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Identification of Aspartate-184 as an Essential Residue in the Catalytic Subunit of cAMP-Dependent Protein Kinase[†]

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ABSTRACT: The hydrophobic carbodiimide dicyclohexylcarbodiimide (DCCD) was previously shown to be an irreversible inhibitor of the catalytic subunit of cAMP-dependent protein kinase, and MgATP protected against inactivation [Toner-Webb, J., & Taylor, S. S. (1987) *Biochemistry* 26, 7371]. This inhibition by DCCD indicated that an essential carboxyl group was present at the active site of the enzyme even though identification of that carboxyl group was not possible. This presumably was because a nucleophile on the protein cross-linked to the electrophilic intermediate formed when the carbodiimide reacted with the carboxyl group. To circumvent this problem, the catalytic subunit first was treated with acetic anhydride to block accessible lysine residues, thus preventing intramolecular cross-linking. The DCCD reaction then was carried out in the presence of [¹⁴C]glycine ethyl ester in order to trap any electrophilic intermediates that were generated by DCCD. The modified protein was treated with trypsin, and the resulting peptides were separated by HPLC. Two major radioactive peptides were isolated as well as one minor peptide. MgATP protected all three peptides from covalent modification. The two major peaks contained the same modified carboxyl group, which corresponded to Asp-184. The minor peak contained a modified glutamic acid, Glu-91. Both of these acidic residues are conserved in all protein kinases, which is consistent with their playing essential roles. The positions of Asp-184 and Glu-91 have been correlated with the overall domain structure of the molecule. Asp-184 may participate as a general base catalyst at the active site. A third carboxyl group, Glu-230, also was identified. This carboxyl group was protected by an inhibitor peptide and MgATP, but not by MgATP alone.

Protein kinases represent a very large and diverse family of enzymes (Krebs, 1985). In spite of this diversity, these enzymes share a homologous catalytic core, implying not only that they have evolved from a common precursor but also that they share many conserved features of secondary and tertiary structure (Hunter, 1987; Taylor et al., 1988). The catalytic (C) subunit of cAMP-dependent protein kinase represents one of the simplest of the protein kinases primarily because the major regulatory elements are part of a distinct regulatory subunit, which dissociates in the presence of the activating ligand cAMP. The C subunit also is one of the best understood protein kinases biochemically. For example, many features of the active site have been defined for the C subunit. Lysine-72 has been shown by affinity labeling to be an essential component of the MgATP binding site (Zoller et al., 1981; Zoller & Taylor, 1979), and Cys-199 has been shown to be in close proximity to the β - and/or γ -phosphates of ATP

(Bhatnagar et al., 1984). Cys-199 also was targeted by affinity labeling with a substrate analogue (Bramson et al., 1982).

In addition to affinity labeling, group-specific labeling has proven to be a successful approach for identifying specific functional residues. In the case of the C subunit, there are several potential roles that might be played by carboxyl groups. For example, several lines of evidence suggest that the mechanism utilized by the C subunit involves a general base catalyst (Yoon & Cook, 1987; Bramson et al., 1984). In addition, carboxyl groups are frequently associated with metal binding sites in other kinases that act on small molecules. Thus, we chose to explore the effects of the hydrophobic carbodiimide dicyclohexylcarbodiimide (DCCD) on the C subunit. The particular intention for choosing a hydrophobic reagent was to identify carboxyl groups that may lie in close proximity to a hydrophobic environment such as the ATP binding site.

DCCD previously was found to be a very good inhibitor of the C subunit. Although a unique peptide was consistently modified as a consequence of treatment with DCCD, it was not possible to identify a specific carboxyl group that was modified by DCCD. The conclusion from those initial studies

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was that the reactive carboxyl group was covalently cross-linking to a nearby nucleophile on the protein (Toner-Webb & Taylor, 1987).

In an effort to circumvent this intrasubunit cross-linking and to identify the specific carboxyl groups that were reacting with DCCD, the C subunit was modified by acetic anhydride prior to treatment with DCCD. This enabled us to identify a major carboxyl group as well as a less reactive carboxyl group, which were covalently modified by DCCD and were specifically protected from modification by MgATP. A third carboxyl group was identified that was protected by MgATP and an inhibitor peptide, but not by MgATP alone. The location of these residues in the overall sequence as well as their possible roles in catalysis is discussed.

