Preparation of an Enzymatically Active Cross-Linked Complex between Brain Cyclic Nucleotide Phosphodiesterase and 3-(2-Pyridyldithio)propionyl-Substituted Calmodulin†

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ABSTRACT: Cyclic nucleotide phosphodiesterase (0.07 nM) was activated by near stoichiometric concentrations of [3-(2-pyridyldithio)propionyl]calmodulin (PDP-CaM) after initial incubation of these proteins at 200-fold higher concentrations; activity in assays with EGTA was 80% of that in the presence of Ca^{2+}. The enzyme incubated with native calmodulin under identical conditions required ~1 nM for half-maximal activation, and no activation was observed in the absence of calcium. These data suggested formation of a covalent complex between phosphodiesterase and PDP-CaM. On high-performance gel-permeation chromatography in the presence of metal chelators, the complex appeared considerably larger than the native enzyme. Incubation of phosphodiesterase with the thiolated (inactivated) form of PDP-CaM did not change its chromatographic behavior, indicating that reactive sulfhydryl groups were involved in complex formation. Although the total activities recovered from chromatography were not significantly different, maximal activation of PDP-CaM–phosphodiesterase complex was only ~20%, whereas the control enzyme was activated 6–8-fold by Ca^{2+} plus calmodulin. Kinetics of cGMP hydrolysis in the presence of EGTA by the isolated complex differed from those of control enzyme but were indistinguishable from those of control enzyme assayed with saturating Ca^{2+} and CaM. The calmodulin antagonists W-7 and trifluoperazine had relatively little effect on activity of the PDP-CaM–phosphodiesterase complex. Incubation of the complex with dithiothreitol dramatically increased its Ca^{2+} and calmodulin responsiveness, suggesting that reduction of the disulfide cross-link released phosphodiesterase from the complex. Because of the covalent, yet reversible, nature of the disulfide linkage, calmodulin–binding protein complexes like that described here may be particularly useful for investigation of the mechanism of calmodulin activation and, in conjunction with selective proteolytic cleavage, may facilitate identification of the interacting domains.

Cross-linking of proteins with reagents such as glutaraldehyde (Quiocho & Richards, 1966), dimethyl suberimidate (Davies & Stark, 1970), and several other bifunctionally reactive molecules [for a review, see Peters & Richards (1977)] has provided valuable information regarding the identity and stoichiometry of interacting molecules. Several inherent difficulties have, however, limited the use of such methods for quantitative investigation of protein interactions and/or enzyme regulation. Frequently, excess reagent is required to achieve cross-linking, and the proportion of components must be carefully adjusted to obtain relevant products and avoid formation of large oligomeric cross-linked complexes. More importantly, many cross-linking conditions may be incompatible with preservation of enzymatic activity.

Cross-linking of the Ca^{2+} binding protein, calmodulin (CaM),[1] to one of its binding proteins, cyclic nucleotide phosphodiesterase, was first reported by LaPorte et al. (1979), who used dimethyl suberimidate and ^{125}I-labeled calmodulin. They thereby demonstrated a direct interaction of CaM with phosphodiesterase and suggested that stimulation of this enzyme was due to formation of an activated tetrameric form, $\alpha_2\beta_2$ (where α and β refer to the phosphodiesterase subunit and calmodulin, respectively). In addition to species with the molecular weight expected for $\alpha_2\beta_2$, forms apparently corresponding to $\alpha\beta$ and $\alpha_2\beta$ were also seen. Activity of the cross-linked enzyme was not reported, and it seems likely that the conditions of cross-linking would have caused inactivation of the phosphodiesterase. In addition, a recent report (Andreasen et al., 1981) indicated that a photoreactive azido-calmodulin derivative may be useful for the specific cross-linking of several calmodulin–binding proteins, including phosphodiesterase. We recently reported the preparation of a novel reactive sulfhydryl derivative of CaM, [3-(2-pyridyldithio)propionyl]calmodulin, that retains biological activity, described its use in affinity chromatography, and mentioned observations suggesting that it was capable of forming a functional cross-linked complex with phosphodiesterase (Kincaid & Vaughan, 1983). The studies presented here demonstrate that a covalent disulfide-linked complex is indeed formed, that the enzyme activity is fully preserved, and that the phosphodiesterase–CaM complex appears to be activated in the absence of added calcium.

