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Cross-Linking of Iodine-125-Labeled, Calcium-Dependent Regulatory Protein to the Ca^{2+}-Sensitive Phosphodiesterase Purified from Bovine Heart†

David C. LaPorte, William A. Toscano, Jr.,‡ and Daniel R. Storm*

ABSTRACT: The calcium-dependent regulatory protein (CDR)·Ca^{2+} sensitive cyclic nucleotide phosphodiesterase was purified to apparent homogeneity from bovine heart by using ammonium sulfate fractionation, DEAE-cellulose chromatography, and CDR–Sepharose affinity chromatography. The enzyme was purified 13 750-fold with a 10% yield and a specific activity of 275 μmol of cAMP min^{-1} mg^{-1}. The purified enzyme ran as a single band during sodium dodecyl sulfate–polyacrylamide gel electrophoresis with an apparent molecular weight of 57 000. Phosphodiesterase activity was stimulated 10-fold by Ca^{2+} and CDR with half-maximal activation occurring at 9 ng/assay. [^{125}I]CDR was cross-linked to the purified phosphodiesterase by using dimethyl suberimidate. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the cross-linked products revealed a number of discrete ^{125}I-labeled bands. The molecular weights of the cross-linked products indicate that the stoichiometry of the phosphodiesterase complex is A_2C_2, where A is the phosphodiesterase catalytic subunit and C is the calcium-dependent regulatory protein.

The calcium-dependent regulatory protein (CDR)[1] was independently discovered by Cheung (Cheung, 1970) and Kakiuchi (Kakiuchi et al., 1970). These investigators discovered that CDR stimulates the Ca^{2+}-sensitive isozyme of cyclic nucleotide phosphodiesterase. It was subsequently demonstrated that CDR binds Ca^{2+} (Teo & Wang, 1973) and both Ca^{2+} and CDR must be present for stimulation of phosphodiesterase activity (Teo & Wang, 1973; Wolff & Brostrom, 1974; Lin et al., 1974; Teo et al., 1973). It is well established that CDR forms a complex with the phosphodiesterase which is stable during Sephadex G-200 chromatography and electrophoresis on nondenaturing gels in the presence of Ca^{2+}, but not in the presence of EGTA (Teshima & Kakiuchi, 1974; Lin et al., 1975; LaPorte & Storm, 1978). These observations support the proposal that CDR binds Ca^{2+}, forms a complex with the phosphodiesterase, and stimulates the enzyme (Kakiuchi et al., 1973).

The Ca^{2+} and CDR sensitive phosphodiesterase has been purified 5250-fold from bovine heart with a 7% yield (Ho et al., 1977). It was estimated that the enzyme was approximately 80% pure. This preparation was quite unstable in the absence of Ca^{2+} and CDR, although less purified preparations were reasonably stable. In this report, we describe a procedure for purification of the Ca^{2+}-sensitive phosphodiesterase which results in a 13 750-fold purification with a 10% yield. This

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[1] Abbreviations used: CDR, calcium-dependent regulatory protein; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate.

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enzyme appears homogeneous on $NaDodSO_4$ gels and exhibits good stability both in the presence and absence of Ca^{2+} and CDR. The stoichiometry for the phosphodiesterase CDR complex was determined by cross-linking of [^{125}I]CDR to the purified phosphodiesterase by using dimethyl suberimidate. It is suggested that this general technique may be useful for studying interactions between CDR and other CDR binding proteins.

Materials and Methods

[^{3}H]cAMP, [^{14}C]AMP, and Na[^{125}I] were purchased from New England Nuclear. DE-52 cellulose was purchased from Whatman and was precycled prior to use according to the manufacturer's instructions. Bovine serum albumin, cross-linked bovine serum albumin, ovalbumin, catalase, aldolase, phenylmethanesulfonyl fluoride ($PhCH_2SO_2F$), *C. atrox* venom, and dimethyl suberimidate were products of Sigma. All other chemicals were reagent grade.

