Feeney, J., Batchelor, J. G., Albrand, J. P., & Roberts, G. C. K. (1979) *J. Magn. Reson. 33*, 519–529.
Forsén, S., Andersson, T., Drakenberg, T., Thulin, E., & Wieloch, T. (1981) in *Advances in Solution Chemistry* (Betini, I., Lunazzi, L., & Dei, A., Eds.) pp 191–208, Plenum, New York.
Fung, C. H., Mildvan, A. S., Allerhand, A., Komoroski, R., & Scrutton, M. C. (1973) *Biochemistry 12*, 620–629.
Jaffe, E. K., & Cohn, M. (1978) *J. Biol. Chem. 253*, 4823–4825.
Johanson, R. A., & Colman, R. F. (1981) *Arch. Biochem. Biophys. 207*, 9–20, 21–31.
Koehler, L. S., Jarnagin, F., Hiskey, R. G., Pederson, L. G., & Koehler, K. A. (1987) *J. Inorg. Biochem. 29*, 153–164.
Kuchel, P. W., Reynolds, C. H., & Dalziel, K. (1980) *Eur. J. Biochem. 110*, 465–473.
Led, J. J., & Neesgaard, E. (1987) *Biochemistry 26*, 183–192.
Levy, R. S., & Villafranca, J. J. (1977a) *Biochemistry 16*, 3293–3301.
Levy, R. S., & Villafranca, J. J. (1977b) *Biochemistry 16*, 3301–3309.
Magnusson, J. A., & Bothner-By, A. A. (1971) in *Magnetic Resonances in Biological Research* (Franconi, C., Ed.) pp 365–376, Gordon and Breach, New York.
Mas, M. T., & Colman, R. F. (1985) *Biochemistry 24*, 1634–1646.
Meiboom, S., & Gill, D. (1958) *Rev. Sci. Instrum. 29*, 688–691.
Mennitt, P. G., Shatlock, M. P., Bartuska, V. J., & Maciel, G. E. (1981) *J. Phys. Chem. 85*, 2087–2091.
O'Leary, M. H., & Limburg, J. A. (1977) *Biochemistry 16*, 1129–1135.
Sillen, L. G., & Martell, A. E. (1971) *Spec. Publ.—R. Soc. Chem. No. 25*, 643.
Sloan, D. L., & Mildvan, A. S. (1976) *J. Biol. Chem. 251*, 2412–2420.
Solomon, I., & Bloembergen, N. J. (1956) *J. Chem. Phys. 25*, 261–266.
Sweetser, P. D., & Bricker, C. E. (1954) *Anal. Chem. 26*, 195–197.
Tsai, M.-D., Drakenberg, T., Thulin, E., & Forsén, S. (1987) *Biochemistry 26*, 3635–3643.
Uhr, M. L., Thompson, V. W., & Cleland, W. W. (1974) *J. Biol. Chem. 249*, 2920–2927.
Villafranca, J. J., & Colman, R. F. (1972) *J. Biol. Chem. 247*, 209–214.
Villafranca, J. J.; & Colman, R. F. (1974) *Biochemistry 13*, 1152–1160.

Dicyclohexylcarbodiimide Cross-Links Two Conserved Residues, Asp-184 and Lys-72, at the Active Site of the Catalytic Subunit of cAMP-Dependent Protein Kinase†

Joseph A. Buechler‡ and Susan S. Taylor*,§
Department of Chemistry, University of California, San Diego, La Jolla, California 92093
Received May 13, 1988; Revised Manuscript Received November 9, 1988

ABSTRACT: In the absence of MgATP, the catalytic subunit of cAMP-dependent protein kinase is irreversibly inhibited by the hydrophobic carbodiimide dicyclohexylcarbodiimide, and this inhibition is most likely due to the formation of a cross-link between a carboxyl group and a lysine residue in the active site (Toner-Webb & Taylor, 1987). In order to identify these cross-linked residues, the catalytic subunit was modified by dicyclohexylcarbodiimide and then treated with acetic anhydride and digested with trypsin. The resulting peptides were resolved by high-performance liquid chromatography. One major absorbing tryptic peptide and one smaller peptide consistently and reproducibly showed a decrease in absorbance after the catalytic subunit had been treated with DCCD. These peptides correspond to residues 166–190 and 57–93, respectively. A unique peptide was isolated from the modified catalytic subunit, and the sequence of this peptide established that the cross-linking occurred between Asp-184 and Lys-72. The cross-linking of these two residues, which were both identified previously as essential residues, confirms the likelihood that each plays a role in the functioning of this enzyme. The fact that Asp-184 and Lys-72 appear to be invariant in all protein kinases further supports the hypothesis that these two residues, located close to one another at the active site of the enzyme, play essential roles in catalysis.

