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Inhibition of the Catalytic Subunit of cAMP-Dependent Protein Kinase by Dicyclohexylcarbodiimide†

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ABSTRACT: The hydrophobic carbodiimide dicyclohexylcarbodiimide (DCCD) has been shown to inhibit the catalytic (C) subunit of adenosine cyclic 3′,5′-phosphate dependent protein kinase (EC 2.7.1.3) in a time-dependent, irreversible manner. The rate of inactivation was first order and showed saturation kinetics with an apparent K_i of 60 μM. Magnesium adenosine 5′-triphosphate (MgATP) was capable of protecting against this inhibition, whereas neither a synthetic peptide substrate nor histone afforded protection. Mg alone afforded some protection. When the catalytic subunit was aggregated with the regulatory subunit in the holoenzyme complex, no inhibition was observed. The inhibition was enhanced at low pH, suggesting that a carboxylic acid group was the target for interaction with DCCD. On the basis of the protection studies, it is most likely that this carboxylic acid group is associated with the MgATP binding site, perhaps serving as a ligand for the metal. Efforts to identify the site that was modified by DCCD included (1) modification with [^{14}C]DCCD, (2) modification by DCCD in the presence of [^{3}H]aniline, and (3) modification with DCCD and [^{14}C]glycine ethyl ester. In no case was radioactivity incorporated into the protein, suggesting that the irreversible inhibition was due to an intramolecular cross-link between a reactive carboxylic acid group and a nearby amino group. Differential peptide mapping identified a single peptide that was consistently lost as a consequence of DCCD inhibition. This peptide (residues 166–189) contained four carboxylic acid residues as well as an internal Lys. Two of these carboxyl groups, Asp-166 and Asp-184, are conserved in all protein kinases, including oncogene transforming proteins and growth factor receptors, and thus are likely to play an essential role.

The active form of the catalytic subunit of adenosine cyclic 3′,5′-phosphate (cAMP) dependent protein kinase is a monomeric protein with a molecular weight of 38 500. The enzyme shows an ordered mechanism of binding where the binding of magnesium adenosine 5′-triphosphate (MgATP) precedes peptide binding (Whitehouse et al., 1983). Synthetic peptides have been used to map the requirements for peptide recognition. These requirements include two basic residues, most frequently arginines in physiological substates, which precede the site of phosphorylation (Kemp et al., 1977). The phosphate acceptor is serine or less frequently threonine.

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Several chemical approaches have been used to probe the active site of this enzyme and to identify residues that may participate in nucleotide and substrate binding or in catalysis. Reagents such as iodoacetamide, *N*-ethylmaleimide (Sugden et al., 1976), and 5,5′-dithiobis(2-nitrobenzoic acid) (Peters et al., 1977; Bechtel et al., 1977; Armstrong & Kaiser, 1978), which specifically modify sulfhydryl groups, were all capable of abolishing catalytic activity, and in each case MgATP protected against this inhibition. [^{14}C]Iodoacetic acid (IAA) was used to specify the sites of modification as Cys-199 and Cys-343 (Nelson & Taylor, 1981). Since holoenzyme protected against inactivation by IAA and did not protect Cys-343 from alkylation, the loss of activity that is seen in the free C-subunit can be attributed to the modification of Cys-199 (Nelson & Taylor, 1983). Affinity labeling with a peptide also led to the covalent modification of Cys-199 which confirms its close proximity to the active site (Bramson et al., 1982). Since cyanylation did not lead to complete loss of catalytic activity of the C-subunit (Peters et al., 1977), it is assumed that a cysteine residue most likely is not essential for activity. Affinity labeling with 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (FSBA) has established that Lys-72 is in close proximity to the MgATP binding site. These two residues, Lys-72 and Cys-199, are the only two groups that have been shown to be close to the active site. Reaction of Cys-199 with *o*-phthalaldehyde leads to subsequent cross-linking with a nearby primary amine (Puri et al., 1985); however, the residue involved in this cross-link was not identified (Puri et al., 1985).

Several additional approaches have suggested other amino acids that may contribute to functional sites. For example, ethoxyformic anhydride is also capable of inhibiting the C-subunit, and once again MgATP protected against this inhibition. The specificity of ethoxyformic anhydride suggests that either a tyrosine or a histidine may be at or near the active site (Witt & Roskoski, 1975b). A water-soluble carbodiimide, 3-[3-(dimethylamino)propyl]-1-ethylcarbodiimide, and [^{14}C]glycine ethyl ester were used to inactivate the C-subunit (Matsuo et al., 1980). Since peptide substrate protected against this inactivation and MgATP did not, it was concluded that the site of modification was at the arginine recognition site of the active center. It was hypothesized that two glutamyl carboxy groups may be components of the arginine recognition portion of the substrate binding site.

The protein kinases represent a large family of related proteins that most likely share a common mechanism although peptide specificities vary. Since there are several conserved carboxylic acid residues within this family of protein kinases, it was of interest to establish whether any other acidic residues play a functional role in the C-subunit. Dicyclohexylcarbodiimide (DCCD) is a hydrophobic carbodiimide that will partition into the hydrophobic environments of proteins. Thus, if a protein possesses a hydrophobic region that can be occupied by DCCD and if this region also contains a DCCD-reactive group such as aspartic or glutamic acid, preferential interaction will take place at this site. Since adenine nucleotide binding sites usually contain a hydrophobic "pocket" that frequently constitutes the primary recognition site for the adenine ring (Hol et al., 1978; Wierenga et al., 1979), it was thought that DCCD may selectively target this region. There is precedence for this specificity since DCCD has been shown to selectively target ATP binding sites in other proteins such as the F_1-ATPase (Pougeois et al., 1979; Satre et al., 1979; Solioz, 1984; Yoshida et al., 1981). The focus of the present study was to establish (1) whether DCCD was capable of irreversibly inhibiting the catalytic subunit of cAMP-dependent protein kinase, (2) whether such inhibition could be correlated with the MgATP binding site, and finally (3) whether any specific DCCD-modified peptides could be identified.