Phosphodiesterase Assay. Phosphodiesterase activity was monitored as previously described (LaPorte & Storm, 1978) except that DEAE-Sephadex A-25 was substituted for IRP-58 as the ion-exchange resin. For assay during the purification, the assay mixture contained 1.2 mM [^{3}H]cAMP (~80 000 cpm), 20 mM Tris-Cl, pH 8.0, 20 mM imidazole, 3 mM $MgCl_2$, 0.1 mM $CaCl_2$, 100 ng of CDR, and [^{14}C]AMP (~8000 cpm) in a total volume of 0.5 mL. For measurement of stimulation of the phosphodiesterase by CDR, the assay mixture contained 0.5 mM [^{3}H]cAMP (~80 000 cpm), 20 mM Tris-Cl, pH 8.0, 20 mM imidazole, 3 mM $MgCl_2$, 100 mM KCl, 0.1 mM $CaCl_2$, [^{14}C]cAMP (~8000 cpm), 0.1 mM $PhCH_2SO_2F$, 0.1 mg/mL BSA, 0.1% Lubrol PX, and varying amounts of CDR. One unit of phosphodiesterase catalyzes the hydrolysis of 1 μmol of cAMP/min at 30 °C in the presence of 1.2 mM cAMP and saturating CDR.

Purification of the CDR-Sensitive Phosphodiesterase. This procedure, through the second DEAE-cellulose column, was a modification of the method of Ho et al. (1976), which gives partial purification of the enzyme. All procedures were carried out at 4 °C unless otherwise specified.

Fresh bovine heart was obtained from a local slaughterhouse. After trimming away fat and connective tissue, 750 g of tissue was sliced into small pieces and ground with a meat grinder. The ground tissue was homogenized in a Waring blender (3 × 10 s bursts) in 2 volumes of cold 20 mM Tris-Cl, pH 7.5, 1 mM EDTA, 0.5 mM $PhCH_2SO_2F$. The homogenate was centrifuged at 3000*g* for 30 min. The resulting supernatant was adjusted to pH 8.0 with 0.5 N NaOH and to 1 mM $PhCH_2SO_2F$. After stirring for 1 h, solid ammonium sulfate was added to 60% saturation. The pellet was collected by centrifugation at 11000*g* for 30 min and the supernatant was discarded. This pellet was resuspended in 200 mL of buffer A (20 mM Tris-Cl, pH 7.5, 1 mM imidazole, 1 mM $MgCl_2$, 15 mM 2-mercaptoethanol) containing 10 μM $CaCl_2$ and dialyzed against 4 L of the same buffer for 10 h with one buffer change. The preparation was then centrifuged at 100000*g* for 1 h.

The 100000*g* supernatant was applied to a DE-52 cellulose column (3.2 × 30 cm) equilibrated with buffer A containing 10 μM $CaCl_2$. The column was washed with 50 mL of equilibration buffer and eluted with a linear gradient (1500 mL) of 0–0.4 M NaCl in the same buffer. Fractions containing the Ca^{2+}·CDR-sensitive phosphodiesterase activity were combined and dialyzed overnight against 3 L of buffer A containing 0.1 mM EGTA.

The dialysate was applied to a second DE-52 cellulose column (2.6 × 30 cm) equilibrated in buffer A containing 0.1 mM EGTA. The column was eluted with a linear gradient (1000 mL) from 0 to 0.4 M NaCl in equilibration buffer. Fractions containing phosphodiesterase activity were combined and dialyzed overnight against buffer B (40 mM Tris-Cl, pH 7.5, 3 mM $MgCl_2$, 0.2 mM $CaCl_2$, 50 mM NaCl, 0.1 mM DTT).

CDR–Sepharose chromatography was carried out in a manner similar to that described by Klee & Krinks (1978). The dialysate was applied to a CDR–Sepharose column (0.8 × 6 cm) equilibrated in buffer B. The column was washed with buffer B containing 200 mM NaCl until the absorbance at 280 nm approached zero. The column was then washed with 10 mL of buffer B and eluted with buffer C (40 mM Tris-Cl, pH 7.5, 1 mM $MgCl_2$, 2 mM EGTA, 50 mM NaCl, 0.1 mM DTT). The phosphodiesterase activity was eluted in a sharp peak. The pooled activity was adjusted to 4 mM $CaCl_2$ and applied to a second CDR–Sepharose column (0.6 × 4 cm) equilibrated in buffer B. The column was washed with 10 mL of buffer B containing 200 mM NaCl, followed by 5 mL of buffer B. Phosphodiesterase activity was eluted with buffer C and the purified enzyme was stored frozen in aliquots at −60 °C.

Purification of CDR. CDR was purified from bovine brain by a method similar to that of Dedman et al. (1977), except that the heat step was not used and Sephadex G-150 was substituted for Ultragel AcA 44.

