitor protein (Cheng et al., 1986), was synthesized on an Applied Biosystems Model 430A peptide synthesizer. The peptide substrate, L-R-R-N-S-I, and the peptide inhibitor, L-R-R-N-A-I, were synthesized at the Peptide and Oligonucleotide Facility at the University of California, San Diego.

Assays. Enzymatic activity was assayed spectrophotometrically according to the method of Cook et al. (1982) using the synthetic peptide substrate L-R-R-N-S-I.

Modification of Carboxyl Residues. Reactions were carried out in 50 mM MES (pH 6.2) and 5% glycerol. Catalytic subunit was incubated on ice for 30 min with additions as indicated. A 5-fold molar excess of glycine ethyl ester over the EDC concentration was added to each reaction mixture except where noted. After addition of EDC (100 mM in H_2O) to the indicated concentrations, the samples were incubated at 37 °C for 90 min and then placed on ice.

Proteolysis. Tryptic digests were carried out at 37 °C with 1:50 (w/w) TPCK-treated trypsin/catalytic subunit following dialysis of the modified catalytic subunit against 50 mM NH_4HCO_3 (pH 8.1). After 2 h, a second equal aliquot of TPCK-treated trypsin was added, and incubation was continued for an additional 2 h. Chymotrypsin digests were carried out under the same conditions as those described above for trypsin. Thermolysin digests were carried out at 37 °C for 24 h in 50 mM NH_4HCO_3 (pH 8.1) with three separate additions of 1:50 (w/w) thermolysin/catalytic subunit. Thermolysin digests also required the addition of an excess of $CaCl_2$ over the amount of phosphate present in pooled HPLC fractions. This led to the formation of the insoluble salt, calcium phosphate, which was centrifuged before the chromatography step.

High-Performance Liquid Chromatography (HPLC). The tryptic peptides were resolved by HPLC using an Altex 3200 system with a Vydac C4 column (0.46 × 25 cm). The buffers employed were (a) 10 mM sodium phosphate (pH 6.9) and (b) CH_3CN. Absorbance was monitored at 219 nm with a 100-30 Hitachi spectrophotometer equipped with a flow-through cell and at 280 nm with a SF 769 Kratos spectrophotometer equipped with a flow-through cell. Separation of chymotrypsin and thermolysin peptides was accomplished with the same system except a Vydac C18 column (0.46 × 25 cm) was used instead of the C4 column. Individual peptides were rechromatographed on a Vydac C18 column with a gradient of 0.1% TFA (pH 2.1) to CH_3CN prior to sequence analysis except where indicated.

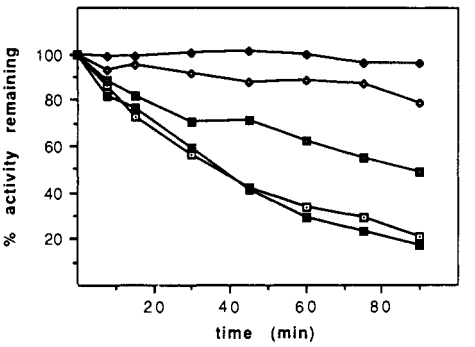

FIGURE 1: Effect of substrates on the inactivation of the catalytic subunit by EDC. The following substrates were incubated with the catalytic subunit (22.4 μM) preceding the addition of EDC to a final concentration of 4 mM: 2 mM $MgCl_2$ (white square with black dot); 4 mM $MgCl_2$ and 2 mM ATP (black square with white dot); 4 mM $MgCl_2$, 2 mM ATP, and 2 mM 6-residue inhibitor peptide (black diamond with white dot); 2 mM $MgCl_2$ and 2 mM 6-residue inhibitor peptide (black square). The reactions were done as described under Experimental Procedures. The control (black diamond) was incubated at 37 °C in the presence of 2 mM $MgCl_2$ and 20 mM glycine ethyl ester. The K_m for the substrate peptide, L-R-R-N-A-I, was 10–15 μM. The 6-residue inhibitor peptide was a competitive inhibitor with an approximate K_I of 100–200 μM.

Sequencing. Gas-phase sequencing was carried out with an Applied Biosystems Model 470A protein sequencer. Phenylthiohydantoin (PTH) amino acids were identified by HPLC with an Applied Biosystems Model 120A analyzer. In addition, aliquots of each step were counted in Cytoscint for radioactivity.