Experimental Procedures

Materials. Dicyclohexyl[^{14}C]carbodiimide (50 mCi/mmol) was purchased from Research Products International. Solutions of [^{14}C]DCCD were prepared in absolute ethanol and were diluted with nonradioactive DCCD, purchased from Aldrich, to the desired specific radioactivity. [^{14}C]Glycine ethyl ester (50 mCi/mmol) was purchased from New England Nuclear. [^{3}H]Aniline and initial portions of [^{14}C]DCCD were a generous gift from Dr. W. S. Allison, University of California, San Diego. Nonradioactive glycine ethyl ester and aniline were from Sigma.

Purification of C-Subunit and R-Subunit. The C-subunit and type II R-subunit were prepared from porcine heart as described previously (Nelson & Taylor, 1983). Holoenzyme was formed by dialyzing the R^{II} subunit with a 10% molar excess of C-subunit followed by removal of the excess C-subunit with (carboxymethyl)cellulose (Nelson & Taylor, 1983).

Assays. Three methods were used to assay enzymatic activity: (1) [γ-^{32}P]ATP phosphorylated histones were precipitated with perchloric acid onto Whatman No. 3 filter disks as described previously (Taylor et al., 1976). (2) Phosphorylated histones were directly spotted onto P81 paper and washed with phosphoric acid according to the method of Witt and Roskoski (1975a). In both cases the washed and dried disks were counted in Cytoscint. (3) Enzymatic activity or the integrity of holoenzyme was assayed spectrophotometrically according to the method of Cook et al. (1982) using the synthetic peptide substrate (L-R-R-W-S-L-G) described by Wright et al. (1981) or Kemptide (L-R-R-A-S-L-G).

Modification of Carboxyl Groups of C-Subunit by Dicyclohexylcarbodiimide. Reactions were carried out in 25 mM 2-morpholinoethanesulfonic acid (MES) (pH 6.3), 3 mM mercaptoethanol, and 5% glycerol. The catalytic subunit (5 μM) was incubated with DCCD (0.4 mM) dissolved in ethanol. Less than 3% total volume of ethanol was added to ensure full activity of the catalytic subunit in control experiments carried out at 37 °C. Conditions varying from the above are noted in the respective figure legends.

Reaction of C-Subunit with DCCD and either [^{14}C]Glycine Ethyl Ester or [^{3}H]Aniline. Reactions were carried out as above with the exception that either radiolabeled glycine ethyl ester or aniline was added to the reaction mixture in a 5-fold molar excess over carbodiimide according to the procedure of Matsuo et al. (1980).

Proteolysis. Proteins were incubated at 37 °C with 1:100 w/w L-1-(tosylamido)-2-phenylethyl chloromethyl ketone–trypsin in 25 mM NH_4HCO_3, pH 8.3, for 4 h. Incubation was continued overnight following an additional equal aliquot of trypsin. Tryptic peptides were resolved by high-performance liquid chromatography (HPLC). Samples were lyophilized to 2 mL when volume reduction was necessary.

High-Performance Liquid Chromatography. Reverse-phase HPLC was performed with an Altex 3200 system with a Vydac C_{18} column (4.6 mm i.d. × 25 cm, 5 μm, 300-Å pore size). The gradients employed were (a) from 10 mM sodium phosphate, pH 6.8–6.9, to CH_3CN (Fisher, HPLC grade) or (b) from 0.1% trifluoroacetic acid (TFA), pH 2.1 (Pierce), to CH_3CN. All buffers were filtered and degassed. The majority of peptides were eluted with a 180-min linear gradient from 0 to 60% CH_3CN at a flow rate of 1 mL/min. Absorbance was monitored at 219 nM with a Hitachi spectro-

photometer equipped with a flow-through cell or at 280 nM with a Kratos spectrophotometer with a flow-through cell.

Peptide or Protein Sequencing. Manual sequencing was carried out by using the dansyl-Edman procedure described by Hartley (1971). Gas-phase sequencing was carried out by using an Applied Biosystems Model 470 A protein sequencer. Samples were applied in 25-μL aliquots to a glass fiber filter pretreated with Biobrene. Phenylthiohydantoin amino acids were identified by HPLC, as described by Hunkapillar and Hood (1983), on an IBM cyano column.

SDS–Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out according to the method of Laemmli (1970) utilizing 10% or 12.5% acrylamide in the lower gels. Proteins were stained overnight with 0.25% Coomassie Blue R-250, 25% isopropyl alcohol, and 10% acetic acid and destained in the same solution without the dye. When autoradiography was necessary, the dried gels were exposed on Kodak X-Omat film.

Protein Determination. Protein was determined according to the method of Bradford (1976) or by the method of Lowry et al. (1951).

Amino Acid Analysis. The lyophilized peptides were hydrolyzed in vacuo at 110 °C in 6 N HCl for 20 h. Analyses were performed on an LKB 4400 automatic amino acid analyzer using a single column system.

Radioactivity. Radioactivity was measured by counting aliquots in Cytoscint (up to 0.2-mL sample) or in Betaphase (up to 0.5-mL sample). Radioactive bands from gels were excised and counted both dry (Cerenkov, 1934) and after Cytoscint was added.