CDR–Sepharose was prepared as described by Westcott et al. (1979).

[^{125}I]CDR. CDR was iodinated and purified as previously published (LaPorte & Storm, 1978).

Cross-Linking of [^{125}I]CDR to PDE. CDR and phosphodiesterase were cross-linked by using dimethyl suberimidate by a method similar to that of Davies & Stark (1970). The reaction mixture contained 250 mM sodium borate, pH 9.8, and 0, 0.04, 0.2, or 1 mg/mL dimethyl suberimidate in a volume of 0.4 mL. Protein samples contained 3 μg/mL phosphodiesterase, diluted from a 26 μg/mL stock solution, 3 × 10^5 cpm of [^{125}I]CDR (approximately 3 μg), 5 mM Tris-Cl, pH 7.5, and 1 mM $CaCl_2$ or 1 mM EGTA in a volume of 0.1 mL. The buffer containing the dimethyl suberimidate was added and the mixture incubated for 2 h at room temperature. The cross-linked samples were desalted on a P-2 column, lyophilized to dryness, and dissolved in 50 μL of 1% $NaDodSO_4$, 1% 2-mercaptoethanol. They were then incubated at 37 °C for 2 h, 15 μL of 67% glycerol, 0.025% bromphenol blue was added, and 25 μL was submitted to $NaDodSO_4$–polyacrylamide gel electrophoresis. Following electrophoresis, the gel was dried onto filter paper and subjected to radioautography at −60 °C by using Kodak BB-1 X-ray film and Cronex Lightning-Plus intensifying screens (Swanstrom & Shank, 1978).

Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gels were run by the method of Laemmli (1970). Molecular weight standards used for $NaDodSO_4$ gel electrophoresis were aldolase (40 000), ovalbumin (43 000), catalase (60 000), bovine serum albumin (67 000), and cross-linked bovine serum albumin (67 000, 134 000, 201 000, 268 000). Protein was determined by the method of Peterson (1977) by using bovine serum albumin as the standard.

Results

Purification of Phosphodiesterase. The Ca^{2+}-sensitive phosphodiesterase was purified from bovine heart by using ammonium sulfate fractionation, DEAE-cellulose chromatography and CDR–Sepharose affinity chromatography. We had previously determined that the partially purified phos-

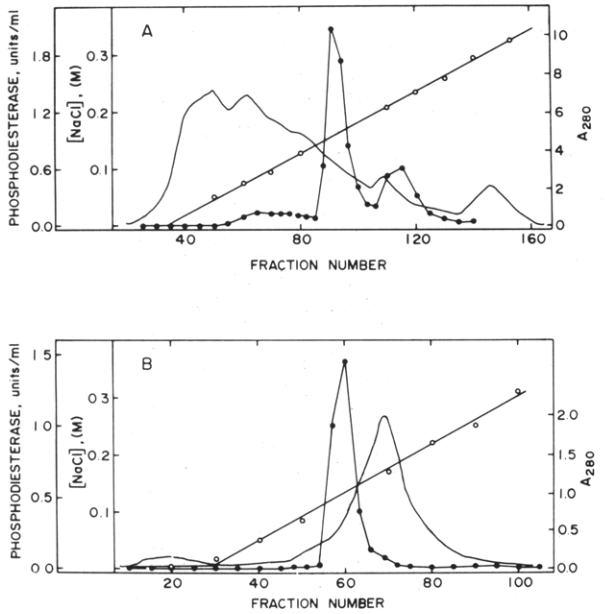

FIGURE 1: Elution profiles from the first and second DEAE-cellulose columns, as described under Materials and Methods. (A) The protein solution resulting from the ammonium sulfate precipitation was applied to a DEAE-cellulose column (3.2 × 30 cm) equilibrated in buffer A containing 10 μM $CaCl_2$. The column was washed with 50 mL of the equilibration buffer and then eluted with a 1500-mL gradient of NaCl from 0 to 0.4 M in the same buffer. (B) The protein resulting from the first DEAE-cellulose was dialyzed against buffer A containing 0.1 mM EGTA and then applied to a DEAE-cellulose column (2.6 × 30 cm) equilibrated in the same buffer. The column was eluted with a gradient of NaCl from 0 to 0.4 M in the EGTA containing equilibration buffer. The symbols are: (●) PDE activity; (○) NaCl gradient; (solid line) absorbance at 280 nm.

phodiesterase prepared by the method of Ho et al. (1976) apparently contained a single CDR-binding component (LaPorte & Storm, 1978). This CDR-binding protein comigrated with phosphodiesterase activity on nondenaturing polyacrylamide gels. Therefore, this purification method was modified and used to prepare partially purified phosphodiesterase suitable for CDR–Sepharose affinity chromatography.