EXPERIMENTAL PROCEDURES

Materials. Reagents were obtained from the following sources: [^{14}C]glycine ethyl ester (50 mCi/mmol), New England Nuclear; acetic anhydride and acetonitrile (HPLC grade), Fisher Scientific; 2-(*N*-morpholino)ethanesulfonic acid (MES), tris(hydroxymethyl)aminomethane (Tris), and glycine ethyl ester, United States Biochemical Corp.; dicyclohexylcarbodiimide (DCCD), Aldrich; adenosine 5'-triphosphate (ATP), L-1-(tosylamino)-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin, and α -chymotrypsin, Sigma; trifluoroacetic acid (TFA, HPLC grade), Pierce; cytosine, Westchem.

Source of Proteins and Peptides. The C subunit was prepared from porcine heart as described previously (Nelson & Taylor, 1983). The inhibitor peptide TTYADFIAS-GRTGRRNAIHD, which represents a fragment of the heat-stable inhibitor protein (Cheng et al., 1986), was synthesized on an Applied Biosystems Model 430A peptide synthesizer.

Modification of Lysine Groups. Reactions were carried out in 0.1 M Tris-HCl (pH 8.0) and 5% glycerol. C subunit (26 μM) was incubated on ice for 30 min with 12 mM MgCl_2 . Acetic anhydride (100 mM in acetonitrile) was added to a final concentration of 3 mM. The reaction was quenched after 2 min by the addition of β -mercaptoethanol to a final concentration of 30 mM.

Modification of Carboxyl Groups. After treatment with acetic anhydride as described above, the C subunit was dialyzed against 50 mM MES (pH 6.2) and 5% glycerol. The C subunit was then incubated on ice for 30 min with additions as indicated. In all cases, [^{14}C]glycine ethyl ester (3.5 mCi/mmol) was added to a concentration of 2 mM. After addition of DCCD (100 mM in acetonitrile) to a final concentration of 0.4 mM, 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/C subunit following dialysis of the modified C 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. 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 \times 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. Peptides were rechromatographed on a Vydac C18 column (0.46 \times 25 cm)

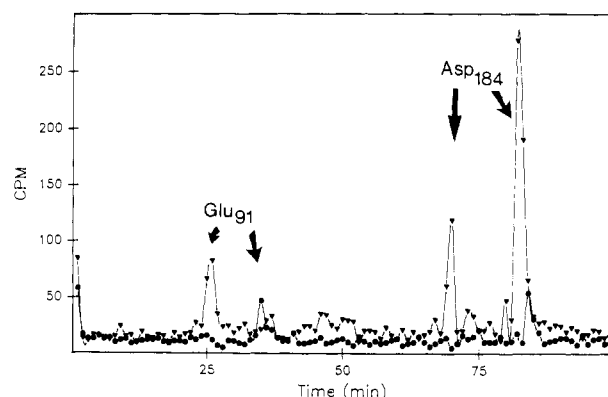


FIGURE 1: Effect of modifying lysine residues on the DCCD-mediated incorporation of [^{14}C]glycine ethyl ester into the C subunit. Catalytic subunit was treated with (\blacktriangledown) or without (\bullet) acetic anhydride followed by modification with DCCD as described under Experimental Procedures. After digestion with TPCK-treated trypsin, the resulting peptides were separated by HPLC as described. The tryptic peptides were eluted with a 136-min linear gradient from 6% to 40% CH_3CN followed by a 10-min linear gradient from 40% to 50% CH_3CN . The radioactivity was determined by counting 10% of each 1-mL fraction in Cytosine.

with a gradient of 0.1% TFA (pH 2.1) to CH_3CN . Chymotryptic peptides were separated in the same manner as the tryptic peptides.

Sequencing. Gas-phase sequencing was done with an Applied Biosystems Model 470A protein sequencer. Phenylthiohydantoin (PTH)-amino acids were identified by HPLC with an Applied Biosystems Model 120A analyzer.