The catalytic (C) subunit of cAMP-dependent protein kinase is a monomeric protein with a molecular weight of 40 900 (Shoji et al., 1983). It is one of the simplest protein kinases, because the major regulatory element is part of a separate subunit that dissociates in the presence of cAMP. In its dissociated, monomeric state, the C-subunit is fully active and transfers phosphate from the γ-position of ATP to the hydroxyl group of either a Ser or a Thr residue on a protein substrate. The phosphorylated residue on the substrate is preceded by two basic amino acids, usually arginines, that are required for recognition (Feramisco et al., 1980; Kemp et al., 1977). There is usually a single intervening residue between the basic residues and the phosphorylated amino acid [for a review, see Bramson et al. (1984)].

† This research was supported in part by USPHS Grant GM 19301 to S.S.T.
* Address correspondence to this author.
‡ Supported by USPHS Training Grant AM07233.
§ Member of the Molecular Genetics Center, University of California, San Diego.

0006-2960/89/0428-2065$01.50/0

Both affinity labeling and group-specific labeling have been used to identify amino acids at the active site of the C-subunit. Two residues in particular have been identified that are thought to be essential for catalytic activity and for MgATP binding. Lys-72 was identified by affinity labeling with an analogue of ATP, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (FSBA), and is thought to be part of the MgATP binding site (Zoller & Taylor, 1979; Zoller et al., 1981). It most likely interacts with a portion of the C-subunit that recognizes the β- and/or γ-phosphate of ATP (Bhatnager et al., 1984). Asp-184 was identified by modification with a hydrophobic carboxyl-specific reagent, dicyclohexylcarbodiimide (DCCD). Treatment of the apoenzyme with DCCD led to irreversible inactivation of the C-subunit, but it was not possible to identify a carboxyl group that was modified by DCCD. Presumably, the reactive carboxyl group cross-linked to a nucleophile on the protein that was in close proximity (Toner-Webb & Taylor, 1987). To circumvent this cross-linking, the C-subunit was treated with acetic anhydride prior to treatment with DCCD. This prevented intramolecular cross-linking and identified the major reactive carboxyl group as Asp-184. Asp-184 also was protected from modification by DCCD in the presence of MgATP (Buechler & Taylor, 1988).

The intention of the studies described here was to identify the cross-linked residues that resulted from treatment of the apoenzyme with DCCD. The identification of these cross-linked residues has allowed us to propose a model for the active-site region of the enzyme and to predict the changes that occur in this region as a consequence of MgATP binding.

Experimental Procedures

Materials. Reagents were obtained from the following sources: [^{3}H]acetic anhydride (100 mCi/mmol), ICN; acetic anhydride and acetonitrile (HPLC grade), Fisher Scientific; dicyclohexylcarbodiimide (DCCD), Aldrich; 2-(*N*-morpholino)ethanesulfonic acid (MES) and tris(hydroxymethyl)aminomethane (Tris), United States Biochemical Corp.; ultrapure urea, Schwarz/Mann Biotech.; L-1-(tosylamido)-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin and α-chymotrypsin, Sigma; trifluoroacetic acid (TFA, HPLC grade), Pierce; Cytoscint, Westchem.

Source of Protein. The C-subunit was prepared from porcine heart as described previously (Nelson & Taylor, 1981).

Modification with DCCD. Reactions were carried out in 50 mM MES (pH 6.2) and 5% glycerol. C-Subunit (29.4 μM) was incubated for 90 min at 37 °C with 1.3 mM DCCD, which was added from a stock solution of 100 mM in acetonitrile. The final concentration of acetonitrile was 1.2% in the control (no DCCD), and in the sample with DCCD. After 90 min, the samples were placed on ice.

Modification of Lysine Residues. After the modification with DCCD as described above, the C-subunit was dialyzed against 0.1 M Tris-HCl (pH 8.3) and then denatured by adding solid urea to a final concentration of 8 M. Lysine residues were stoichiometrically modified by incubating with aliquots (75 μL) of [^{3}H]acetic anhydride (1 mCi/mmol, 100 mM in acetonitrile) at room temperature. The aliquots were added over a 40-min period. Prior to proteolysis, the modified C-subunit was dialyzed extensively against 50 mM NH_4HCO_3, pH 8.1, to remove excess reagent.