The sample obtained from the ammonium sulfate fractionation was dialyzed against buffer A (see Materials and Methods) containing 10 μM $CaCl_2$ and submitted to DEAE-cellulose chromatography in the presence of $CaCl_2$. Phosphodiesterase activity was eluted from this column in two major peaks (Figure 1A). The first, and most prominent, peak contained the Ca^{2+}- and CDR-sensitive isozyme. This observation differs somewhat from the results reported by Ho et al. (1977). They found that the phosphodiesterase activity eluted from a DEAE-cellulose column, run under similar conditions, as a single broad peak.

The Ca^{2+}-sensitive phosphodiesterase obtained from the first DEAE-cellulose column was applied to a second DEAE-cellulose column and chromatographed in the presence of EGTA (Figure 1B). The enzyme eluted at a lower NaCl concentration under these conditions since it chromatographs as the CDR-free enzyme.

The Ca^{2+}-sensitive phosphodiesterase activity obtained from the second DEAE-cellulose column was then dialyzed into a Ca^{2+}-containing buffer (buffer B), applied to a CDR–Sepharose column, washed, and eluted as described under Materials and Methods. The enzyme was eluted as a sharp peak, easily observable by monitoring the absorbance at 280 nm. Although the phosphodiesterase was highly purified at this stage, $NaDodSO_4$ gel electrophoresis revealed a number of minor protein bands. These minor contaminants were removed by chromatography of the enzyme on a second CDR–Sepharose column.

Table I: Purification of the Ca^{2+}-Sensitive Phosphodiesterase from Heart

purifcn	total protein (mg)	sp act. (units/mg)	total act.	% yield	purifcn
homogenate	15340	0.02	307	100	
$(NH_4)_2SO_4$ precipitation	6154	0.04	246	80	2
DEAE-cellulose I	546	0.28	153	50	14
DEAE-cellulose II	57	1.43	82	27	72
CDR–Sepharose	0.11	275	30	10	13750

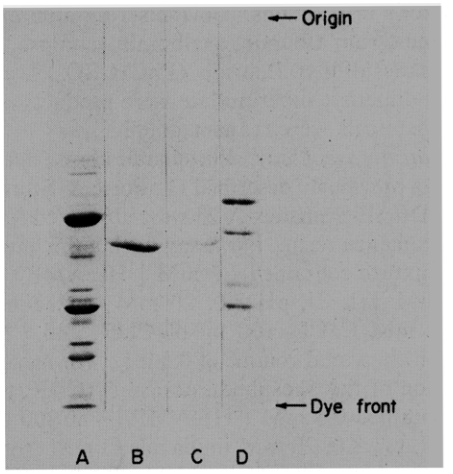

FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoresis of crude extract, purified phosphodiesterase, and molecular weight standards on a 7.5% $NaDodSO_4$–polyacrylamide gel stained with Coomassie brilliant blue. (A) Thirty micrograms of crude bovine heart extract; (B and C) 4 μg and 0.75 μg of purified phosphodiesterase; (D) 1 μg each of bovine serum albumin, bovine liver catalase, hen egg ovalbumin, and rabbit muscle aldolase.

The final purification of the enzyme was 13 750-fold with a 10% yield (Table I). These values are expressed relative to total phosphodiesterase activity in the crude extract which contains a mixture of phosphodiesterase isozymes. We estimate that approximately 60% of the total phosphodiesterase activity in crude extracts is contributed by the Ca^{2+}-sensitive isozyme. Therefore, the actual purification and yield were approximately 23 000-fold and 16%, respectively. With three independent preparations, the specific activity of the purified enzyme varied from 270 to 280 units/mg. Electrophoresis on 7.5% $NaDodSO_4$–polyacrylamide gels revealed one Coomassie blue staining band with a molecular weight of 57 000 (Figure 2). Electrophoresis on 15% $NaDodSO_4$–polyacrylamide gels also gave a single band (mol wt 57 000) with the absence of smaller molecular weight polypeptides. The purified enzyme was also labeled with ^{125}I by using Iodogen (Fraker & Speck, 1978), submitted to $NaDodSO_4$ gel electrophoresis and autoradiographed. Again, only one protein band was detected (data not shown).