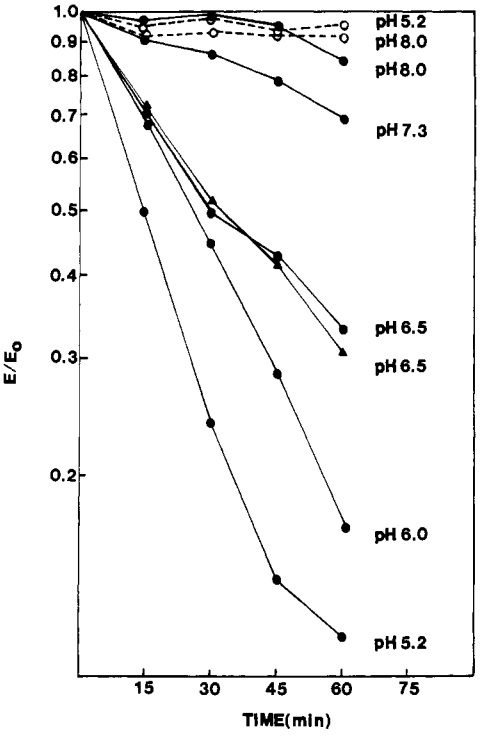

FIGURE 1: pH dependency of C-subunit inactivation by DCCD. Each sample contained 0.8 μM C-subunit and 1.2 mM DCCD in 50 mM buffers at the pHs indicated. MES was used for pH 5.2 and 6.5 buffers. Potassium phosphate was used for pH 6.5–8.0 buffers. Closed symbols indicate incubation with DCCD. Open symbols indicate control incubations with ethanol at the extreme pHs.

RESULTS

Derivatization of protein carboxyl groups by carbodiimides is very efficient at moderately acidic pHs in the region 4–5, but still proceeds at a reasonable rate in the pH range 6.0–7.0. Incubation of C-subunit with DCCD at 37 °C resulted in linear, time-dependent inactivation. The pH dependency of this inactivation is indicated in Figure 1. Since the optimum pH range for activity of the catalytic subunit is rather broad and does not fluctuate much between pH 6 and 8, pH 6.0 was selected for the optimum derivatization of carboxyl groups. The rate of inactivation was first order and obeyed saturation kinetics according to

$$E + I \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} EI \xrightarrow{k_2} E_i \qquad (1)$$

where E = enzyme, I = DCCD, EI = complex of enzyme and DCCD, and E_i = irreversibly inactivated enzyme. The observed rate of inhibition can, therefore, be expressed according to

$$k_{obsd} = k_2/(K_i/[I] + 1) \qquad (2)$$

where $K_i = (k_{-1} + k_2)/k_1$. When the reciprocal of $1/k_{obsd}$ was plotted vs 1/DCCD, the K_i for the inhibition was calculated to be 60 μM, and the rate constant, k_2, for inhibition at saturation was 1.8×10^{-2} min^{-1}.

A potential side reaction during modification of the carboxyl groups of proteins by carbodiimides is covalent cross-linking between adjacent subunits or between aggregated subunits (Carraway et al., 1972). To establish whether such intersubunit cross-linking had occurred, C-subunit modified by DCCD was subjected to SDS–polyacrylamide gel electrophoresis. Since the modified sample migrated as a single band (M_r ~38 500) that was indistinguishable from the control, untreated sample, there was no evidence that intersubunit cross-linking had occurred. Protein determinations established that the C-subunit was not precipitated following inactivation by DCCD. The modification of the C-subunit by DCCD was irreversible, since extensive dialysis against 4 L of 50 mM MES (pH 6.0) containing 2 mM ethylenediaminetetraacetic acid (EDTA) and 5 mM 2-mercaptoethanol for 24 h at 4 °C did not restore any activity.

Effects of MgATP, Peptide Substrate, and Regulatory Subunit on the Rate of Inactivation of the C-Subunit by DCCD. As can be seen in Figure 2, the inactivation of C-subunit by DCCD was completely protected against by preincubation with MgATP. The addition of ATP alone offered little protection (Figure 2, left panel), and Mg^{2+} alone gave only slight protection at higher concentrations (Figure 2, right panel). The fact that MgATP protected better than either Mg^{2+} or ATP alone suggested that DCCD may be specifically interacting at the MgATP binding portion of the active site. The ability of DCCD to inhibit catalytic activity when the C-subunit was part of the holoenzyme complex also was investigated. Holoenzyme was formed by reassociation of porcine heart R^{II} with C-subunit as described under Experimental Procedures. Intact holoenzyme retained full activity in the presence of DCCD, whereas when the holoenzyme was dissociated by cAMP prior to reaction with DCCD, inactivation proceeded at a rate comparable to that of the free C-subunit (Figure 3, left). Neither histone nor synthetic peptide substrate was capable of protecting the C-subunit from inactivation by DCCD (Figure 3, right).