Proteolysis. Tryptic digests were carried out at 37 °C with 1:50 w/w TPCK-treated trypsin/C-subunit in 50 mM NH_4HCO_3 (pH 8.1). After 2 h, an equal aliquot of TPCK-treated trypsin was added, and incubation was continued for an additional 2 h. Chymotryptic digests were carried out under the same conditions as those described above for trypsin.

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 chymotryptic peptides was accomplished with the same system except that a Vydac C18 column (0.46 × 25 cm) was used instead of the C4 column. Pooled fractions from the original elutions of tryptic and chymotryptic peptides were rechromatographed on a Vydac C18 column with a gradient of 0.1% TFA (pH 2.1) to CH_3CN prior to sequencing.

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

Results

The irreversible inhibition of the C-subunit by DCCD in the absence of MgATP suggested that a cross-linking reaction was occurring between a reactive carboxyl group and a nearby lysine residue (Toner-Webb & Taylor, 1987). Pretreatment of the C-subunit with acetic anhydride prevented cross-linking and identified Asp-184 as the major site of covalent modification by DCCD and Glu-91 as a minor site. Modification of both residues was prevented by MgATP (Buechler & Taylor, 1988). In this study, the C-subunit was first treated with DCCD under conditions that would allow the cross-linking reaction to occur. After dialysis, the C-subunit was denatured with 8 M urea and then treated with [^{3}H]acetic anhydride so that all the lysine side chains were stoichiometrically modified. The acetic anhydride reaction was carried out in order to minimize the total number of tryptic peptides. In a related study, all of these lysine-containing tryptic peptides have been identified (Buechler et al., 1989). This facilitated the identification and isolation of any cross-linked peptides. In addition, radiolabeling each lysine after DCCD treatment meant that any lysine side chain that participated in an intramolecular cross-link would not be radiolabeled, thus providing an additional means of confirming the existence of a cross-link. The intramolecular nature of this cross-link was established by gel electrophoresis carried out in the presence of SDS, since only monomeric protein was observed after treatment with DCCD.

Eleven lysine peptides were obtained after proteolytic cleavage with trypsin, and these peptides were resolved by HPLC. Figure 1, lower panel, shows the HPLC profile of the tryptic peptides resulting from incubation of the C-subunit with 1.2% acetonitrile in the absence of DCCD, while the elution profile of tryptic peptides obtained from the C-subunit incubated in the presence of DCCD is shown in Figure 1, upper panel. The elution of the tryptic peptide containing Asp-184 (residues 166–190) when the C-subunit was not treated with DCCD prior to tryptic digestion is indicated in Figure 1, lower panel. The absorbance of this peptide decreased significantly following treatment with DCCD (Figure 1, upper panel), indicating that an amino acid from this peptide may be one of the participants in the cross-linking reaction. One additional peptide that eluted slightly before the large peak at 78 min (Figure 1) showed a decrease in absorbance following treatment with DCCD (not marked). Although this peptide absorbs less than the peptide containing Asp-184, the decrease

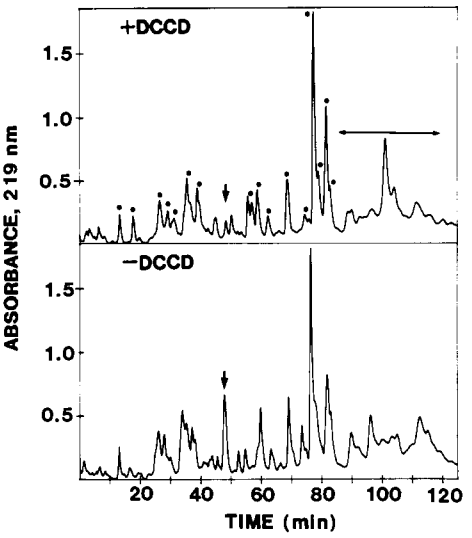

FIGURE 1: Tryptic peptides isolated from the C-subunit with and without DCCD treatment. C-Subunit was treated with (upper panel) or without DCCD (lower panel) as described under Experimental Procedures. After digestion with TPCK-treated trypsin, the resulting peptides were separated by HPLC as described. The tryptic peptides were eluted with a 120-min linear gradient from 10% to 40% CH_3CN followed by a 10-min linear gradient from 40% to 50% CH_3CN. The tryptic peptide containing Asp-184 is marked by the arrow. The dots above several peaks in the upper panel indicate regions that were rechromatographed in the upper and lower panels while searching for the cross-linked peptide.