It was found in previous studies that the highly purified Ca^{2+} sensitive phosphodiesterase had a stringent requirement for Ca^{2+} and CDR to stabilize the enzyme (Ho et al., 1977). The same investigators noted that the partially purified enzyme exhibited good stability even in the presence of EGTA. The stability of our purified preparation was examined at 4 and 30 °C in the absence of CDR and in the presence of 2 mM EGTA. At 4 °C, there was no apparent loss in activity during a 2-week period. At 30 °C, there was no loss in Ca^{2+} or CDR

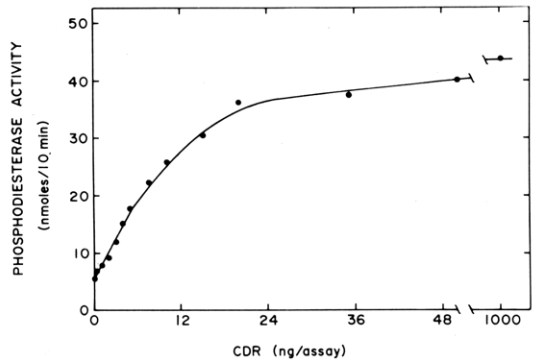

FIGURE 3: CDR stimulation of purified Ca^{2+}-sensitive phosphodiesterase. The phosphodiesterase was purified as described under Materials and Methods and assayed as a function of CDR as described under Materials and Methods. Each assay contained 20 ng of purified phosphodiesterase.

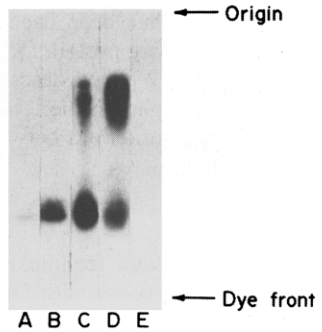

FIGURE 4: Electrophoresis of the products obtained by cross-linking of [^{125}I]CDR to phosphodiesterase on 6% polyacrylamide gels. [^{125}I]CDR was cross-linked to phosphodiesterase with various concentrations of dimethyl suberimidate in the presence of Ca^{2+} or EGTA. The products were submitted to $NaDodSO_4$ gel electrophoresis on 6% gels. The gels were dried onto filter paper and autoradiographed for 24 h, as described under Materials and Methods. A through D were cross-linked in the presence of 0.2 mM $CaCl_2$ and (A) 0, (B) 0.04, (C) 0.2, and (D) 1 mg/mL dimethyl suberimidate. (E) The cross-linking medium contained 0.2 mM EGTA and 1 mg/mL dimethyl suberimidate.

sensitivity during a 40-min incubation period, although there was a 10% reduction in maximal activity. The purified enzyme was also quite stable in the presence of Ca^{2+} and CDR.

Stimulation of the Purified Phosphodiesterase by CDR and Ca^{2+}. The concentration dependence for activation of the phosphodiesterase by CDR is reported in Figure 3. The phosphodiesterase was stimulated tenfold by CDR, with half-maximal stimulation achieved at 9 ng of CDR/assay. CDR activation had an absolute requirement for Ca^{2+}. Data discussed below indicate that the phosphodiesterase binds 2 mol of CDR/mol of enzyme. Therefore, we estimate that the K_d for the complex is approximately 6×10^{-10} M under these assay conditions.

Cross-Linking of [^{125}I]CDR to the Purified Phosphodiesterase. ^{125}I-Labeled CDR was covalently cross-linked to the Ca^{2+}-sensitive phosphodiesterase in order to determine the stoichiometry between the two protein subunits in the native complex. The CDR-free enzyme was incubated with [^{125}I]CDR in the presence or absence of Ca^{2+} and then treated with the bifunctional cross-linking reagent, dimethyl suberimidate, as described under Materials and Methods. The CDR·phosphodiesterase complex was stable under the cross-linking conditions described under Materials and Methods. The cross-linked samples were then submitted to $NaDodSO_4$ gel electrophoresis and autoradiographed. The results from a typical experiment are shown in Figure 4. In