Results

To determine the role of solvent-accessible carboxyl groups in the catalytic subunit, conditions were established that led to inhibition of enzymatic activity following exposure to the water-soluble carbodiimide, EDC. As discussed previously (Toner-Webb & Taylor, 1987), carbodiimides react very well with carboxylic acid residues in the pH range 4.0–6.5, while the reaction of the radiolabeled nucleophile with the activated carbodiimide is more favorable at higher pH values. Thus, all the reactions were done at pH 6.2, which was the same pH used for the modification of the catalytic subunit by the hydrophobic carbodiimide, DCCD, and [^{14}C]glycine ethyl ester (Buechler & Taylor, 1988). Modification of the catalytic subunit in the absence of substrates with 4 mM EDC resulted in the loss of 85% of the initial activity after 90 min (Figure 1). This inhibition was first order with respect to time (not shown). The addition of 2 mM MgATP appeared to partially protect the catalytic subunit, while the rate of inactivation in the presence of the inhibitor peptide, L-R-R-N-A-I, was identical with that in the free catalytic subunit (Figure 1). The substrate peptide, L-R-R-N-S-I (2 mM), also did not protect the catalytic subunit from inhibition by EDC (data now shown). In contrast, the catalytic subunit was totally protected

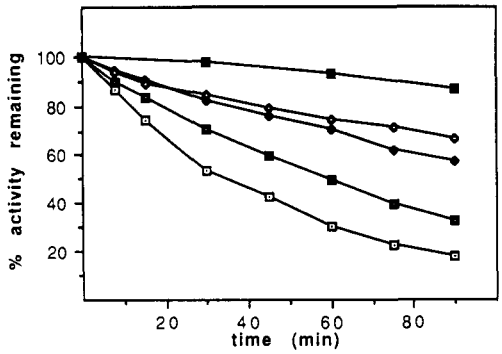

FIGURE 2: Effect of regulatory subunit on the inactivation of the catalytic subunit by EDC. Type I holoenzyme (19.2 μM) was formed by dialyzing a 50% excess of regulatory subunit with catalytic subunit at room temperature for 3 days in the absence of MgATP. The following were added to each sample before the addition of EDC: 0.12 mM ATP, 0.25 mM $MgCl_2$, and 0.2 mM cAMP (black square with white dot); 0.2 mM cAMP and 0.25 mM $MgCl_2$ (white square with black dot); 0.25 mM $MgCl_2$ (black diamond); 0.12 mM ATP and 0.25 mM $MgCl_2$ (black diamond with white dot). The reactions were done as described under Experimental Procedures with a final EDC concentration of 4 mM. The control (black square) was incubated at 37 °C in the presence of 20 mM glycine ethyl ester. It should be noted that some cross-linking occurred when the holoenzyme was incubated with 4 mM EDC (unpublished results), and this may account for the low loss of activity that is seen in the absence of cAMP. This cross-linking was very significant in the absence of glycine ethyl ester but was reduced to about 10–15% in the presence of 20 mM glycine ethyl ester on the basis of SDS gel electrophoresis. The precise nature of the cross-linking was not characterized.

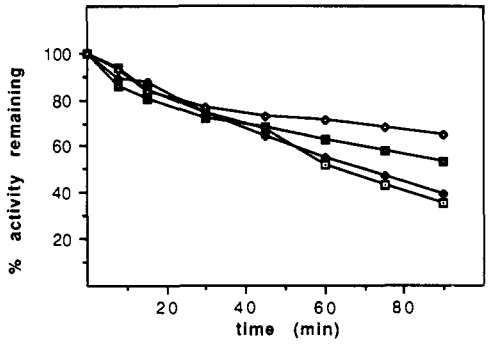

FIGURE 3: Effect of MgATP on the inhibition of the catalytic subunit by EDC. The catalytic subunit (25.3 μM) was incubated on ice for 30 min prior to the addition of EDC with the following: 2 mM $MgCl_2$ (white square with black dot); 0.4 mM $MgCl_2$ and 0.2 mM ATP (black diamond); 2 mM $MgCl_2$ and 1 mM ATP (black square with white dot); 10 mM $MgCl_2$ and 5 mM ATP (black diamond with white dot). The reactions were done as described under Experimental Procedures with a final EDC concentration of 4 mM.

against inactivation by EDC in the presence of MgATP (200 μM or 2 mM) and the 6-residue inhibitor peptide (Figure 1). The regulatory subunit, when bound to the catalytic subunit as part of the holoenzyme complex, also protected the catalytic subunit from inhibition by EDC, even in the absence of MgATP (Figure 2).