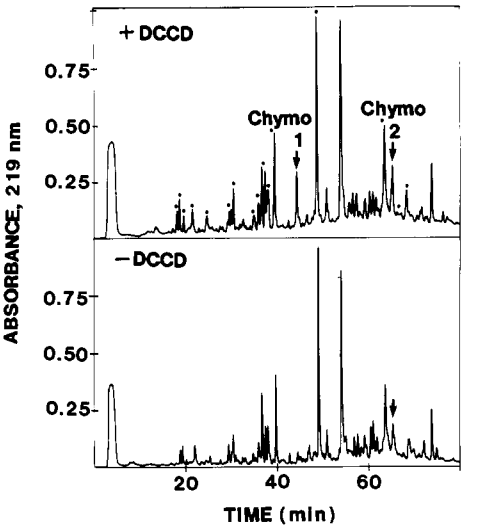

FIGURE 2: Identification of unique peptides resulting from DCCD treatment. Fractions 90–125 from the tryptic peptides of the C-subunit with (upper panel) or without DCCD (lower panel) were pooled, lyophilized, and digested with chymotrypsin as described under Experimental Procedures. The chymotryptic peptides were separated with an 80-min linear gradient from 0 to 30% CH_3CN. The locations of chymo 1 and chymo 2 are indicated (upper panel), while the region corresponding to chymo 2 is marked by an arrow in the lower panel. The dots above several peaks in the upper panel indicate regions that were rechromatographed in the upper and lower panels while searching for the cross-linked peptide.

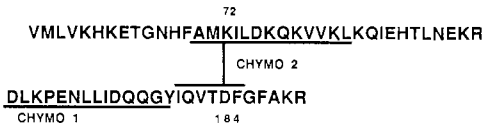

FIGURE 3: Sequences of the tryptic peptides that are cross-linked by DCCD. Two chymotryptic peptides that were sequenced from this region, chymo 1 and 2, are underlined.

in absorbance was reproducibly observed in several digests. This peptide corresponded to residues 57–93, and it contained eight lysines. It was assumed that the cross-linked peptide eluted after 90 min since a novel, major absorbing peptide was not apparent among the peptides eluting prior to 90 min. It should be noted that the retention time of several peptides varied somewhat between HPLC runs (Buechler et al., 1989); thus, several peaks showed differences when the upper and lower panels in Figure 1 were compared. None of these differences were caused by the formation of cross-linked peptides. This was confirmed by rechromatographing the indicated peaks and comparing the retention times with earlier results (Buechler et al., 1989). Previous attempts to identify a cross-linked peptide from a total tryptic digest also had failed to identify a new major absorbing peptide in the well-resolved region of the elution profile (Toner-Webb & Taylor, 1987). Consequently, the poorly resolved peaks of absorbance that eluted after 90 min were pooled and digested with chymotrypsin in an effort to locate a cross-linked peptide. The peptides from this pooled fraction were resolved by HPLC (Figure 2).

When these chymotryptic peptides from the untreated C-subunit were compared to those resulting from the DCCD-treated C-subunit, a novel peptide was immediately identified that eluted at 45–46 min in the chymotryptic profile of the DCCD-treated C-subunit (Figure 2, chymo 1). This peptide was purified by rechromatographing with a different elution gradient and then sequenced. The sequence, as indicated in Table I, corresponded to residues 166–179, which is the chymotryptic peptide immediately preceding the predicted chymotryptic peptide that would contain Asp-184 (Figure 3). Identification of this peptide verified that the original tryptic peptide (residues 166–190) was located in the chymotryptic digest and, in addition, confirmed that it had been modified in the DCCD-treated protein, presumably accounting for its dramatically altered elution properties. Several other differences in the chymotryptic profile were observed; however, these differences were due primarily to incomplete digestion with chymotrypsin. The elution profile of the chymotryptic digest was then scanned at 219 and 280 nm, and the difference ratio was determined for each peak (data not shown), since neither the chymotryptic peptide containing Asp-184 nor the other tryptic peptide that was missing after DCCD treatment (residues 57–93) contains any amino acids that would absorb at 280 nm. A significant decrease in absorbance at 280 nm was noted at a retention time of 66–67 min when the DCCD-treated protein was compared to the

Table I: Amino Acid Sequence of Chymo 1 and Chymo 2[a]

	amino acid assignment			
step	chymo 1	chymo 2		cpm
1	Asp	Ile	Ala	50
2	Leu	Gln	Met	119
3	Lys	Val		97
4	Pro	Thr	Ile	75
5	Glu		Leu	121
6	Asn	Phe	Asp	97
7	Leu		Lys	657
8	Leu		Gln	378
9	Ile		Lys	411
10	Asp		Val	179
11	Gln		Val	91
12	Gln		Lys	121
13	Gly		Leu	58
14	Tyr			

[a] Peptides were purified and sequenced as described under Experimental Procedures. Aliquots of each sequencer step were counted in Cytoscint. cpm refers only to the radioactivity associated with chymo 2.