Table II: Molecular Weights of ^{125}I-Labeled Polypeptides Obtained upon Cross-Linking of [^{125}I]CDR and Phosphodiesterase

subunit composition[a]	calcd mol wt[b]	obsd mol wt[c]
AC	75 500	71 000
A_2C	132 500	142 000
A_2C_2	151 000	155 000

[a] A, phosphodiesterase subunit; C, CDR. [b] Calculated by using molecular weights of 18 500 for C, determined by electrophoresis on 15% $NaDodSO_4$ gels, and 57 000 for A, from Figure 2. [c] Data obtained from Figure 4 by using molecular weight standards as described under Materials and Methods.

the absence of free Ca^{2+}, no cross-linked products were detected. In the presence of Ca^{2+} and dimethyl suberimidate, three ^{125}I-labeled bands were detected on $NaDodSO_4$ gels. The ^{125}I-labeled bands became less distinct at higher concentrations of dimethyl suberimidate, probably as a result of increased heterogeneity of the cross-linked species produced by extensive intramolecular cross-linking. The apparent molecular weights of the cross-linked products were estimated from standards run simultaneously on the slab gels. The molecular weights of the cross-linked proteins are compared with the calculated molecular weights for various combinations of CDR and the phosphodiesterase subunit in Table II. Electrophoresis of the cross-linked products was performed on $NaDodSO_4$ gels of 5, 6, and 7.5% acrylamide in order to optimize molecular weight estimates for each of the species. The estimates made from these gels were found to be in excellent agreement with one another. The molecular weights of the cross-linked peptides are consistent with the formation of A–C, A–A–C, and C–A–A–C where A is the phosphodiesterase subunit and C is CDR. These data suggest that each phosphodiesterase subunit can bind one CDR and that the stoichiometry of the complex is A_2C_2. The molecular weight of the CDR-free enzyme estimated by Sephadex G-200 chromatography was 155 000 (Ho et al., 1977). These data indicate that the CDR-free enzyme is a dimer of identical subunits and are consistent with the proposal that the CDR–phosphodiesterase complex has a composition of A_2C_2.

The cross-linking experiments with [^{125}I]CDR yielded one unanticipated result (Figure 4A). When the phosphodiesterase was incubated with Ca^{2+} and [^{125}I]CDR in the *absence* of dimethyl suberimidate, a single ^{125}I-labeled band with a molecular weight of 71 000 was formed. The intensity of this band was considerably less than the 71 000-dalton cross-linked product observed in the presence of dimethyl suberimidate; however, it was observed in three separate experiments. It should be noted that this band was not produced when the incubation mixture contained excess EGTA (Figure 4E) or in the absence of phosphodiesterase. The molecular weight of the product was not consistent with simple exchange of ^{125}I between [^{125}I]CDR and A. These observations indicate that 1 mol of A and CDR form a complex in the presence of Ca^{2+} that is stable during $NaDodSO_4$ gel electrophoresis.

Discussion

A number of laboratories have made significant progress in purifying the Ca^{2+}-CDR-sensitive phosphodiesterase. Ho et al. have extensively purified the enzyme; however, the preparation was not homogeneous and was unstable in the absence of Ca^{2+} or CDR (Ho et al., 1977). A major contribution has been made by the introduction of CDR–Sepharose for the purification of this enzyme (Watterson & Vanaman, 1976; Miyaki et al., 1977; Klee & Krinks, 1978). Successful application of CDR–Sepharose for the purification

of the Ca^{2+}·CDR-sensitive phosphodiesterase requires partially purified preparations that are free of other CDR-binding proteins. Our previous binding studies with [^{125}I]CDR (LaPorte & Storm, 1978) indicated that the partially purified phosphodiesterase, prepared by the method of Ho et al. (1976), contained a single CDR-binding protein which comigrated with phosphodiesterase on nondenaturing gels. Therefore, we employed a similar procedure to prepare partially purified phosphodiesterase suitable for CDR–Sepharose affinity chromatography.