RESULTS

DCCD was previously shown to irreversibly inactivate the C subunit, and MgATP was able to protect against inhibition (Toner-Webb & Taylor, 1987). Intramolecular cross-linking, however, presumably prevented the identification of any specific carboxyl groups that were modified by DCCD. In order to hinder potential cross-linking, the C subunit was treated with acetic anhydride to modify reactive lysine residues prior to treatment with DCCD. Incubation of the C subunit with 3 mM acetic anhydride resulted in the rapid loss of 55% of the initial activity in less than 1 min. When MgATP (2 mM ATP/12 mM MgCl_2) was included in the incubation mixture, the inhibition was reduced to 15% after 5 min. Thus, the reaction of C subunit with acetic anhydride, in the absence of MgATP, appeared to modify at least one residue in close proximity to the ATP binding site. The modified C subunit remained soluble and was not denatured under these relatively mild conditions for treatment with acetic anhydride.

Following treatment with acetic anhydride, the modified C subunit was treated with DCCD in the presence of [^{14}C]glycine ethyl ester in an effort to radiolabel any reactive carboxyl groups. A comparison of the amount of [^{14}C]glycine ethyl ester incorporated into the tryptic peptides of the C subunit with and without first modifying lysines with acetic anhydride is shown in Figure 1. The profile of the tryptic peptides showed three main peaks of radioactivity when the lysines were modified with acetic anhydride prior to treatment with DCCD, and two much smaller peaks when the lysines were not modified prior to DCCD treatment. The three peaks of radioactivity from Figure 1 were purified by rechromatographing with different elution gradients (Figure 2), and the purified peptides were sequenced.

The major peak of radioactivity (fractions 82–84) corresponded to residues 166–189, and the site of modification was identified as Asp-184 (Figure 2, lower panel). A unique

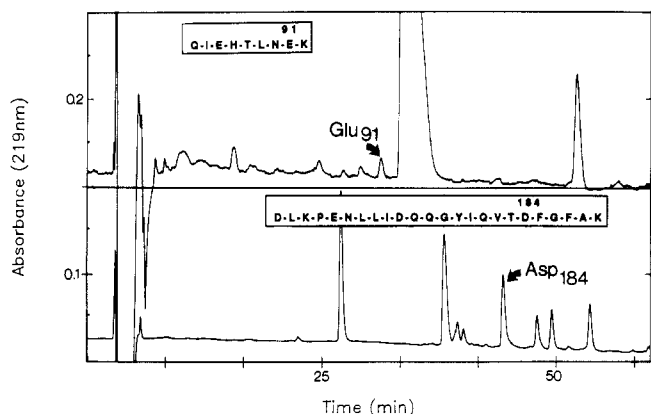


FIGURE 2: Purification of tryptic peptides labeled with [^{14}C]glycine ethyl ester. The radioactive peaks shown in Figure 1 were rechromatographed as described under Experimental Procedures. (Top) The Glu-91 peptide was eluted with a 60-min linear gradient from 9% to 14% CH_3CN . (Bottom) The Asp-184 peptide (fractions 82–84) was eluted with a 60-min linear gradient from 20% to 33% CH_3CN . The radioactive peptide was determined in each case by counting 5% of each 1-mL sample in Cytosint.

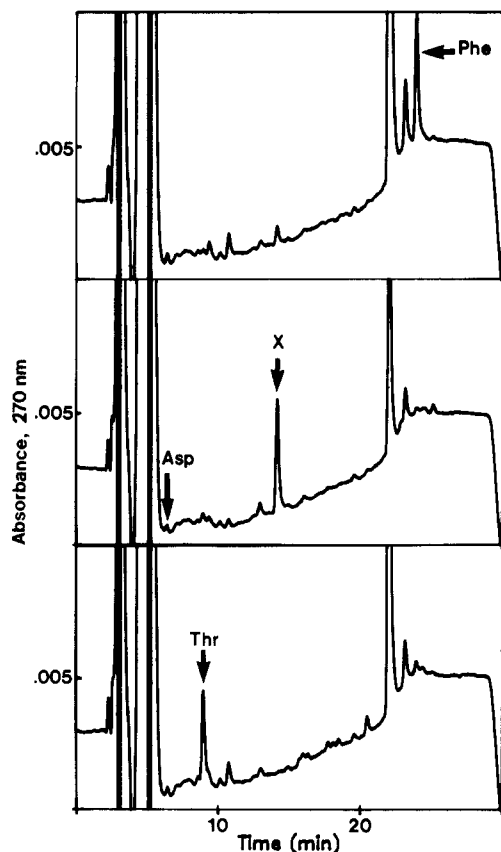


FIGURE 3: Identification of PTH-amino acids. The peptide from the bottom panel of Figure 2 was sequenced, and the PTH-amino acids were identified by HPLC as described under Experimental Procedures. The residues immediately before and after Asp-184, Thr-183, and Phe-185 are indicated as well as the typical elution position of PTH-aspartic acid. The modified residue corresponding to Asp-184, denoted by an X, clearly eluted as a unique PTH-amino acid.