The inactivation of the catalytic subunit in the presence of different concentrations of MgATP was done to determine the basis for the partial protection from inhibition by 2 mM MgATP shown in Figure 1. At high concentrations of MgATP, the rate of inhibition appeared to be slower than in the absence of nucleotide; however, low amounts of MgATP did not protect against inactivation (Figure 3). In addition, the initial rate of inhibition was similar from 0 to 5 mM ATP, indicating that MgATP bound to the catalytic subunit was not protecting the enzyme from modification by EDC. An explanation for the apparent protection of activity in the presence of nucleotide is that excess MgATP reacts with EDC,

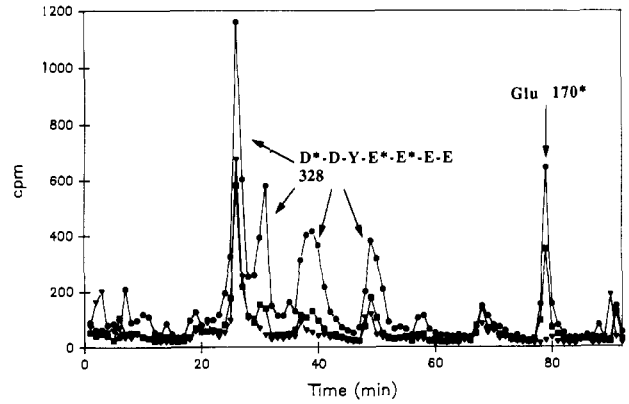

FIGURE 4: Effect of substrates on the incorporation of [^{14}C]glycine ethyl ester into the catalytic subunit. Catalytic subunit (31 μM) was incubated on ice for 30 min with the following substrates prior to the addition of EDC: (a) 2 mM $MgCl_2$ (black circle); (b) 4 mM $MgCl_2$ and 2 mM ATP (black square); (c) 4 mM $MgCl_2$, 2 mM ATP, and 35 μM 20-residue inhibitor peptide (black triangle). After modification with 0.8 mM EDC and 2.8 mM [^{14}C]glycine ethyl ester (3.3 mCi/mmol), the catalytic subunit was digested with TPCK-treated trypsin, and the resulting peptides were separated by HPLC as described under Experimental Procedures. The tryptic peptides were eluted with a 100-min linear gradient from 6% to 31% CH_3CN followed by a 15-min linear gradient from 31% to 50% CH_3CN (not shown). Radioactivity was determined by counting 10% of each 1.0-mL fraction in Cytoscint. Peptides containing Glu 170 and the cluster of acidic residues near the carboxy terminus, D-D-Y-E-E-E-E are indicated by the corresponding arrows. The entire tryptic peptides containing these residues are shown in Table I. The most reactive carboxyl groups in the apoenzyme are marked with an asterisk.

leading to a decrease in the actual amount of EDC available for modification of the catalytic subunit. To test this hypothesis, EDC was incubated with MgATP at 37 °C for 45 min. This preincubation destroyed the ability of EDC to inhibit the catalytic subunit compared to the control where MgATP and EDC were incubated separately prior to being added to the protein solution (data not shown). A separate study also showed that EDC has a significantly lower half life in the presence of MgATP (Gilles et al., 1989).

To identify any specific carboxylic acids that might account for the inhibition of catalytic activity, the catalytic subunit was next modified with EDC and [^{14}C]glycine ethyl ester under three different conditions: (1) in the presence of $MgCl_2$ alone, (2) in the presence of MgATP, and (3) in the presence of MgATP plus a 20-residue inhibitor peptide. The 20-residue inhibitor peptide was used because its K_I is 2 nM (Cheng et al., 1986) compared to approximately 100–200 μM for the 6-residue inhibitor peptide; therefore, a tighter ternary complex with the catalytic subunit and MgATP would be formed. The catalytic subunit was inhibited 40% by 0.8 mM EDC after 90 min, while only 18% inactivation was observed when 2 mM MgATP was included in the reaction mixture. A lower concentration of EDC was used here compared to the experiment in Figure 1 so that conformational changes induced in the protein by the chemical modification would be kept to a minimum. A comparison between the amounts of [^{14}C]glycine ethyl ester incorporated into the catalytic subunit in the presence of the indicated substrates is shown in Figure 4. Each peak of radioactivity in Figure 4 was purified by rechromatographing with different elution gradients, and the purified peptides were sequenced. Unique PTH amino acids corresponding to all the modified residues that will be described were observed at their respective sequencer steps and radioactivity eluted at those positions in the sequences.