In this study, the Ca^{2+}·CDR-sensitive phosphodiesterase from bovine heart was purified 13 750-fold with a 10% yield. The purified protein ran as a single band on $NaDodSO_4$–polyacrylamide gels with a molecular weight of 57 000. The specific activity of the purified phosphodiesterase was 275 μmol of cAMP min^{-1} mg^{-1} and the enzyme exhibited good stability both in the presence and absence of Ca^{2+}·CDR. CDR stimulated the phosphodiesterase approximately 10-fold and the CDR concentration dependence for stimulation was hyperbolic. Assuming that fractional stimulation of the phosphodiesterase by CDR parallels complex formation, we estimate that the dissociation constant for the complex is approximately 6×10^{-10} M. This calculation also assumes that each phosphodiesterase monomer is stimulated only by direct interaction with CDR with no cooperativity between the enzyme subunits. It must also be emphasized that the apparent affinity of the phosphodiesterase for CDR is dependent upon assay conditions (Teo et al., 1973; Wolff & Brostrom, 1974; Wickson et al., 1975; Brostrom & Wolff, 1976). Thus, the estimate for K_d is valid only under these assay conditions.

The stoichiometry for CDR binding to the phosphodiesterase was examined by cross-linking of [^{125}I]CDR to the CDR-free enzyme by using the bifunctional reagent, dimethyl suberimidate. The data presented indicate that the stoichiometry of the phosphodiesterase·CDR complex is A_2C_2 where A is the catalytic subunit and C is CDR. This conclusion is consistent with previous studies which have suggested, on the basis of indirect evidence, that more than 1 mol of CDR is bound per mol of phosphodiesterase (Teshima & Kakiuchi, 1974; Dedman et al., 1977). The molecular weight of the CDR free phosphodiesterase was estimated as 155 000 by Sephadex G-200 chromatography (Ho et al., 1977). This suggests that the enzyme exists as a dimer of two identical subunits in the absence of CDR. Our data from $NaDodSO_4$ gels indicate that the dimer would have a molecular weight of 114 000. These molecular weight estimates differ because gel filtration provides a measure of Stoke's radius, and not molecular weight. This difference may result from either an increase in asymmetry or degree of hydration of the phosphodiesterase relative to the standards used to calibrate the Sephadex G-200 column. Klee and Krinks have reported that the Ca^{2+}·CDR-sensitive phosphodiesterase from bovine brain may contain subunits of 61 000, 59 000, and 15 000 daltons (Klee & Krinks, 1978). It is quite possible that the heart and brain enzyme may have different quaternary structures. However, these investigators have reported that their phosphodiesterase preparation is contaminated by the modulator binding protein, which has protein subunits of 61 000 and 15 000 daltons (Richman & Klee, 1978). A structural comparison between the brain and heart phosphodiesterase will require a homogeneous preparation of the brain enzyme.

Cross-linking studies with [^{125}I]CDR and the purified phosphodiesterase indicated that the two proteins form a complex in the absence of dimethyl suberimidate which is stable during $NaDodSO_4$–polyacrylamide gel electrophoresis. This product was not formed in the presence of EGTA or in the absence of the phosphodiesterase. Furthermore, the molecular weight of this complex (71 000) was identical with the major product formed in the presence of dimethyl suberimidate. This molecular weight is consistent with the formation of CDR–A and could not be due to direct transfer of ^{125}I from [^{125}I]CDR to the enzyme. Although the nature of the forces maintaining this complex is not defined, a covalent bond between CDR and A seems likely since the complex was not dissociated by $NaDodSO_4$. Since brain CDR does not contain cysteine (Watterson et al., 1976), formation of a disulfide bond between the two proteins is not possible. The physiological significance of this observation is not clear, and the spontaneous cross-linking of CDR to the phosphodiesterase may occur only under the conditions of these experiments.

Bifunctional cross-linking reagents are powerful tools for defining the quaternary structure of multisubunit proteins (Davies & Stark, 1970; Peters & Richards, 1977). For example, dimethyl suberimidate has been used to study the structure of the modulator binding protein (Klee & Krinks, 1978; Richman & Klee, 1978). We have described a method for cross-linking of [^{125}I]CDR to phosphodiesterase which may be of general use in studying interactions between CDR and other CDR-binding proteins.

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Stereochemical Course of the Reaction Catalyzed by 5′-Nucleotide Phosphodiesterase from Snake Venom†

Floyd R. Bryant and Stephen J. Benkovic*

ABSTRACT: The hydrolysis reaction of ATPαS by snake venom phosphodiesterase is highly specific for the B diastereomer and proceeds with 88% retention of configuration at phosphorus. Since this enzyme also catalyzes the hydrolysis of the *S* enantiomer of *O-p*-nitrophenyl phenylphosphonothioate, the absolute configuration at P_α of ATPαS (B) is assigned as the *R* configuration provided the two substrates are processed identically. A mechanism for the hydrolysis reactions catalyzed by the venom phosphodiesterase involving at least a single covalent phosphoryl-enzyme intermediate is in accord with this result.