PTH-amino acid was seen at step 19 of the sequence, which corresponded to the modified aspartic acid (Figure 3). In addition, the majority of the radioactivity eluted at step 19. The peak of radioactivity comprising fractions 67–70 also corresponded to residues 166–189, and the site of modification was again identified as Asp-184.

The third peak of radioactivity shown in Figure 1 corresponded to fractions 29–31. When this peak was pooled and

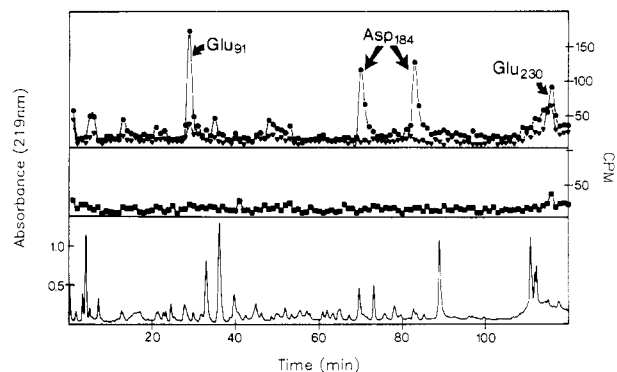


FIGURE 4: Effect of substrates on the incorporation of [^{14}C]glycine ethyl ester into the C subunit. Acetic anhydride treated C subunit was incubated with the following substrates: (a) none (\bullet); (b) 10 mM ATP/20 mM MgCl_2 (\blacktriangledown); (c) 10 mM ATP, 20 mM MgCl_2 , and 35 μM inhibitor peptide (\blacksquare). After modification with DCCD, the C subunit was digested with TPKC-treated trypsin, and the resulting peptides were separated by HPLC as described under Experimental Procedures. The tryptic peptides were eluted with a 100-min gradient from 6% to 31% CH_3CN followed by a 15-min gradient from 31% to 50% CH_3CN (bottom). Radioactivity was determined by counting 10% of each 1.0-mL fraction in Cytosint (top and center).

rechromatographed, as indicated in Figure 2 (upper panel), a single radioactive peak was observed, which when sequenced corresponded to residues 84–92. The site of modification in this peptide was identified as Glu-91 (Figure 2). The unique PTH-amino acid seen at step 8 of the sequence corresponded to the modified glutamic acid (data not shown). In addition, most of the radioactivity eluted at step 8. Although the amount of radiolabel that was incorporated into Asp-184 and Glu-91 varied slightly with each experiment, in general, the covalent modification of Asp-184 was approximately 3 times greater than the modification of Glu-91.

The two peaks of radioactivity from the reaction in which the lysines were not modified also were rechromatographed, and the purified peptides were partially sequenced. The first peak (fraction 35) corresponded to residues 82–92, and the site of modification was presumed to be Glu-91. The second peak (fractions 84 and 85) corresponded to residues 166–189, and the site of modification was presumed to be Asp-184. A definitive assignment of the labeled carboxyl group in each of those peaks was not possible because the quantity of the radiolabeled peptide was not sufficient to sequence the required number of steps. The assignments were made as discussed above because the retention times of the peaks were similar to the radioactive peptides that were isolated from the acetic anhydride treated C subunit, and also on the basis of partial sequencing.

Since the modification of lysine residues enhanced the incorporation of radiolabel into the C subunit, further DCCD reactions were carried out after the C subunit had been treated with acetic anhydride to determine if substrates could protect against the incorporation of [^{14}C]glycine ethyl ester. In order to distinguish which, if any, carboxyl groups were protected by MgATP , the modified C subunit was incubated with varying quantities of MgATP before the DCCD reaction. The protection that was afforded by MgATP is shown in Figure 4 (upper panel). The incorporation of [^{14}C]glycine ethyl ester into Glu-91 and Asp-184 as a function of MgATP concentration is shown in Figure 5. It should be noted that the modification of lysine residues by acetic anhydride decreased the affinity of the C subunit for MgATP somewhat. However, 10 mM MgATP was sufficient to prevent the modification of both Glu-91 and Asp-184 by DCCD and [^{14}C]glycine ethyl ester.