The broad region of radioactivity extending from fraction 25 to fraction 50 in Figure 4 that is present when the catalytic

Table I: Amino Acid Sequences of the Peptides That Were Modified with EDC and [^{14}C]Glycine Ethyl Ester[a]

peptide	
	328
1	F-K-G-P-G-D-T-S-N-F-D-D-Y-E-E-E-E-E-I-R-V-S-I-N-E-K
	170
2	D-L-K-P-E-N-L-L-I-D-Q-Q-G-Y-I-Q-V-T-D-F-G-F-A-K
	107
3	L-E-Y-S-F-K
	241
4	A-D-Q-P-I-Q-I-Y

[a] The underlined portion of peptide 1 is the radiolabeled peptide that was obtained after digestion with thermolysin.

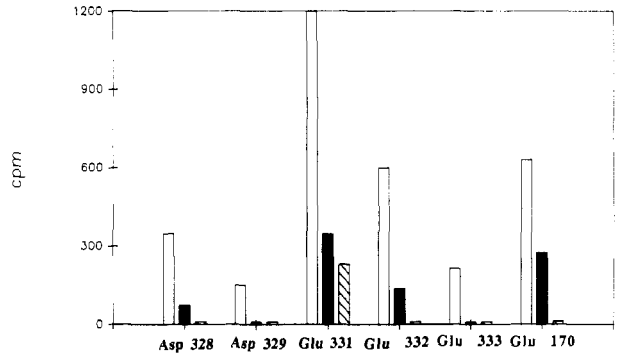

FIGURE 5: Relative amount of radioactivity incorporated into the carboxyl groups of peptides 1 and 2.

subunit was modified with EDC in the presence of $MgCl_2$ alone consisted primarily of peptide 1 (Table I). Two different procedures were developed to identify the labeled amino acids in this peptide. Since the yield of peptide 1 from the HPLC column was about 10% when the aqueous phase was at pH 2.1, morpholine was added to the 0.1% TFA buffer until the pH was 6.8. Morpholine and TFA were also added to the CH_3CN. This significantly increased the yield of the peptide to about 80% and allowed it to be purified prior to sequencing. After sequencing, the sites of covalent modification in the apoenzyme were identified as Asp 328, Asp 329, Glu 331, Glu 332, and Glu 333. Because the tryptic peptide containing those carboxylic acid residues was long and the first site of modification, Asp 328, was at step 11 of the sequence, it was difficult to unambiguously identify the modified amino acids. Consequently, a second procedure for isolating the labeled amino acids in peptide 1 was developed. For this method, the fractions containing peptide 1 from the elution of tryptic peptides were pooled, lyophilized, and digested with thermolysin as described under Experimental Procedures. The thermolysin peptides were separated by HPLC (not shown), and each of the peaks of radioactivity were rechromatographed with a TFA gradient to purify the peptides. The sequenced radiolabeled peptides started with Phe 327 and ended at Glu 334 (Table I). The advantage to this procedure is a shorter peptide was sequenced, leading to less carry-over of the PTH amino acids into the following steps. Because of the difficulty encountered in purifying peptide 1, it was very hard to quantitate the amount of radioactivity incorporated into each carboxyl group in peptide 1; however, it was possible to identify the labeled carboxyl groups in each case and to estimate the relative reactivity of each residue. The most reactive side chain in peptide 1 was Glu 331. Glu 332 and Asp 328 showed an intermediate level of reactivity, while Asp 329 and Glu 333 reacted to a low extent (Figure 5). The amount of radioactivity incorporated into peptide 1 in the presence of MgATP was about 25% of the free catalytic subunit, but Asp 328, Glu 331, and Glu 332 were still modified (Figure 5). Because ATP reacted with EDC, it was not possible to determine whether the decreased reactivity seen in the presence of MgATP is due to conformational changes induced by ATP or to a simple decrease in the EDC concentration because of the side reaction with nucleotide. With both MgATP and the inhibitor peptide bound to the catalytic subunit, the amount of radiolabel incorporated was somewhat lower than with just MgATP present, but two residues, Asp 328 and Glu 332, were protected completely from modification. Only Glu 331 in peptide 1 reacted with EDC in the ternary complex (Figure 5).