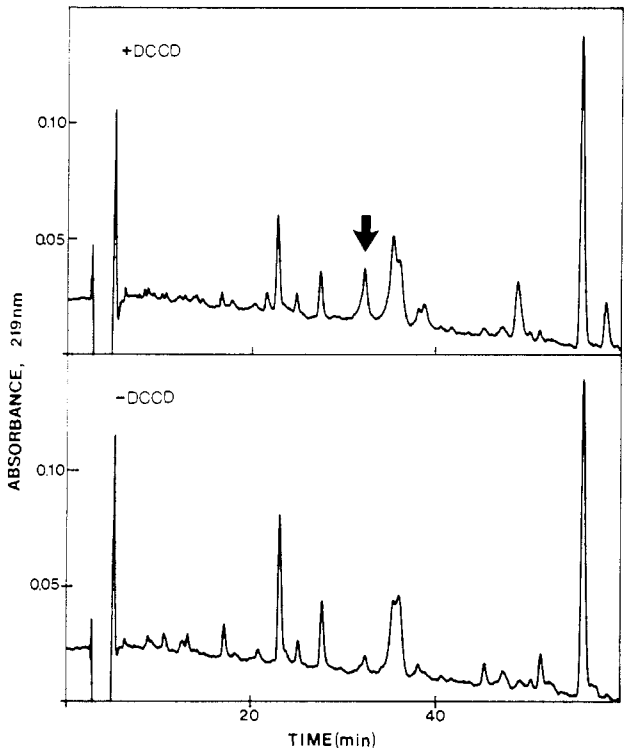

FIGURE 4: Purification of the cross-linked chymotryptic peptide. Fractions 65–67 (Figure 2) were rechromatographed as described under Experimental Procedures. The peptides were eluted with a linear gradient from 22% to 34% CH_3CN. The location of chymo 2 is indicated by an arrow in the upper panel. It should be noted that the small peak seen in the lower panel at about the same retention time as chymo 2 represented a separate peptide. This minor peptide was resolved sufficiently from chymo 2 (upper panel) to obtain the sequence shown in Table I.

control C-subunit, even though the 219-nm profile looked essentially the same. Consequently, fractions 65–67 were rechromatographed, as shown in Figure 4. A unique peptide was found in the sample derived from C-subunit that had been treated with DCCD (Figure 4).

When this peptide (chymo 2) was analyzed, two sequences were observed simultaneously, corresponding to residues 70–82 and residues 180–185 (Table I, Figure 3). Two PTH-amino acids were absent from the sequence of this peptide when compared to the analogous sequences of the two individual peptides from the unmodified C-subunit. Valine was the only PTH-amino acid to elute at step 3, and leucine was the only PTH-amino acid observed at step 5. Thus, the PTH-amino acids of Lys-72 (step 3) and Asp-184 (step 5) were not seen in the sequence of the unique peptide. In addition, radioactivity did not elute at step 3. In contrast, ^3H radiolabel eluted at positions corresponding to Lys-76, Lys-78, and Lys-81 (Table I). On this basis, it was concluded that modification of Asp-184, which is the major site that is labeled by DCCD in the apoenzyme, leads to the formation of a cross-link with the side chain of Lys-72.