Materials and Methods

Materials. Sources of chromatographic media, derivatization reagents, and assay components were as previously described (Kincaid & Vaughan, 1983; Kincaid et al., 1982). Three times crystallized ovalbumin was purchased from Sigma, crystalline bovine serum albumin from Miles, soybean trypsin inhibitor from Worthington, and the TSK SW-3000 column from Beckman.

Assay of Phosphodiesterase Activity. Enzyme activity was assayed essentially as described previously (Kincaid et al., 1981) with 0.5 μM [^3H]cGMP as substrate. Basal activity is that assayed in the presence of 100 μM EGTA; total activity

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[1] Abbreviations: CaM, calmodulin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonium]-1-propanesulfonate; QAE-Sephadex, [diethyl(2-hydroxypropyl)amino]ethyl-Sephadex; PDP-CaM, [3-(2-pyridyldithio)propionyl]calmodulin; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; BES, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; PDE, phosphodiesterase; SDS, sodium dodecyl sulfate.

is that assayed with 330 μM $CaCl_2$ and 50 nM CaM.

Purification of Phosphodiesterase. Bovine brain phosphodiesterase was purified from frozen tissue as described previously (Kincaid & Vaughan, 1983) up to elution from disulfide-linked CaM–Sepharose, which was carried out as follows. The column was eluted with three bed volumes of buffer A (50 mM ammonium bicarbonate, pH 8.3, containing 200 mM NaCl, 1 mM $MgCl_2$, 2 mM EGTA, 10% glycerol, and 20 mM dithiothreitol). After addition of $^1/_{20}$ volume ovalbumin (10 mg/mL) plus soybean trypsin inhibitor (0.5 mg/mL), the eluate was applied to a column of QAE-Sephadex A-25 (dimensions identical with those of the PDP-CaM–Sepharose column) equilibrated with buffer A. The QAE column was washed with one bed volume of buffer A, and the entire effluent (initial unretained sample plus wash) was collected and adjusted to 4 mM in Ca^{2+}. The phosphodiesterase was then purified on CaM–Sepharose and organomercurial agarose (Affi-Gel 501, Bio-Rad) as described by Kincaid & Vaughan (1983). Phosphodiesterase was judged to be homogeneous by SDS gel electrophoresis, and on high-performance gel-permeation chromatography, the specific activity was constant across the protein peak. A subunit molecular weight of 59 000 was used to calculate phosphodiesterase concentrations.

Preparation of Dansyl- and PDP-CaM. Calmodulin prepared as described (Kincaid et al., 1982) was homogeneous by electrophoretic and spectrophotometric criteria. [^{3}H]-Dansylcalmodulin was prepared essentially as described by Kincaid et al. (1982) with [^{3}H]dansylchloride (200 mCi/mmol, New England Nuclear) in place of unlabeled material. [^{3}H]Dansyl-CaM (50 μCi/μmol) was exhaustively dialyzed against sodium acetate, pH 5.3, containing 100 mM NaCl and stored at −20 °C. The hydrodynamic behavior of dansyl-CaM on high-performance gel-permeation chromatography was identical with that of the unmodified protein. PDP-CaM was prepared as described; the degree of substitution was quantified by release of 2-thiopyridone in the presence of excess dithiothreitol (Kincaid & Vaughan, 1983). All preparations used in experiments reported here contained ~2.7 mol of PDP/mol of CaM. The activated derivative stimulated phosphodiesterase with affinity and maximal effectiveness very similar to those to native CaM as previously observed for the reduced form (Kincaid & Vaughan, 1983). The apparent affinities of several preparations were 2–4 nM vs. 1–2 nM for native CaM.