Part of the radioactivity in Figure 4 eluting at 68 min and the major peak at 79 min were from peptide 2 (Table I). After this peptide was purified and sequenced, the modified amino acid was identified as Glu 170. The amount of radiolabel incorporated into Glu 170 in the presence of MgATP was about 50% of the quantity incorporated into the free catalytic subunit and was reduced to nothing with MgATP and inhibitor peptide bound to the catalytic subunit (Figures 4 and 5).

Two additional minor sites of covalent modification were identified. The reactivity of these two residues was not altered significantly by substrate binding. The first, corresponding to peptide 3 (Table I), eluted at 58 min and also at 68 min along with peptide 2 (Figure 4). After purification and sequencing, the labeled amino acid was shown to be Glu 107. The second reactive carboxyl group that was not affected by substrates eluted at about 90 min (Figure 4) and also in a broad peak from 100 to 110 min (not shown). These fractions were pooled, lyophilized, and digested with chymotrypsin as described under Experimental Procedures (not shown). Two peaks of radioactivity were found in the apoenzyme and MgATP profiles, while only one peak was seen in the MgATP/inhibitor peptide profile. The site of modification not present in the MgATP plus inhibitor profile corresponded to Glu 170. The other peptide common to all three profiles was peptide 4 (Table I). After purification, the peptide was sequenced, and the site of modification was identified as Asp 241. The radioactivity incorporated into Asp 241 was not significantly different in the presence and absence of substrates.

The catalytic subunit also was modified with EDC (0.8 mM) and radiolabeled nucleophile (2.8 mM) in the presence of the 6-residue inhibitor peptide (2 mM) and MgATP (4 mM $MgCl_2$, 2 mM ATP). The type I holoenzyme with MgATP (0.6 mM $MgCl_2$, 0.12 mM ATP) plus and minus cAMP was labeled in the same manner. The HPLC radioactivity profiles from the catalytic subunit/6-residue inhibitor peptide/MgATP and the holoenzyme minus cAMP reactions were similar to the profile shown in Figure 4 for the catalytic subunit/MgATP/20-residue inhibitor peptide (data not shown). The same three amino acids, Glu 170, Asp 328, and Glu 332, were protected from modification by EDC. When the holoenzyme was labeled in the presence of cAMP, the same amino acids radiolabeled in the free catalytic subunit were modified including Asp 329 and Glu 333. This differs from the catalytic subunit that was modified in the presence of 2 mM ATP (Figure 4), where Asp 329 and Glu 333 were not reactive.

Discussion

Treatment of the catalytic subunit of cAMP-dependent protein kinase with EDC led to the irreversible inhibition of activity. Neither MgATP nor an inhibitor peptide alone was sufficient to protect against inactivation by EDC, but the ternary complex of enzyme with MgATP and peptide blocked the inhibition completely. Modification of the free catalytic subunit with EDC and [^{14}C]glycine ethyl ester identified several reactive carboxyl groups. A cluster of acidic residues

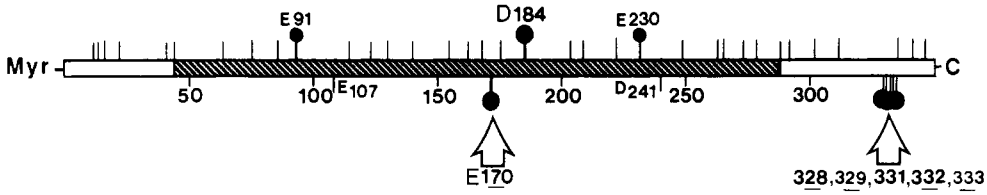

FIGURE 6: Amino acids in the catalytic subunit that are modified by EDC and DCCD. The residues that are modified with DCCD are marked on top, while the carboxyl groups that are labeled with EDC are shown below the bar. The carboxylic acid residues that are not modified by either carbodiimide are represented by a small line extending out of the bar. The shaded region of the bar represents the conserved catalytic core in the catalytic subunit that is shared between the protein kinases (Hanks et al., 1988).

at the carboxy terminus was the most reactive region of the protein. Of the five labeled amino acids in this region, only Glu 331 was modified under all conditions. Glu 333 and Asp 329 were labeled to a low extent only in the free catalytic subunit. Asp 328 and Glu 332 reacted in the free catalytic subunit and with MgATP present, but were protected completely in the presence of MgATP and an inhibitor peptide. Glu 170 was the other amino acid that was completely protected when MgATP and inhibitor peptide were added to the catalytic subunit prior to modification by EDC and [^{14}C]-glycine ethyl ester. The reactivity of two other carboxyl groups, Glu 107 and Asp 241, was not significantly affected by substrate binding.