Identification of Modified Tryptic Peptides. Radiolabeled [^{14}C]DCCD (10 Ci/mol) was used to inactivate the C-subunit to determine if a stable *N*-acylisourea cross-link could be generated. Although the inactivation proceeded normally and was complete in 90 min, no incorporation of ^{14}C into the C-subunit was detected after the protein was subjected to polyacrylamide gel electrophoresis. Likewise, no radioactivity was retained following dialysis of the [^{14}C]DCCD-treated

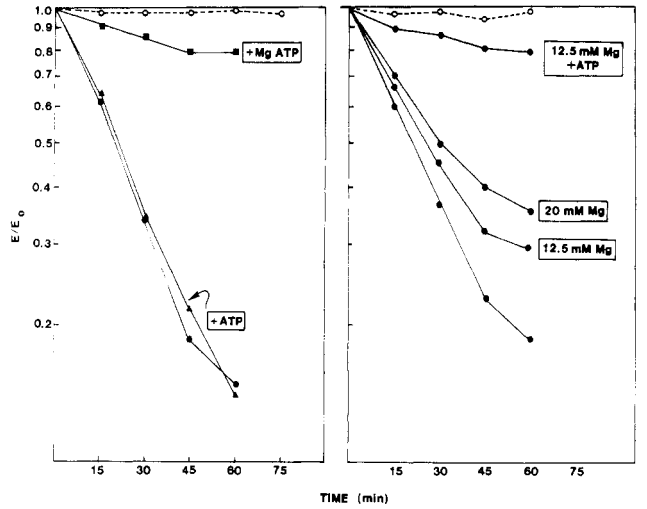

FIGURE 2: Effect of Mg^{2+}, ATP, and MgATP on the inactivation of C-subunit by DCCD. C-subunit (0.82 μM) was reacted with 0.87 mM DCCD in MES buffer (pH 6.0). (Left panel) C-Subunit was preincubated with 2 mM ATP (▲) or with both 2 mM ATP and 12.5 mM Mg^{2+} (■) prior to the addition of DCCD. (Righ panel) C-Subunit was preincubated with the concentrations of Mg^{2+} indicated; ATP indicates 2 mM ATP was added to the preincubation. For both panels the open circles represent C-subunit incubated with ethanol alone and unmarked closed circles represent C-subunit incubated with DCCD alone.

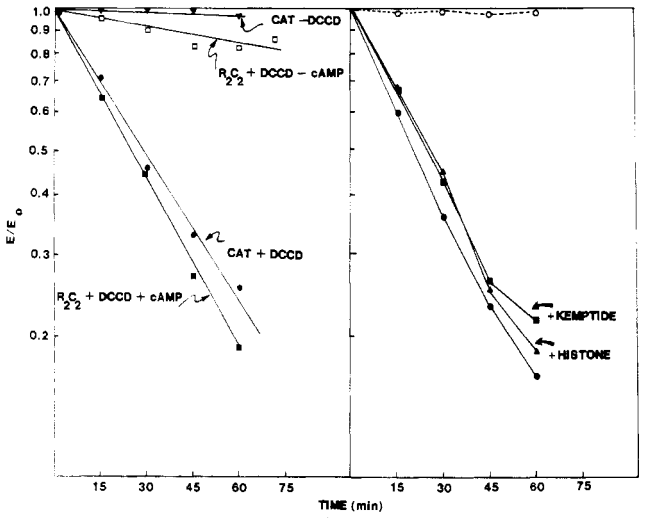

FIGURE 3: Effect of regulatory subunit, histone, and Kemptide on the inactivation of C-subunit by DCCD. (Left panel) C-Subunit alone (2.0 μM) or as part of a holoenzyme complex (1.0 μM) was reacted with 1.0 mM DCCD in the presence or absence of 30 μM cAMP. Reactions were initiated by the addition of DCCD after preincubation for 5 min at 37 °C. (Right panel) C-Subunit (0.82 μM) was incubated at pH 6.0 with 1.5 mM DCCD in 50 mM MES containing 5 mM Mg^{2+}. Preincubations with 0.4 mM histone or Kemptide preceded the addition of DCCD where indicated. Control reactions of C-subunit with or without DCCD are indicated by closed and open circles, respectively.

protein. Finally, no radiolabeled peptides could be identified following HPLC resolution of the tryptic peptides from [^{14}C]DCCD-treated C-subunit.

Since these results indicated that a stable rearrangement product was not being formed, attempts were made to react an *O*-acylisourea intermediate with a radiolabeled nucleophile. Two nucleophiles were used: [^{3}H]aniline or [^{14}C]glycine ethyl ester. Either nucleophile was incubated with DCCD and C-subunit under typical reaction conditions. In both cases, inactivation proceeded normally, but no radioactivity was incorporated into the C-subunit as shown by gel electrophoresis and HPLC of tryptic peptides. These combined results implied