Results

Samples of phosphodiesterase (13 nM) were incubated with native or PDP-CaM (7–335 nM) and 500 μM Ca^{2+} and then diluted and assayed at an enzyme concentration of 0.07 nM (Figure 1). In assays of the enzyme that had been incubated with PDP-CaM, half-maximal activation was observed with ~0.1 nM in the presence of Ca^{2+}, whereas with enzyme that had been incubated in the same way with native CaM, ~1 nM was required for half-maximal activation. The absolute specific activities of both enzyme preparations under maximally stimulated conditions were, however, indistinguishable. For enzyme incubated with PDP-CaM, activity assayed with EGTA was ~80% of that in assays with added Ca^{2+} at each concentration of PDP-CaM (Figure 1); there was no significant activation of enzyme incubated with native CaM and assayed with EGTA. The phosphodiesterase preparation displaying high activity in the absence of added Ca^{2+} was not observed if the initial incubation with PDP-CaM were carried out in the presence of 2 mM EGTA, indicating that formation of this species was Ca^{2+} dependent (unpublished data).

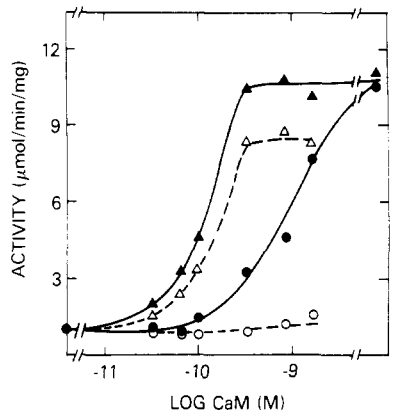

FIGURE 1: Activity of phosphodiesterase as a function of CaM concentration in assay after incubation with 200 times that concentration of native CaM or PDP-CaM. Phosphodiesterase (~13 nM) was incubated with native (○, ●) or PDP-CaM (△, ▲) at concentrations of 7, 13, 20, 67, 168, and 335 nM in 10 mM BES, pH 7.0, containing 0.2 mM EDTA, 0.6 mM $CaCl_2$, bovine serum albumin (0.5 mg/mL), and soybean trypsin inhibitor (20 μg/mL) (total volume 30 μL). After 10 min at 22 °C, 5 μL of 40 mM EGTA was added followed by 270 μL of the above buffer containing 20% glycerol and lacking $CaCl_2$. Samples were then frozen and stored at −60 °C until assayed in the presence of 1 mM $CaCl_2$ (●, ▲) or 0.3 mM EGTA (○, △). Two points at the extreme right are means of activities of samples incubated with different concentrations of native CaM (●) or PDP-CaM (▲) assayed with 1 mM $CaCl_2$ and 50 nM CaM.

Table I: Effects of Ca^{2+}, CaM, and W-7 on Activity of Control Phosphodiesterase and Enzyme Linked to CaM[a]

	phosphodiesterase act. (μmol min^{-1} mg^{-1})			
	expt 1		expt 2	
addition	control PDE	PDP-CaM-PDE	control PDE	PDP-CaM-PDE
none	1.2	6.7	1.6	10.9
W-7	1.6	5.6		
$CaCl_2$	2.9	7.7	1.7	13.4
$CaCl_2$ and CaM	9.1	8.9	12.4	13.3
$CaCl_2$, CaM, and W-7	2.8	5.7	1.6	11.5

[a] For experiment 1, fractions of peak enzyme activity from chromatography of control and PDP-CaM-linked phosphodiesterase were pooled and frozen after dilution as described in Figure 2. For experiment 2, a single peak fraction was diluted and frozen. Samples were later assayed without or with 100 μM W-7, 330 μM $CaCl_2$, and 50 nM CaM as indicated.