Some qualitative features of substrate binding can be deduced upon examination of the EDC inhibition characteristics that were observed for the catalytic subunit. For example, the 6-residue inhibitor peptide alone had no effect on the inactivation by EDC, while the presence of MgATP appeared to enhance the binding of the 6-residue inhibitor peptide to the catalytic subunit, since MgATP and the peptide afforded complete protection from the EDC-mediated effects (Figure 1). The 6-residue substrate peptide alone also did not block the inhibition. If the substrate or inhibitor peptides were productively binding to the catalytic subunit in the absence of nucleotide, than they should have protected the enzymatic activity on the basis of the results shown in Figure 2 with the holoenzyme complexes. Although a detailed kinetic analysis of the peptides used in this study was not done, the results suggest that binding of the peptides to the catalytic subunit is enhanced in the presence of nucleotide. These results are consistent with the kinetic studies of Whitehouse et al. (1983).

The results described here for the modification of the catalytic subunit with EDC differ markedly from previous studies with the hydrophobic carbodiimide, DCCD. First, MgATP alone was capable of protecting the catalytic subunit against inactivation by DCCD (Toner-Webb & Taylor, 1987). In addition, the reactive carboxyl groups that were protected from modification by DCCD when MgATP was bound to the enzyme, Asp 184 and Glu 91 (Buechler & Taylor, 1988), were not labeled by EDC (Figure 6). These differences can be attributed to the physical properties of the two carbodiimides. DCCD typically partitions into hydrophobic areas of the protein, while EDC, because of its hydrophilic properties, generally reacts with carboxylic acids exposed to the aqueous solvent (Solioz, 1984).

A potential function for a solvent-accessible carboxylic acid group in the catalytic subunit would be to interact with the basic amino acids preceding the phosphorylation site in the protein substrates, since the basic residues are the primary determinants for substrate specificity. Thus, the three carboxyl groups that are protected from modification by EDC in the presence of MgATP and inhibitor peptide, Glu 170, Asp 328, and Glu 332, may contribute to the recognition of the basic residues in the protein substrates. Although it is possible that a conformational change induced by the binding of the inhibitor peptide to the catalytic subunit–MgATP complex could be responsible for the protection of the three carboxyl groups from modification by EDC, the following discussion will focus on the potential interactions with the substrate.

Table II: Sequence Comparisons of the Regions Containing Glu 170 in Other Protein Kinases and the Substrate Specificities of the Indicated Kinases[a]

kinase	sequence flanking Glu 170	substrate specificity
	170	
C subunit	R-D-L-K-P-E-N-L-L-	basic
cG kinase	R-D-L-K-P-E-N-L-I-	
MLCK	L-D-L-K-P-E-N-I-L-	
protein kinase C	R-D-L-K-L-D-N-V-M-	basic
phosphorylase kinase	R-D-L-K-P-E-N-I-L-	
casein kinase II	R-D-V-K-P-H-N-V-M-	acidic
c-src	R-D-L-R-A-A-N-I-L-	acidic
EGFR	R-D-L-A-A-R-N-V-L	

[a] Abbreviations: C subunit, catalytic subunit; cG kinase, cGMP-dependent protein kinase; MLCK, myosin light chain kinase from rabbit skeletal muscle; EGFR, epidermal growth factor receptor. The amino acid sequences were taken from the recent comparison by Hanks et al. (1988).