Discussion

The C-subunit of cAMP-dependent protein kinase is irreversibly inhibited by the hydrophobic carbodiimide DCCD. MgATP afforded protection against this inactivation, and it was predicted that the inhibition was a multistep process based on the following observations. First, in spite of the irreversible inhibition, it was not possible to radiolabel the protein using [^{14}C]DCCD. Second, it was not possible to trap a reactive intermediate with radioactive amines such as [^3H]aniline or [^{14}C]glycine ethyl ester (Toner-Webb & Taylor, 1987). On these bases, it was predicted that the first step in the DCCD-mediated inhibition of the C-subunit was the reaction of a specific carboxyl group in a hydrophobic region of the C-subunit with DCCD. Activation of the carboxyl group was then followed by the formation of an intramolecular cross-link with a nearby nucleophile. Cysteine or lysine residues were likely candidates for this cross-linking reaction. Pretreatment of the C-subunit with acetic anhydride, which specifically reacts with lysine side chains under the conditions employed, was carried out in an attempt to incorporate radiolabel into the C-subunit by blocking potential nucleophiles. When the acetic anhydride treated C-subunit was modified with DCCD in the presence of [^{14}C]glycine ethyl ester, two carboxyl groups were radiolabeled, and MgATP blocked both modifications. The major labeled carboxyl group was identified as Asp-184 and the minor as Glu-91 (Buechler & Taylor, 1988). At this point, treatment of the apoenzyme directly with DCCD was reinvestigated in an effort to identify the residues involved in the cross-linking reaction accounting for the irreversible inhibition of the apoenzyme. The amino acid side chain that covalently cross-links to Asp-184 has been identified here as Lys-72. A cross-linked peptide containing Glu-91 was not found, possibly because Glu-91 was not as reactive toward DCCD as Asp-184. Alternatively, cross-linking of Asp-184 to Lys-72 may interfere with or prevent modification of Glu-91 by DCCD.

The evidence for placing the side chain of Lys-72 in close proximity to the β- and/or γ-phosphate of MgATP is convincing. Modification of the C-subunit with FSBA first identified Lys-72 as an essential residue (Zoller & Taylor, 1979; Zoller et al., 1981). Although Jacobson and Colman (1984) demonstrated that the reactive (fluorosulfonyl)benzoyl group in FSBA may actually lie close to the adenine ring in solution, the alkylating group of FSBA likely occupies the region of the protein that recognizes the β- and/or γ-phosphate portion of ATP when it is bound to the active site of the C-subunit. In particular, Bhatnagar et al. (1984) characterized the nucleotide binding properties of the C-subunit after it had been modified with FSBA and subjected to mild hydrolysis to remove the adenine moiety. They demonstrated that the binding of fluorescent analogues of MgATP and MgADP to the FSBA-modified C-subunit was severely impaired but that fluorescent analogues of AMP and adenosine bound in a similar manner to both the native C-subunit and the FSBA-treated C-subunit. This led to the prediction that modification by FSBA specifically interfered with the region of the enzyme recognizing the β- and/or γ-phosphate of ATP. Lys-72 also is invariant in every known protein kinase where sequence information is available (Hanks et al., 1988), providing further support for an essential role. Final evidence for the importance of this residue comes from site-directed mutagenesis, since replacement of the homologous lysine in several kinases, such as $pp60^{src}$ (Kamps & Sefton, 1986; Snyder et al., 1985) and the EGF receptor (Chen et al., 1987), resulted in the loss of kinase activity.

The cross-linking reaction described here places Asp-184 in the active site of the C-subunit. Indirect evidence supporting an essential role for Asp-184 derives from the previous DCCD modification studies (Toner-Webb & Taylor, 1987; Buechler & Taylor, 1988). These studies showed that the modification of a carboxyl group by DCCD inhibited C-subunit activity, and Asp-184 was the major site of modification in the acetic anhydride treated enzyme. The inhibition of the C-subunit by DCCD and the modification of Asp-184 in the acetic anhydride treated protein also are blocked in the presence of

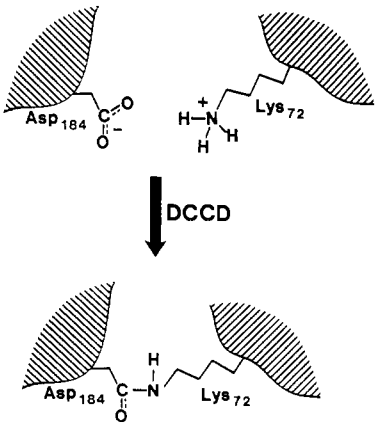

FIGURE 5: Residues that are cross-linked following treatment of the free C-subunit with DCCD.