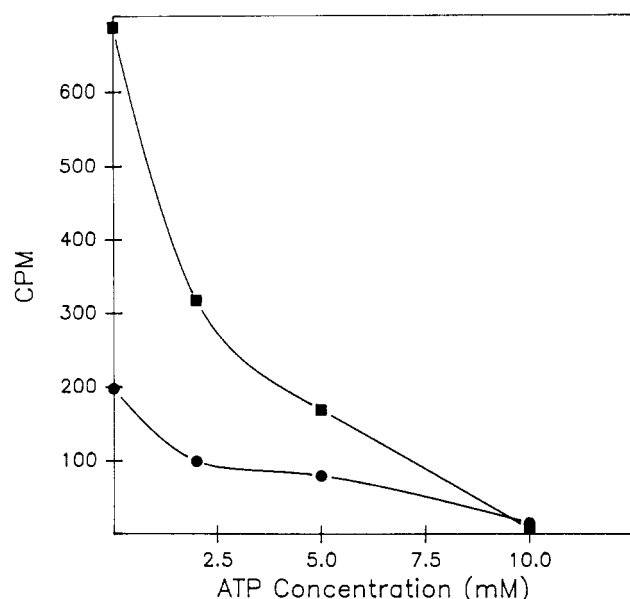


FIGURE 5: Effect of MgATP on the modification of Glu-91 (●) and Asp-184 (■). Following treatment with acetic anhydride, C subunit was incubated with various concentrations of MgATP and then modified by DCCD as described under Experimental Procedures. Three concentrations of MgATP were used: (a) 2 mM ATP/12 mM MgCl_2 ; (b) 5 mM ATP/10 mM MgCl_2 ; (c) 10 mM ATP/20 mM MgCl_2 . The amount of radioactivity was determined by counting 10% of each 1.0-mL fraction in Cytosint.

The elution of the tryptic peptides from the HPLC column for the ATP protection study was changed from the initial gradient. Peptides having a retention time greater than 100 min eluted over a span of 20 fractions instead of being spread out over 50 fractions. Under those conditions, a fourth broad region of radioactivity was observed (Figure 4). The fractions containing this radioactivity were pooled and lyophilized, and the resulting peptides were digested with chymotrypsin. When the chymotryptic peptides were separated by HPLC, two major peaks of radioactivity were seen. One of these peaks corresponded to the chymotryptic peptide containing Asp-184 and most likely resulted from incomplete digestion with trypsin. The other radioactive peptide corresponded to residues 230–247, and the site of modification in this peptide was identified as Glu-230. The PTH-amino acid for the modified Glu-230 eluted at step 1 of the sequence and was identical with the residue that was observed for the modified Glu-91. Radioactivity also eluted at step 1. In the presence of MgATP, no radiolabeled peptide containing Asp-184 was found in this region of the chromatogram. On the other hand, MgATP did not protect Glu-230 from modification by DCCD and [^{14}C]glycine ethyl ester.

An additional reaction was carried out to determine whether or not MgATP and an inhibitor peptide together were capable of blocking the incorporation of radiolabel into Glu-230. Figure 4 (center and bottom panels) shows the HPLC elution profile of the tryptic peptides and the accompanying radioactivity profiles. The inhibitor peptide and MgATP almost completely blocked the labeling of Glu-230.

Mg^{2+} binds weakly to the C subunit, and there is some evidence suggesting that a carboxyl group may be involved as a chelator of a metal. For example, Mg^{2+} was shown to slightly protect the C subunit from inactivation by DCCD (Toner-Webb & Taylor, 1987). DCCD reactions were done in the presence of two concentrations of Mg^{2+} (12 and 20 mM), but the amount of radioactivity incorporated into all three of the modified carboxyl groups did not differ significantly from that of the apoenzyme.