Glu 170 is located in the central region of the protein and is flanked by segments that are highly conserved in all protein kinases (Table II). Unlike Asp 184 and Lys 72, which are thought to be important for catalysis and/or MgATP binding and are invariant in every protein kinase, Glu 170 is not conserved (Hanks et al., 1988). Furthermore, a correlation can be made between the residue at the position corresponding to Glu 170 in other kinases and the amino acids that are required for substrate recognition by each kinase. For example, as summarized in Table II, a carboxyl group is conserved in protein kinases having a known specificity for basic residues preceding the phosphorylation site, such as phosphorylase kinase (Chan et al., 1982), protein kinase C (House et al., 1987), and myosin light chain kinase (Kemp & Pearson, 1985; Michnoff et al., 1986). On the other hand, casein kinase II, which prefers substrates with acidic residues clustered around the phosphorylation site (Meggio et al., 1984), has a histidine at the homologous position. Several tyrosine kinases also show a specificity for carboxyl groups in their substrates (Patschinsky et al., 1982: Hunter, 1982; Cooper et al., 1984). These kinases have an arginine either in the position corresponding to Glu 170, as in the epidermal growth factor receptor, or two residues on the N-terminal side of that position, as in pp60^{src}. It must be emphasized that correlating the substrate specificity of several kinases with the amino acid located in the homologous position to Glu 170 does not prove that this region contributes to substrate specificity, but it does add indirect evidence to support the conclusion that Glu 170 may be important for recognizing the basis residues in the protein substrate.

The reactivity of the cluster of acidic residues near the carboxy terminus also is drastically altered in the presence of MgATP and inhibitor peptide, especially Asp 328 and Glu 332.

These amino acids are located outside the catalytic core shared by all eucaryotic protein kinases, but they are conserved in all the cyclic nucleotide dependent kinases, including the yeast (Toda et al., 1987) and *Drosophila* (Foster et al., 1988) catalytic subunits, and the cGMP-dependent protein kinase (Takio et al., 1984). This conservation implies that these carboxyl groups may play an important role in structure or function for this subfamily of protein kinases. The data in this study suggest a potential interaction of Asp 328 and/or Glu 332 with the arginine residues in the protein substrate. These residues may differentiate the protein substrates of the catalytic subunit from the substrates of other kinases that recognize basic residues, since the region containing these carboxyl groups is not highly conserved in other protein kinases. On the other hand, the amount of radioactivity incorporated into this cluster of acidic residues seemed to decrease more in the presence of MgATP relative to Glu 170, suggesting that MgATP binding may induce conformational changes in this region of the protein. If this were the case, then a second conformational change induced by peptide binding could potentially account for the complete loss of reactivity with these carboxylic acid residues. Substrate-induced conformational changes are known to occur for other kinases, such as hexokinase (Bennett & Steitz, 1978), and may be associated with this kinase as well. This region of the protein may also be important for facilitating substrate recognition, as suggested by Mobashery and Kaiser (1988) on the basis of their affinity-labeling results. The carboxyl group that was labeled by their peptide analogues, Glu 346, did not react with EDC and glycine ethyl ester under the conditions that were used here.

In many regards, the protein kinases share many analogies with the trypsin-like serine proteases. Sequence similarities throughout a conserved catalytic core strongly suggest that the eucaryotic protein kinases and the trypsin-like family of proteases both represent examples of divergent evolution. Amino acids thought to be essential for catalysis in each family of enzymes are conserved, such as Lys 72 and Asp 184, in the catalytic subunit, and the catalytic triad, Ser 195, His 57, and Asp 102, in trypsin. Like the protein kinases, the serine proteases also have different protein substrate specificities, which are determined in the serine proteases by the amino acids making up their substrate recognition pockets (Kraut 1977). For example, the carboxylic acid residue in trypsin that interacts with the positively charged lysine or arginine in the protein substrates of trypsin is not conserved in other proteases. Interestingly, this carboxyl group, Asp 189, was modified with EDC, leading to the loss of activity. This effect of EDC on trypsin was blocked in the presence of an inhibitor of trypsin (Eyl & Inagahi, 1971). Similarly, different members of the protein kinase family may also have specific amino acids lining their substrate recognition pockets, and thus the amino acids that were protected from modification by EDC in the presence of MgATP and inhibitor peptide, Glu 170, Asp 328, and Glu 332, may provide important contacts with the basic residues in the protein substrates of the catalytic subunit.

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Registry No. L-R-R-N-S-I, 124755-17-5; L-R-R-N-A-I, 124755-18-6; Glu, 56-86-0; Asp, 56-84-8; protein kinase, 9026-43-1.