MgATP, further localizing this amino acid to a region close to the MgATP binding site. In addition, the sequence surrounding Asp-184, Asp-Phe-Gly, is a highly conserved feature of all protein kinases, and Asp-184, like Lys-72, is invariant (Barker & Dayhoff, 1982; Hunter & Cooper, 1986; Hanks et al., 1988). Several roles are possible for Asp-184. The presence of a general base at the active site of the enzyme is predicted from the pH dependency of the reaction, which indicates that a residue with a pK_a of 6.2 is required for catalysis and that this group must be deprotonated for the enzyme to be active (Yoon & Cook, 1987; Bramson et al., 1984). Asp-184 is a likely candidate for this general base. Alternatively, Asp-184 could serve as a ligand of Mg^{2+} in the MgATP complex. Since Asp-184 is covalently modified by a hydrophobic carbodiimide, it is probably near a relatively hydrophobic region of the protein. Essential carboxyl groups in hydrophobic environments have been identified in other proteins by covalent modification with DCCD (Yoshida et al., 1981; Satre et al., 1979). The hydrophobic environment may correspond to the adenine binding site, or it may simply reflect the immediate environment of the carboxyl group. In either case, the hydrophobic nature of the region surrounding Asp-184 would tend to raise the pK_a of the carboxylic acid. The data presented here do not allow us to distinguish the precise function of Asp-184; however, as discussed previously, the evidence for Asp-184 being the general base in catalysis is somewhat more convincing than for other functions, such as binding Mg^{2+} in the MgATP complex (Buechler & Taylor, 1988).

The identification of a covalent cross-link between Asp-184 and Lys-72 allows one to predict more precisely the topography of the active-site region in the apoenzyme. As indicated in Figure 5, the side chains of Asp-184 and Lys-72 in the apoenzyme must lie close to one another so they can be covalently cross-linked by DCCD, with the concomitant formation of an amide bond between the two residues. This close proximity must exist in spite of the fact that these two residues are widely separated in the linear sequence. Lys-72 is predicted to lie in a nucleotide fold structure and has been localized to the region that links the second β-strand to an α-helix in this nucleotide fold (Taylor, 1987; Taylor et al., 1988). Asp-184, on the other hand, lies well beyond this region, perhaps even outside the nucleotide fold motif (Taylor, 1987). The active-site region where catalysis occurs may therefore contain segments from different regions of the protein that come into close contact.

The locations of Asp-184 and Lys-72 in the active site, along with the following previously published results, enable us to propose two possible models for the steps leading to the phosphorylation of the substrate. The kinetic and equilibrium binding studies of Whitehouse et al. (1983) predicted that the C-subunit obeys a mechanistic pathway where MgATP preferentially binds first. In the presence of MgATP, a high-affinity binding site for the peptide is available. For example, the substrate peptide Leu-Arg-Arg-Ala-Ser-Gly-Leu binds to the apoenzyme with a K_d of about 200 μM in the absence of MgATP. In the presence of MgATP, the K_m for the substrate peptide is 16 μM. This evidence suggests that the binding of MgATP leads to specific changes in the active site, which not only facilitate the binding of MgATP but also subsequently enhance the binding of the peptide substrate.

The results described here provide a potential molecular basis for the steps that are likely to occur in catalysis. These steps could occur through two possible pathways. The first assumes that Asp-184 is the general base in catalysis, while the second model has Asp-184 participating as a ligand for Mg^{2+} in the MgATP complex. In either model, the side chains of Lys-72 and Asp-184 lie in close proximity in the apoenzyme with a proton possibly being shared between them. The binding of MgATP would disrupt this interaction, and in the presence of nucleotide, the side chain of Lys-72 could now interact primarily with one of the oxygens of the γ-phosphate of ATP, drawing electrons away from the phosphorus and making it more susceptible to nucelophilic attack. This sequence of events, as outlined in Figure 6, now leaves the side chain of Asp-184 exposed and free to interact with the peptide substrate. Its most likely role here is to serve as a general base catalyst by hydrogen bonding to the hydroxyl side chain of the substrate, pulling the proton away from the oxygen and consequently making the oxygen more nucleophilic. The ordered binding pathway described by Whitehouse et al. is explained readily by this mechanism. The second model differs from the sequence of events in Figure 6 in that when MgATP binds to the catalytic subunit, the side chain of Asp-184 would bind to Mg^{2+} of the MgATP complex. This interaction would occur either through direct contact between the carboxyl group of Asp-184 and Mg^{2+} or with a H_2O molecule bridging Asp-184 with Mg^{2+}. The interaction between Asp-184 and Mg^{2+} would potentially help orient the γ-phosphate of ATP for transfer to the peptide substrate. This second model requires that another residue functions as the general base.