DISCUSSION

DCCD was previously found to inhibit C-subunit activity irreversibly, the rate of inactivation was first order, and MgATP was capable of protecting against inhibition. Nevertheless, despite extensive efforts, it was not possible to radiolabel the C subunit either in a direct manner with [^{14}C]DCCD or by trapping the reactive carboxyl groups with an excess of radiolabeled aniline or glycine ethyl ester. Thus, although a peptide was identified that was altered following inhibition by DCCD, the modified carboxyl group could not be identified (Toner-Webb & Taylor, 1987). It was assumed that a nucleophile on the protein cross-linked to the electrophilic intermediate that formed when DCCD reacted with the carboxyl group, and lysine or cysteine residues were the most probable candidates for such a nucleophile. In order to circumvent this cross-linking, the C subunit was treated with acetic anhydride prior to modification with DCCD. The catalytic activity of the C subunit was inhibited by acetic anhydride, and MgATP protected the C subunit from this inhibition. Therefore, at least one potential nucleophile was being blocked in the active site by acetic anhydride.

Consequently, the DCCD reaction was repeated with C subunit that had been pretreated with acetic anhydride. The reaction was carried out in the presence of an excess of [^{14}C]glycine ethyl ester in order to trap and radiolabel any reactive carboxyl groups. The amount of radiolabel incorporated into the C subunit was increased substantially when lysine residues were modified with acetic anhydride prior to DCCD treatment. However, the same sites of covalent modification also were observed in the unmodified subunit, even though the level of radioactivity that was incorporated was extremely low. The two major carboxylic acid residues that were radiolabeled with [^{14}C]glycine ethyl ester were both protected from modification in the presence of MgATP. Therefore, although pretreatment with acetic anhydride inhibited the C subunit, it did not denature the enzyme since the modified C subunit could still bind MgATP.

The major site of covalent modification by DCCD was identified as Asp-184. This residue is contained within the peptide that was previously shown to be modified by DCCD (Toner-Webb & Taylor, 1987). Because of its hydrophobic properties, DCCD selectively partitions into hydrophobic areas of the protein (Solioz, 1984). In this regard, it is a highly selective reagent that does not react with most of the carboxyl groups in hydrophilic environments. The modification of Asp-184 by DCCD leads to the prediction that this carboxyl group lies near an accessible, hydrophobic part of the molecule. Protection by MgATP makes it likely that this hydrophobic region is in close proximity to the active site. Presumably, MgATP blocks inhibition because it competes for the same hydrophobic pocket as DCCD.

Since Asp-184 is conserved in all protein kinases, it most likely has a conserved role in catalysis. For this reason, in particular, it would not be expected to be involved in the recognition of the protein substrate because the recognition sequence is variable between protein kinases. Two potential roles for Asp-184 are to serve either as a chelator of Mg^{2+} in the MgATP complex or as a general base for the removal of the proton from the hydroxyl group of the substrate.

Several lines of evidence indicate that the C subunit has a requirement for a general base in the catalytic mechanism. The most convincing evidence comes from the pH dependency of the reaction. Yoon and Cook (1987) showed that the $V/K_{\text{ser-pep}}$ had a biphasic pH dependency requiring that one group with a pK_a of 6.2 be in its deprotonated form and that

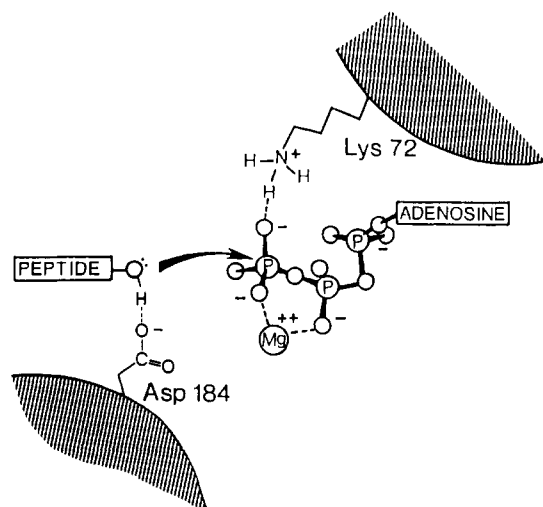


FIGURE 6: Proposed model of the active site of the C subunit. This model, based in part on the data presented here, predicts that Lys-72 will be hydrogen bonded to the γ -phosphate of ATP and Asp-184 will act as a general base toward the hydroxyl group of the substrate.