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Chromophore Topography and Secondary Structure of 124-Kilodalton *Avena* Phytochrome Probed by Zn^{2+}-Induced Chromophore Modification†

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ABSTRACT: The relative extent of chromophore exposure of the red-absorbing (P_r) and far-red-absorbing (P_{fr}) forms of 124-kDa oat phytochrome and the secondary structure of the phytochrome apoprotein have been investigated by using zinc-induced modification of the phytochrome chromophore. The absence of bleaching of P_r in the presence of a 1:1 stoichiometric ratio of zinc ions, in contrast to extensive spectral bleaching of the P_{fr} form, confirms previous reports of differential exposure of the P_{fr} chromophore relative to the P_r chromophore [Hahn et al. (1984) *Plant Physiol. 74*, 755–758]. The emission of orange fluorescence by zinc-chelated P_{fr} indicates that the P_{fr} chromophore has been modified from its native extended/semi-extended conformation to a cyclohelical conformation. Circular dichroism (CD) analyses of native phytochrome in 20 mM Tris buffer suggests that the P_r-to-P_{fr} phototransformation is accompanied by a photoreversible change in the far-UV region consistent with an increase in the α-helical folding of the apoprotein. The secondary structure of phytochrome in Tris buffer, as determined by CD, differs slightly from that of phytochrome in phosphate buffer, suggesting that phytochrome is a conformationally flexible molecule. Upon the addition of a 1:1 molar ratio of zinc ions to phytochrome, a dramatic change in the CD of the P_{fr} form is observed, while the CD spectrum of the P_f form is unaffected. Analysis of the bleached P_{fr} CD spectrum by the method of Chang et al. (1978) reveals that chelation with zinc ions significantly alters the secondary structure of the phytochrome molecule, specifically by increasing the β-sheet content primarily at the expense of α-helical folding. Further evidence of a zinc-induced conformational change in phytochrome has been obtained with proteolytic digestions of chelated and nonchelated P_{fr}. We propose that chelation with zinc ions at the phytochrome chromophore cyclizes the semiextended P_{fr} chromophore, which reduces/eliminates the interactive forces between the chromophore and the polypeptide. Once these forces are attenuated, the phytochrome molecule undergoes a conformational rearrangement, resulting in a secondary structure similar to that of the phytochrome apoprotein, which appears to differ substantially from that of the holoprotein.

Phytochrome is a tetrapyrrolic chromoprotein that serves as the primary photoreceptor for several light-mediated developmental responses in plants. There are two photoreversible forms of phytochrome, the red light absorbing P_r[1] form and the physiologically active far-red-absorbing P_{fr} form. Phytochrome undergoes the following photoreversible transformations upon irradiation with red or far-red light [for reviews, see Schäfer and Briggs (1986), Quail et al. (1986), and Furuya (1987)]:

$$P_{r(\text{red-absorbing form})} \underset{730\text{ nm}}{\overset{660\text{ nm}}{\rightleftharpoons}} P_{fr(\text{far-red-absorbing form})} \rightarrow \text{morphogenic and developmental responses}$$

Although the exact structure of native phytochrome and the mechanism of its action have not yet been elucidated, the chromophore topography and conformational differences between the two forms have been studied extensively [for detailed reviews, see Lagarias (1985) and Song (1988)]. Although considerable progress has been made in these areas, major discrepancies have arisen in attempts to quantitatively assess the accessibility of the chromophore and to predict the secondary structure of phytochrome. For example, while methods based on the amino acid sequence of phytochrome predict a substantial amount of β-pleated sheet structures, particularly at the chromophore attachment site, methods based on CD analyses have frequently failed to predict any β-sheet structures in the phytochrome conformation.

The present study used zinc ions, which are known to chelate the tetrapyrroles in phycocyanins and phytochrome (O'hEocha, 1963; Furuya et al., 1965; Lisansky & Galston, 1974), to probe

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[1] Abbreviations: HA, hydroxyapatite; CD, circular dichroism; P_r, red-absorbing form of phytochrome; P_{fr}, far-red-absorbing form of phytochrome; kDa, kilodaton(s); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; ME, 2-mercaptoethanol; PMSF, phenylmethanesulfonyl fluoride; PEI, poly(ethylenimine); EG, ethylene glycol; KPB, potassium phosphate buffer; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; UV–visible, ultraviolet–visible; ANS, 8-anilinonaphthalene-1-sulfonate.

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