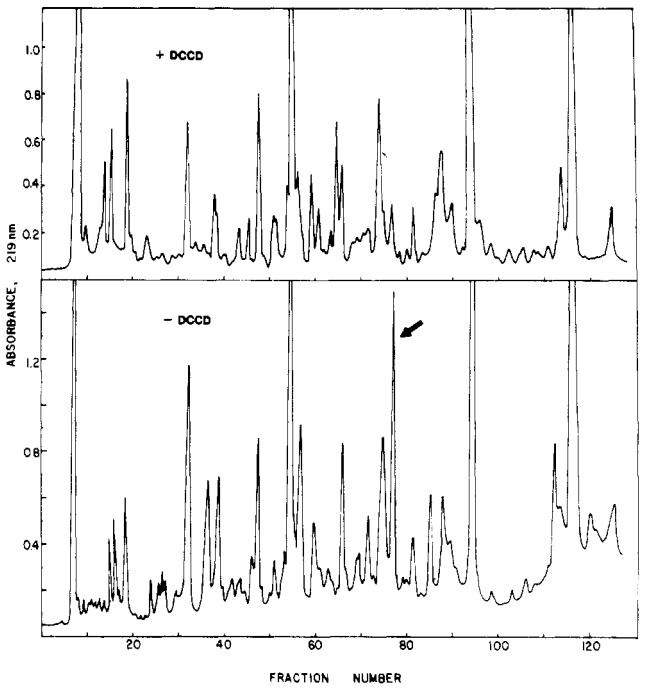

FIGURE 4: High-performance liquid chromatography separation of the tryptic peptides from C-subunit with and without treatment with DCCD. C-Subunit (3 mg) was incubated in 50 mM MES with or without 1.0 mM DCCD for 90 min at 37 °C. The samples were dialyzed against 50 mM NH_4HCO_3 (pH 8.3), digested with trypsin, and chromatographed as discussed under Experimental Procedures. The gradient used was from 10 mM sodium phosphate (pH 6.9) to 60% CH_3CN in 180 min. Absorbance was monitored at 219 nm. (Top) HPLC of C-subunit treated with DCCD. (Bottom) HPLC of untreated C-subunit. Arrow indicates the peptide that is missing following inhibition by DCCD.

that an intramolecular bond was being formed between a reactive carboxylic acid group and a nearby nucleophile. The emphasis therefore shifted to characterization of total tryptic peptides in an effort to identify a cross-linked peptide by comparing differences in HPLC profiles of tryptic peptides from native and DCCD-treated C-subunit.

The tryptic peptides of C-subunit modified with DCCD were chromatographed by HPLC using a sodium phosphate/acetonitrile gradient as described under Experimental Procedures and Figure 4. Two control reactions were carried out: (1) C-subunit incubated with ethanol alone prior to digestion and (2) C-subunit incubated with DCCD in the presence of MgATP. Tryptic peptides from all three samples were resolved by HPLC and compared. Numerous profiles of tryptic peptides from DCCD-inhibited C-subunit were compared to those of control digests, and these consistently showed one difference: the profiles of tryptic peptides from DCCD-inactivated C-subunit were always missing the single peak indicated in the lower panel of Figure 4. The peptide that was contained in this peak in the control samples was sequenced by gas-phase methods and corresponds to residues 166–189 in the linear sequence. This peptide contains four carboxyl groups, and any one of these groups could be targets for initial modification by DCCD. This peptide was readily identified from both untreated C-subunit and C-subunit that was treated with DCCD in the presence of MgATP.

An exhaustive search was made to locate the modified peptide. This search included (1) N-terminal determinations of all major tryptic peptides to find and sequence any new peptide resulting from the modification of the peptide shown in Figure 4; (2) scanning the OD 219-nm and OD 280-nm profiles to identify any differences in absorbance and se-

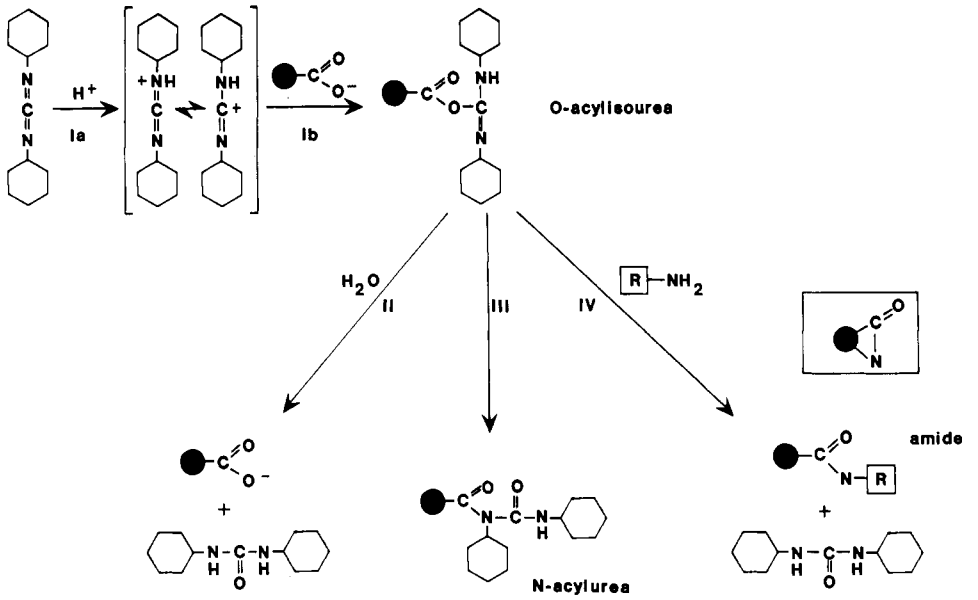

FIGURE 5: Possible reaction pathways for the interaction of DCCD with a carboxyl group on the C-subunit. The protein molecule is indicated by the solid black circle; R indicates the side chain of aniline or glycine ethyl ester.

quencing the appropriate peptides; and (3) sequencing the major absorbing peptides that might be capable of masking the missing peptide. In addition, since samples were centrifuged before loading onto the HPLC column, the possibility that an insoluble fraction might contain this modified peptide was investigated. Following formic acid treatment to solubilize any remaining peptides in either the control or the DCCD-treated C-subunit, these fractions were immediately loaded onto the gas-phase sequencer. The major peptide in the insoluble fraction was found by sequencing to be the hydrophobic tryptic peptide (Phe-145–Arg-165), immediately prior to the peptide of interest (Asp-166–Lys-189). The possibility that these peptides were not cleaved as a consequence of cross-linking was eliminated by carrying out the sequencing of the hydrophobic peptide for 30 residues. The sequence stopped abruptly after Arg-165, indicating that cleavage between Arg-165 and Asp-166 had occurred. Alternatively, the tryptic peptides from control and DCCD-treated C-subunit were separated by HPLC using a pH 2.1 TFA to acetonitrile gradient and differences were investigated. No modified peptide was identified by any of these methods.