a second group with a pK_a of 8.5 be in its protonated form. The residue with the pK_a of 6.2 was presumed to be a general base. This was consistent with the earlier pH study of Bramson et al. (1984). Although the pK_a of the β -carboxyl group of aspartic acid is typically about 3.9, Asp-184 is probably located in a hydrophobic environment on the basis of its reactivity with DCCD. This hydrophobic environment would make ionization less favorable by excluding the aqueous solvent. Thus, Asp-184 could easily have an elevated pK_a , similar to other carboxyl groups that are known to be in close proximity to active-site regions. For example, several small molecule kinases, such as yeast hexokinase (Anderson et al., 1978) and bacterial phosphofructokinase (Evans et al., 1981), are predicted to have an Asp at the active site that functions as a general base. In the case of hexokinase, the pH dependency of the reaction has established that the residue participating as a general base has a pK_a of 6.2 (Viola & Cleland, 1978).

The location of Asp-184 in the polypeptide chain also makes it a good candidate for playing an essential role in catalysis. Many kinases show a two-domain structure with one domain constituting a nucleotide binding fold. The adjacent domain frequently contains essential residues for catalysis (Anderson et al., 1979). In the case of the C subunit, the nucleotide binding site has been localized at the amino terminus, and this region is predicted to have a nucleotide-fold motif similar to the one that was predicted by Rossman et al. (1974) for all adenine nucleotide binding proteins (Taylor, 1987; Taylor et al., 1988; Sternberg & Taylor, 1984; Mildvan & Frye, 1987). This model places the peptide containing Asp-184, residues 165–189, near the end of the nucleotide fold, allowing it to serve as a "linker" between the nucleotide binding site and the rest of the molecule. This is followed by another region of the protein that also is thought to be in close proximity to the active site. This is the region flanking Cys-199, which was targeted by a peptide affinity analogue (Bramson et al., 1982). Thus, it is reasonable to predict that Asp-184 functions as a general base in the catalytic mechanism by hydrogen bonding to the proton on the substrate hydroxyl group; consequently, the oxygen would become more nucleophilic and a better attacking group toward the γ -phosphate of ATP (Figure 6).

Asp-184 could alternatively serve as a chelator of Mg^{2+} . Although the arguments to support this are less convincing, the data presented here cannot rule out this possibility. Metal binding studies with the ligand substitution-inert complex

$Co^{3+}(NH_3)_4ATP$ led to the conclusion that the metal binds as an enzyme-ATP-metal complex (Armstrong et al., 1979). This result suggested that residues in the C subunit do not directly chelate the metal in the metal-ATP complex. This conclusion was supported by the observation that $Co^{3+}(NH_3)_4ATP$ serves as a substrate, albeit a poor one, for the C subunit (Granot et al., 1979). These results, however, do not preclude that H_2O , which could replace the NH_3 ligands in the $MgATP$ complex, bridges an enzyme residue to the Mg^{2+} , similar to what has been proposed for adenylate kinase Dreusicke et al., 1988). A protein residue also could contribute to the inhibitory metal binding site (Armstrong et al., 1979).

A second carboxylic acid residue, Glu-91, was labeled by DCCD and protected by $MgATP$. On the basis of its location in the polypeptide chain, Glu-91 is probably an integral part of the nucleotide binding domain, possibly involved in binding the adenosine portion of ATP. Mapping of the ATP binding site with nucleotide analogues has demonstrated that the amino group at position 6 of the adenine ring is important for the tight binding of $MgATP$ (Bhatnagar et al., 1983; Flockhart et al., 1984) and this amino group may be hydrogen bonded to the protein (Bhatnagar et al., 1983). The region of the C subunit near the α -phosphate of ATP also may contain a negatively charged residue (Cook et al., 1982). Glu-91 could contribute at either of these sites.

Although the protein kinases are known to include a large family of diverse enzymes, the high conservation of sequence throughout the catalytic core predicts that they will share many common features of secondary and tertiary structure. It is likely that this conservation also extends to the enzyme mechanism, and thus residues essential for $MgATP$ binding or for catalysis should be conserved in every protein kinase. Glu-91 and Asp-184 are invariant in every protein kinase, further supporting the likelihood that these carboxyl groups play essential roles. Asp-184 and the two following residues, Phe-185 and Gly-186, are also recognized as a highly conserved region in all protein kinases (Hunter, 1987; Taylor, 1987; Taylor et al., 1988).