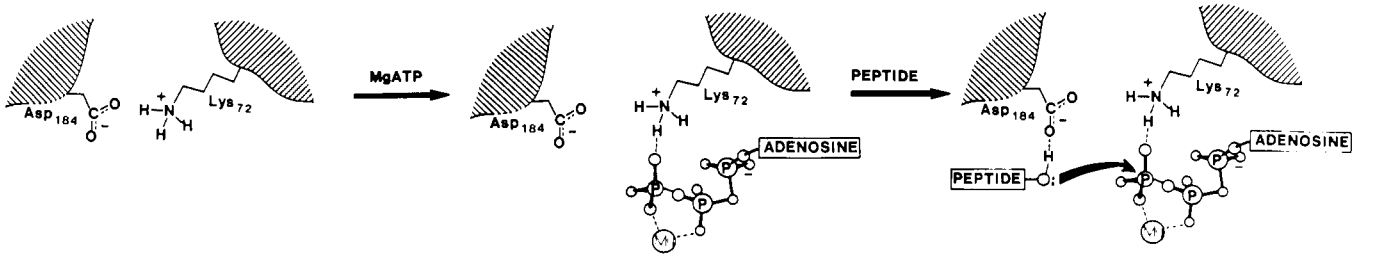

FIGURE 6: Proposed mechanism for the reaction that is catalyzed by the C-subunit.

Neither model can be ruled out until a high-resolution crystal structure is available.

Registry No. DCCD, 538-75-0; L-Lys, 56-87-1; L-Asp, 56-84-8; protein kinase, 9026-43-1.

REFERENCES

Barker, W. C., & Dayhoff, M. O. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2836–2839.

Bhatnagar, D., Hartle, F. T., Roskoski, R., Jr., Lessor, R. A., & Leonard, N. J. (1984) *Biochemistry 23*, 4350–4357.

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

Buechler, J. A., & Taylor, S. S. (1988) *Biochemistry 27*, 7356–7361.

Buechler, J. A., Vedvick, T. S., & Taylor, S. S. (1989) *Biochemistry* (in press).

Chen, W. S., Lazar, C. S., Poenie, M., Tsien, R. Y., Gill, G. N., & Rosenfeld, M. G. (1987) *Nature 328*, 820–823.

Feramisco, J. R., Glass, D. B., & Krebs, E. G. (1980) *J. Biol. Chem. 255*, 4240–4245.

Hanks, S. K., Quinn, A. M., & Hunter, T. (1988) *Science 241*, 42–52.

Hunter, T., & Cooper, J. A. (1986) *Enzymes (3rd Ed.) 17*, 191–246.

Jacobson, M. A., & Colman, R. F. (1984) *J. Biol. Chem. 259*, 1454–1460.

Kamps, M. P., & Sefton, B. M. (1986) *Mol. Cell. Biol. 6*, 751–752.

Kemp, B. E., Graves, D. J., Benjamini, E., & Krebs, E. G. (1977) *J. Biol. Chem. 252*, 4888–4894.

Nelson, N., & Taylor, S. S. (1981) *J. Biol. Chem. 256*, 3743–3750.

Satre, M., Lunardi, J., Pougeois, R., & Vignais, P. V. (1979) *Biochemistry 18*, 3134–3140.

Shoji, S., Ericsson, L. H., Walsh, K. A., Fischer, E. H., & Titani, K. (1983) *Biochemistry 22*, 3702–3709.

Snyder, M., Bishop, J. M., McGrath, J., & Levinson, A. (1985) *Mol. Cell. Biol. 5*, 1772–1779.

Taylor, S. S. (1987) *BioEssays 7*, 24–29.

Taylor, S. S., Bubis, J., Toner-Webb, J., Saraswat, L. D., First, E. A., Buechler, J. A., Knighton, D. R., & Sowadski, J. (1988) *FASEB J. 2*, 2677–2685.

Toner-Webb, J., & Taylor, S. S. (1987) *Biochemistry 26*, 7371–7378.

Walsh, D. A., Perkins, J. P., & Krebs, E. G. (1968) *J. Biol. Chem. 243*, 3763–3765.

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

Yoon, M. Y., & Cook, R. F. (1987) *Biochemistry 26*, 4118–4125.

Yoshida, M., Poser, J. W., Allison, W. S., & Esch, F. S. (1981) *J. Biol. Chem. 256*, 148–153.

Zoller, M. J., & Taylor, S. S. (1979) *J. Biol. Chem. 254*, 8363–8368.

Zoller, M. J., Nelson, N. C., & Taylor, S. S. (1981) *J. Biol. Chem. 256*, 10837–10842.