Discussion

Dicyclohexylcarbodiimide inactivated the C-subunit of cAMP-dependent protein kinase in an irreversible manner. The inhibition was first order, showed saturation kinetics, and had a K_i of 60 μM. Incubation with a peptide substrate provided no protection, and protein substrates such as histone also were not capable of protecting against DCCD inhibition. On the other hand, MgATP was capable of protecting against this inactivation. These results are consistent with the hydrophobic DCCD specifically binding to and subsequently modifying a reactive group that is in close proximity to the MgATP binding site. Since free Mg^{2+} did provide some protection, whereas ATP alone did not, it is possible that the modification targeted the Mg^{2+} binding portion of the nucleotide binding site and that the reactive group may serve as a ligand for the metal.

DCCD theoretically could react with Asp, Glu, Cys, and Tyr (Carraway & Koshland, 1972); however, only carboxyl residues have been found to be implicated in specific DCCD–protein interactions (Solioz, 1984). If DCCD interacts with a carboxyl residue, a stable *N*-acylurea adduct may form by a rearrangement reaction as designated in Figure 5 (I and II). Radiolabeled DCCD (*N*,*N*′-dicyclohexyl[^{14}C]carbodiimide) can be used to quantitate this potential reaction pathway. Alternatively, the initial *O*-acylisourea that is formed may interact with a nucleophile (Figure 5, I and IV). Under these conditions inhibition that is mediated by [^{14}C]DCCD will result in no radiolabeling of the protein. The protein also remains unmodified if the nucleophile is water (Figure 5, I and II); however, in this instance inhibition should be reversible. If, however, the nucleophile is a nearby amino group of an amino acid side chain, an inter- or intramolecular "zero-length" cross-link may be formed (Figure 5, box) (Herz & Packer, 1981; Pennington & Fisher, 1981). In cases where the *O*-acylisourea is reacting with a solvent nucleophile, it may be possible to covalently trap this intermediate by the addition of a radiolabeled nucleophile such as [^{3}H]aniline or [^{14}C]-glycine ethyl ester (Matsuo et al., 1980) and in this way quantitate the extent of interaction of the carbodiimide with the protein.

The inactivation of C-subunit proceeded more rapidly as the pH decreased from 8.0 to 5.0. The increased rate of inactivation occurring at a lower pH is due probably to increased concentration of the protonated carbodiimide, since this is the species of carbodiimide that reacts preferentially with a nucleophilic group (Smith et al., 1958). Also, as the pH is lowered from 8 to 5, it is clear that the best available choice of a nucleophile is a carboxyl group. Therefore, it is most likely that the reactive group being modified in the C-subunit is a carboxylic acid.

All efforts to trap a radiolabeled derivative in the DCCD-inactivated subunit were unsuccessful. Since the inhibition was irreversible, it was concluded that inhibition of C-subunit by DCCD was due to the formation of an intramolecular cross-link between a reactive carboxylic acid group and a nearby primary amine. Attempts were made to identify this peptide by comparative peptide mapping. Such mapping demonstrated that a single tryptic peptide selectively disappeared in conjunction with inactivation by DCCD. The tryptic peptide that disappeared as a consequence of DCCD treatment corresponds to residues 166–189 in the linear sequence and is shown in Figure 6. This peptide contains four carboxyl groups, which could each be candidates for interaction with DCCD.

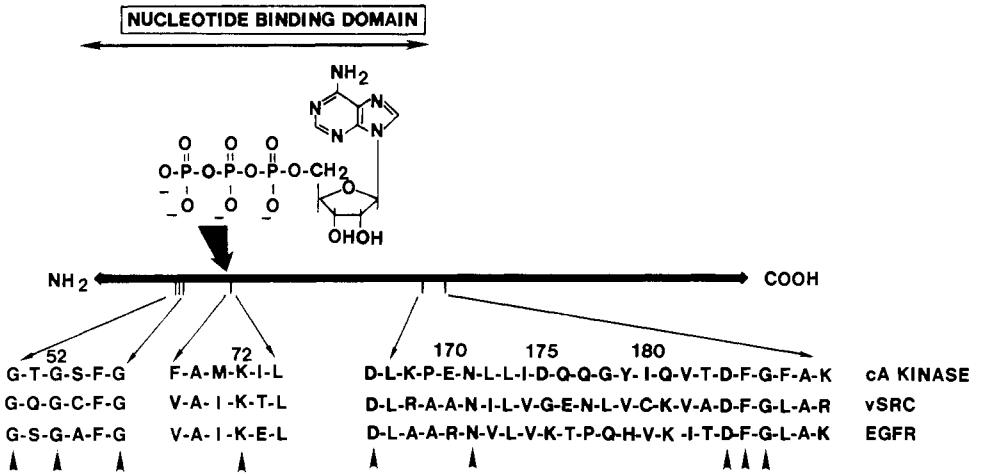

FIGURE 6: Sequences of conserved regions of various kinases including the potential DCCD-modified peptide. Sequences for the C-subunit (cA kinase), the Rous sarcoma virus transforming protein (vSRC), and the epidermal growth factor receptor (EGFR) are included as representative kinases. The conserved regions include the triad of glycines (50, 52, and 55 in the C-subunit), which are a conserved feature of most adenine nucleotide binding sites, Lys-72, which is covalently modified by FSBA, and the tryptic peptide (166–189), which is lost as a consequence of inactivation by DCCD. Residues that are invariant in all protein kinases are indicated by arrows.