The third carboxyl group that was radiolabeled, Glu-230, was protected from modification by DCCD with an inhibitor peptide and $MgATP$, but not by $MgATP$ alone. Glu-230 is not likely to be essential for catalysis since $MgATP$, which protected the C subunit against inhibition by DCCD, had no effect on Glu-230. In addition, Glu-230 is the only one of the three modified carboxyl groups that is not conserved in all protein kinases. The inhibitor peptide TTYADFIAS-GRTGRRNAIHD obviously binds to the C subunit in ways that differ from the substrate (LRRASLG) used in the assays for measuring C-subunit activity (Toner-Webb & Taylor, 1987). This is based not only on the difference in size of the two peptides but also on the difference in binding affinities, since the inhibitor peptide has a K_i of 2 nM (Cheng et al., 1986) in contrast to a K_m of 16 μM for the substrate (Kemp et al., 1977). Thus, Glu-230 could interact with a region of the inhibitor peptide that is not common to the substrate.

The proposed locations and functions of Asp-184, Glu-91, and Glu-230 discussed here must eventually be confirmed by X-ray crystallographic analysis of the C subunit, and the function of each hopefully can be verified further by site-directed mutagenesis.

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Structure-Activity Relationship of Synthetic Branched-Chain Distearoylglycerol (Distearin) as Protein Kinase C Activators[†]

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ABSTRACT: Several representative branched-chain analogues of distearin (DS) were synthesized and tested for their abilities to activate protein kinase C (PKC) and to compete for the binding of [³H]phorbol 12,13-dibutyrate (PDBu) to the enzyme. Substitutions of stearoyl moieties at *sn*-1 and *sn*-2 with 8-methylstearate decreased activities on these parameters, relative to those of the parental diacylglycerol DS, a weak PKC activator. Substitutions with 8-butyl, 4-butyl, or 8-phenyl derivatives, on the other hand, increased activities of the resulting analogues to levels comparable to those seen for diolein (DO), a diacylglycerol prototype shown to be a potent PKC activator. Kinetic analysis indicated that 8-methyldistearin (8-MeDS) acted by decreasing, whereas 8-butyldistearin (8-BuDS) and 8-phenyldistearin (8-PhDS) acted by increasing, the affinities of PKC for phosphatidylserine (PS, a phospholipid cofactor) and Ca²⁺ compared to the values seen in the absence or presence of DS. The stimulatory effect of 8-BuDS and 8-PhDS on PKC, as DO, was additive to that of 1,2-(8-butyl)distearoylphosphatidylcholine [1,2(8-Bu)DSPC] and, moreover, they abolished the marked inhibition of the enzyme activity caused by high concentrations of 1,2(8-Bu)DSPC. The present findings demonstrated a structure-activity relationship of the branched-chain DS analogues in the regulation of PKC, perhaps related to their abilities to specifically modify interactions of PKC with PS and/or Ca²⁺ critically involved in enzyme activation/inactivation.

Earlier studies have shown that unsaturated long-chain fatty acids at *sn*-1 and/or *sn*-2 of glycerol are required for diacylglycerol derivatives to be PKC¹ activators (Kishimoto et al., 1980; Mori et al., 1982). For example, diarachidonin (di 20:4), DO (di 18:1), and 1-stearoyl-2-oleoylglycerol (18:0;

18:1) are active but DS (di 18:0) or dipalmitin (di 16:0) are less active. The cell-permeable diacylglycerol, 1-oleoyl-2-acetyl-glycerol, is also active (Fujita et al., 1984), indicating that presence of a short-chain acyl moiety does not impair the

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¹ Abbreviations: PKC, protein kinase C; DO, diolein; DS, distearin; PS, phosphatidylserine; PC, phosphatidylcholine; DSPC, 1,2-distearoylphosphatidylcholine; 1,2(8-Bu)DSPC, *sn*-1,2-(8-butyl)DSPC; PDBu, phorbol 12,13-dibutyrate; TLC, thin-layer chromatograph. The methyl (Me), butyl (Bu), and phenyl (Ph) analogues of branched-chain DS are abbreviated as follows: for example, 8-MeDS and 8-BuDS indicate that stearoyl moieties at *sn*-1 and *sn*-2 in DS were substituted by 8-methylstearic acid and 8-butylstearic acid, respectively (see Figure 1 for structures).