The stereochemical course of an enzyme-catalyzed phosphoryl transfer reaction at either the mono- or diester level has been a subject of considerable recent interest (Richard et al., 1978; Orr et al., 1978; Midelfort & Sarton-Miller, 1978; Saenger et al., 1974; Eckstein, 1975; Usher et al., 1972) since its elucidation furnishes information concerning the geometry of the transition state as well as the possible intermediacy of a covalent phosphoryl-enzyme species. The observation of net retention is in accord with a single or odd number of intermediates or lacking such species an adjacent spatial alignment between donor and acceptor substrates during phosphoryl transfer presuming permutational isomerism to be absent. Alternatively, net inversion is most simply interpreted as a direct displacement process through an in-line orientation (Benkovic & Schray, 1973). In view of the predominant observation of inversion stereochemistry at phosphorus for simple nonenzymic nucleophilic displacement processes (Benkovic & Schray, 1978) and, by inference, enzyme-catalyzed transfer processes, perhaps the more important use of this mechanistic probe will be in its detection of transient covalent intermediates in cases not readily amenable to kinetic or isolation methods. We wish to report our findings for such an enzyme, venom phosphodiesterase.

Experimental Procedures

Materials

Phosphodiesterase I (*Crotalus adamanteus venom*), inorganic pyrophosphatase (Bakers yeast), adenylate kinase (rabbit muscle), pyruvate kinase (rabbit muscle), hexokinase (yeast, type F-300), lactate dehydrogenase (rabbit muscle), and glucose-6-phosphate dehydrogenase (yeast) were purchased from Sigma. ATP (Na^+ salt), $NADP^+$, NADH, and phosphoenolpyruvate (monocyclohexylammonium salt) also were from Sigma. Adenosine was purchased from Aldrich. PEI-cellulose plates were obtained from Scientific Products, DEAE-Sephadex A-25 was from Pharmacia, DEAE-cellulose DE-52 was from Whatman, and Dowex 50-X8 was from Bio-Rad. Thiophosphoryl chloride was purchased from Alfa Inorganics and diphenyl phosphorochloridate from Aldrich. All other organic reagents, buffers, and inorganic salts were reagent grade. Doubly deionized distilled water was used throughout. The deuterium oxide (99.7%) was obtained from MSD Isotopes and the $H_2{}^{18}O$ (99%) was from KOR Isotopes.

The syntheses of ADPαS[1] (A + B)[2] and ATPαS (A + B) were accomplished according to the method of Eckstein & Goody (1976) as modified by P. Frey (personal communication). The AMPS was synthesized according to the method of Murray & Atkinson (1968). The purification of approximately 0.5 mmol of crude ATPαS (A + B) (dissolved in 200 mL of water with the pH adjusted to 9 with triethylamine) was by chromatography on a DEAE-Sephadex A-25 column (3.0 × 55 cm) by using a 5-L linear 0.1 M (2.5 L) to 0.75 M (2.5 L) gradient of ammonium bicarbonate. The concentration of the nucleotides was estimated throughout from A_{260} by employing ϵ 15 000 M^{-1} cm^{-1} (Eckstein & Grindl, 1970). The elution resulted in the separation of the A and B diastereomers. There were 350 fractions of approximately 15 mL volume collected. The ATPαS (A) eluted in fractions 280–294, whereas ATPαS (B) eluted in fractions 295–314 monitoring the effluent at A_{260}. The fractions containing the individual diastereomers were pooled separately and evaporated under vacuum to dryness. The residue was dissolved in water

† From the Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802. *Received February 28, 1979.* This investigation was supported by a grant from the National Institutes of Health (GM 13306).

[1] Abbreviations used: ATPαS, adenosine 5′-*O*-(1-thiotriphosphate); ADPαS, adenosine 5′-*O*-(1-thiodiphosphate); AMPS, adenosine 5′-*O*-thiophosphate; EDTA, ethylenediaminetetraacetic acid; P_i, inorganic phosphate; PP_i, inorganic pyrophosphate; PEP, phosphoenolpyruvate; TEA, triethanolamine; Tris, tris(hydroxymethyl)aminomethane.

[2] The A and B designations for the diastereomers differing in configuration at P_α are those suggested by Eckstein (1975).

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