There are several potential roles that carboxylate groups may play in the mechanism of action of the C-subunit. These roles include (1) interaction with the Arg-Arg portion of the peptide substrate, (2) actual involvement in catalysis through interaction at the Ser-OH group of the substrate, and (3) participation in binding MgATP either by serving as a ligand for the Mg^{2+} or by interacting with the N^6-position on the adenine ring. Evidence that carboxyl groups are located in the peptide recognition site was obtained by Matsuo et al. (1980), who used a water-soluble carbodiimide to inactivate the C-subunit. Those carboxyl groups were distinct from the group(s) modified by DCCD, since in the latter case the substrates did not protect the C-subunit from inactivation.

Precedence for carboxyl groups participating in the catalytic mechanism comes from an analysis of some of the kinases that act on small molecules. For example, comparisons of sequence data with the crystal structures of hexokinase, adenylate kinase, and phosphofructokinase all indicate that an aspartate is involved in hydrogen bonding to the substrate phosphoryl acceptor, and the hypothesis is that this aspartate probably functions as a general base catalyst (Anderson et al., 1978; Schulz et al., 1982; Evans & Hudson, 1979). The fact that DCCD inactivation of the C-subunit was not prevented by incubation of the C-subunit with substrate implies that the group which was attacked by DCCD may not participate directly in catalysis, although this possibility cannot be ruled out.

The third role that carboxyl groups may play in the active site is that of nucleotide binding. There is no evidence as yet that a carboxyl group interacts with the 6-NH_2 position of the adenine ring in nucleotide binding proteins. From ATP analogue studies it is clear that the 6-amino group is essential for high-affinity binding of ATP to the cAMP and cGMP kinases (Hoppe et al., 1977; Hoppe & Wagner, 1979; Flockhart et al., 1984). There is, however, disagreement as to whether the NH_2 group is more likely to be involved as a hydrogen donor (Hoppe et al., 1977) or as a hydrogen acceptor (Flockhart et al., 1984). Evidence that a carboxyl group is involved in binding MgATP to phosphofructokinase came from analysis of the crystal structure of that enzyme, which has an aspartic acid residue positioned near the Mg^{2+} atom of bound MgATP (Evans & Hudson, 1979). Furthermore, a structure analysis of elongation factor Tu (EF-Tu) from *Escherichia coli* indicated that guanosine diphosphate (GDP) in the EF-Tu–GDP crystal was linked to the protein via a Mg^{2+} ion, which forms a salt bridge with an Asp side chain (Jurnak, 1985). As discussed earlier, the inhibitory effect of DCCD on the C-subunit was protected against by MgATP and partially blocked by Mg^{2+} alone. From the above arguments, it is likely that a carboxylate group of the C-subunit is stabilizing the Mg^{2+} portion of the nucleotide–metal complex. The NMR data of Granot et al. (1979) indicate that two metals bind at the active site and that the second inhibitory site bridges the phosphoryl groups of ATP to the protein.

The protein kinases represent a large family of related proteins that clearly have evolved from a common precursor. This family includes other serine-specific kinases such as phosphorylase kinase (Reimann et al., 1984), myosin light chain kinase (Takio et al., 1985), and protein kinase C (Parker et al., 1986). It also includes tyrosine-specific kinases such as the transforming protein from Rous sarcoma virus, $pp60^{v\text{-}src}$ (Barket & Dayhoff, 1982), and growth factor receptors such as the epidermal growth factor (Ullrich et al., 1984). All of these kinases are homologous proteins that share a conserved catalytic core which corresponds to the catalytic subunit of cAMP-dependent protein kinase. Lys-72, which has been shown to be essential for the activity of the C-subunit and for $pp60^{v\text{-}src}$ (Zoller & Taylor, 1979; Kamps et al., 1984; Kamps & Sefton, 1986), is invariant in all of these protein kinases. Likewise, it is anticipated that other residues which participate in MgATP binding and in catalysis will be conserved throughout the protein kinase family. Those residues that determine peptide specificity obviously will differ.

Selected regions that show a high degree of homology between various members of the kinase family also are indicated in Figure 6. The series of three glycine residues corresponding to Gly-50, Gly-52, and Gly-55 in the C-subunit are a conserved feature of most adenine nucleotide binding proteins (Hol et al., 1978; Wierenga et al., 1986). These constitute part of a secondary structure known as a "nucleotide fold", which Rossmann et al. (1974) first recognized from a comparison of the crystal structures of lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase. They hypothesized that this structure, which consists of six parallel β-sheets between two α-helices above and below the plane of the β-sheets, would be conserved in all adenine nucleotide binding proteins, and this prediction has proved to be remarkably accurate as more crystal structures of adenine nucleotide binding proteins

are solved. Although generally it is only the secondary structure that is conserved in adenine nucleotide binding sites, the conserved glycine residues are an exception, and the prevailing theory based on crystal structure and computer modeling is that they represent a turn between a β-sheet and an α-helix which comes close to the nucleotide (Rossmann et al., 1974; Wierenga et al., 1986). In general, this nucleotide fold includes approximately 150 residues. Several lines of evidence for the C-subunit place the nucleotide binding portion in the first half of the molecule, which is consistent with this general trend. The invariant Lys-72, also indicated in Figure 6, lies within this region as well.

Given the homology that persists throughout the catalytic domain, it is likely that a common mechanism is conserved in all of the protein kinases. Therefore, if certain carboxylic acid groups are essential for kinase activity, they should be invariant. A comparison of sequences indicates that there are four carboxylic acid groups which are invariant in the protein kinase family. Two of these invariant carboxyl groups, which correspond to Asp-166 and Asp-184 in the C-subunit, are located in the tryptic peptide that is thought to be modified by DCCD. This tryptic peptide, shown in Figure 6, is located at the end of the proposed nucleotide-fold region, which, by analogy with other nucleotide binding proteins, is where residues associated with catalysis and substrate binding are frequently located. The results presented here have indicated that one of four carboxyl side chains is modified by DCCD and most likely undergoes an intramolecular cross-link, possibly to another residue in that same peptide or to another amino group that is in close proximity to the reactive carboxyl group. It is probable that the DCCD modification leading to inactivation involves one of the invariant carboxyl acids in this sequence. Thus, it is proposed from these studies that one of the invariant carboxyl groups, Asp-166 or Asp-184, most likely is involved in binding MgATP in the active site of the C-subunit or alternatively participates directly in catalysis.

Registry No. DCCD, 538-75-0; MgATP, 1476-84-2; Asp, 56-84-8; EC 2.7.1.3, 9026-43-1.

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Evidence from Nitrogen-15 and Solvent Deuterium Isotope Effects on the Chemical Mechanism of Adenosine Deaminase†

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ABSTRACT: We have determined ^{15}N isotope effects and solvent deuterium isotope effects for adenosine deaminase using both adenosine and the slow alternate substrate 7,8-dihydro-8-oxoadenosine. With adenosine, ^{15}N isotope effects were 1.0040 in H_2O and 1.0023 in D_2O, and the solvent deuterium isotope effect was 0.77. With 7,8-dihydro-8-oxoadenosine, ^{15}N isotope effects were 1.015 in H_2O and 1.0131 in D_2O, and the solvent deuterium isotope effect was 0.45. The inverse solvent deuterium isotope effect shows that the fractionation factor of a proton, which is originally <0.6, increases to near unity during formation of the tetrahedral intermediate from which ammonia is released. Proton inventories for $1/V$ and $1/(V/K)$ vs percent D_2O are linear, indicating that a single proton has its fractionation factor altered during the reaction. We conclude that a sulfhydryl group on the enzyme donates its proton to oxygen or nitrogen during this step. pH profiles with 7,8-dihydro-8-oxoadenosine suggest that the pK of this sulfhydryl group is 8.45. The inhibition of adenosine deaminase by cadmium also shows a pK of ~9 from the pK_i profile. Quantitative analysis of the isotope effects suggests an intrinsic ^{15}N isotope effect for the release of ammonia from the tetrahedral intermediate of ~1.03 for both substrates; however, the partition ratio of this intermediate for release of ammonia as opposed to back-reaction is 14 times greater for adenosine (1.4) than for 7,8-dihydro-8-oxoadenosine (0.1). The most likely chemical mechanism for adenosine deaminase involves protonation of N-1 by the sulfhydryl group while water adds to C-6 with general base assistance by a histidine with pK = 5.24. The pK values for 7,8-dihydro-8-oxoadenosine are 3.0 and 8.8 and for 7,8-dihydro-8-oxoinosine 7.8 and 11.1. The shape of a plot of $(V/K)K_i$ vs pH is shown to be a very sensitive criterion for the stickiness of a substrate, and by this test adenosine is not sticky.

Adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) catalyzes the hydrolytic cleavage of adenosine to inosine and ammonia, as well as the elimination of a number of other groups from the 6-position of purine ribonucleosides. Two chemical mechanisms have been proposed for adenosine deaminase. Orsi et al. (1972) suggested that direct addition of an enzymic sulfhydryl group to C-6 of adenosine was the first step, with ammonia release from this tetrahedral intermediate leaving an enzyme-bound intermediate that was in turn displaced by water to yield inosine. Kurz and Frieden (1983) suggest protonation of N-1 by the enzyme sulfhydyl group during attack at C-6 by water, with ammonia release from the tetrahedral intermediate giving inosine directly.

To help gain evidence on the chemical mechanism, and in particular the participation of a sulfhydryl group, we have measured the ^{15}N kinetic isotope effects in H_2O and D_2O and the solvent deuterium isotope effect for both the normal substrate adenosine and the slow alternate substrate 7,8-dihydro-8-oxoadenosine. The larger isotope effects observed with the latter facilitate quantitative analysis and have allowed us to establish without doubt that a sulfhydryl group is involved in the mechanism and transfers its proton to oxygen or nitrogen prior to ammonia release.

Materials and Methods

Materials. Adenosine deaminase from calf intestinal mucosa in glycerol, glutamate dehydrogenase from bovine liver in glycerol, α-ketoglutarate, reduced β-nicotinamide adenine dinucleotide (DPNH), and adenosine were from Sigma. NaOD and *N*-bromoacetamide were from Aldrich. D_2O (99.9 atom % D) was from Cambridge Isotope Laboratories. Assay of ammonia was with the Nessler's-based Sigma Ammonia Color Reagent. AG 50W-X8 cation-exchange resin was from Bio-Rad.

7,8-Dihydro-8-oxoadenosine (6-Amino-9-β-D-ribofuranosyl-9H-purin-8(7H)-one). Tri-*O*-acetyladenosine (II) was prepared according to Bredereck and Martini (1947). A total of 50 g (187 mmol) of adenosine (I) was dissolved in 740 mL of dry pyridine. To this was added 530 mL of acetic anhydride, and this solution was allowed to stir at 25 °C for 2.5 h. Solvent was removed in vacuo and the residue washed twice with 300 mL of ethanol. The yellowish syrup was recrystallized from 150 mL of cold ethanol at 4 °C. The white crystals were collected and dried under vacuum to yield 47 g of II (64% yield from I). Thin-layer chromatography of this

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