Phosphodiesterase (5.6 μM) incubated with 17 μM PDP-CaM in the presence of saturating Ca^{2+} and subjected to high-performance gel-permeation chromatography exhibited a single, symmetrical peak of activity with the hydrodynamic behavior of a molecule considerably larger than native phosphodiesterase (Figure 2, upper panel). The "basal" activity of the cross-linked species was ~70–90% of that assayed in the presence of Ca^{2+} and CaM for all fractions across the peak (Figure 2, upper panel), consistent with the quantitative conversion of phosphodiesterase to a larger, activated form. Phosphodiesterase incubated with PDP-CaM that had been reduced with dithiothreitol (control enzyme) eluted in the same position as the native enzyme (Figure 2, lower panel), suggesting that formation of the larger cross-linked species required the reactive sulfhydryl groups of PDP-CaM. Activation by Ca^{2+} and CaM of enzyme in these column fractions was 6–8-fold.

Peak fractions of enzyme activity from each of the two columns were pooled and their enzymatic properties compared

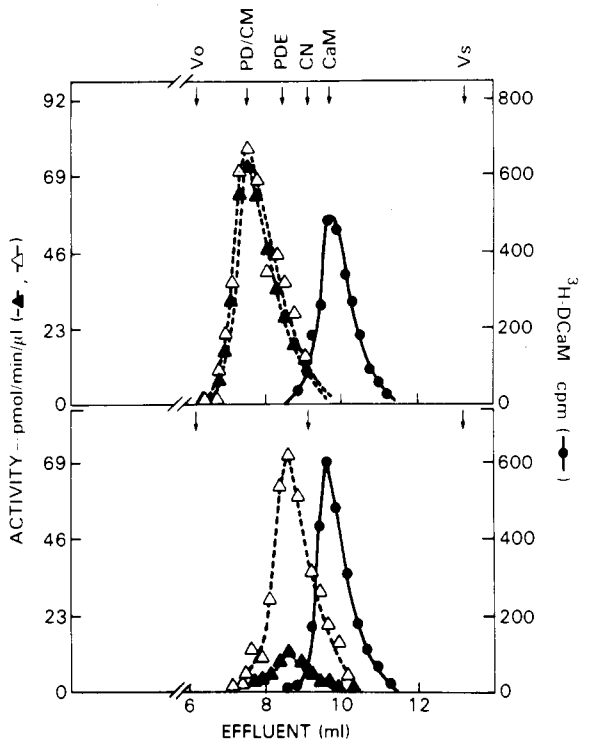

FIGURE 2: High-performance gel-permeation chromatography of the PDP-CaM–phosphodiesterase complex and of control enzyme. (Upper panel) Phosphodiesterase (36 μg in 90 μL) was mixed with PDP-CaM (33 μg in 20 μL) in 40 mM Tris-HCl, pH 8.0, containing 10 mM $CaCl_2$; 5 μL of [^{3}H]dansyl-CaM (20 000 cpm) was added as an internal standard. Final concentrations of phosphodiesterase and PDP-CaM were ~5.6 and 17 μM, respectively. After incubation for 10 min at 22 °C, 10 μL of 200 mM EGTA was added. The mixture was centrifuged for 1 min in a table-top Microfuge, and ~100 μL was injected onto a SW-3000 column (7.5 × 300 mm). The column was eluted with 20 mM BES (pH 7.2) containing 100 mM NaCl, 0.2 mM EDTA, 0.1 mM EGTA, and 10% glycerol (flow rate ~0.32 mL/min, Spectraphysics Model 3500 liquid chromatograph). Fractions (250 μL) were collected; samples (20 μL) were frozen after dilution with 180 μL of buffer containing 20% glycerol and later assayed for basal (▲) and total (△) phosphodiesterase activity. Portions (50 μL) of each fraction were also taken for radioassay of [^{3}H]-dansyl-CaM (●). Arrows indicate elution positions of dansyl-CaM, phosphodiesterase (PDE), calcineurin (CN), and the PDP-CaM–phosphodiesterase complex (PD/CM). Void volume (V_0) and total column volume (V_s) were determined by using thyroglobulin and L-tryptophan, respectively. (Lower panel) Procedure was identical with that described for the upper panel except that PDP-CaM was reduced by incubation for 10 min at 22 °C with 50 mM dithiothreitol before addition to phosphodiesterase. The preparation is referred to as the control enzyme. Recoveries of enzyme activity and [^{3}H]-dansyl-CaM in upper and lower panels were ~65–70%.

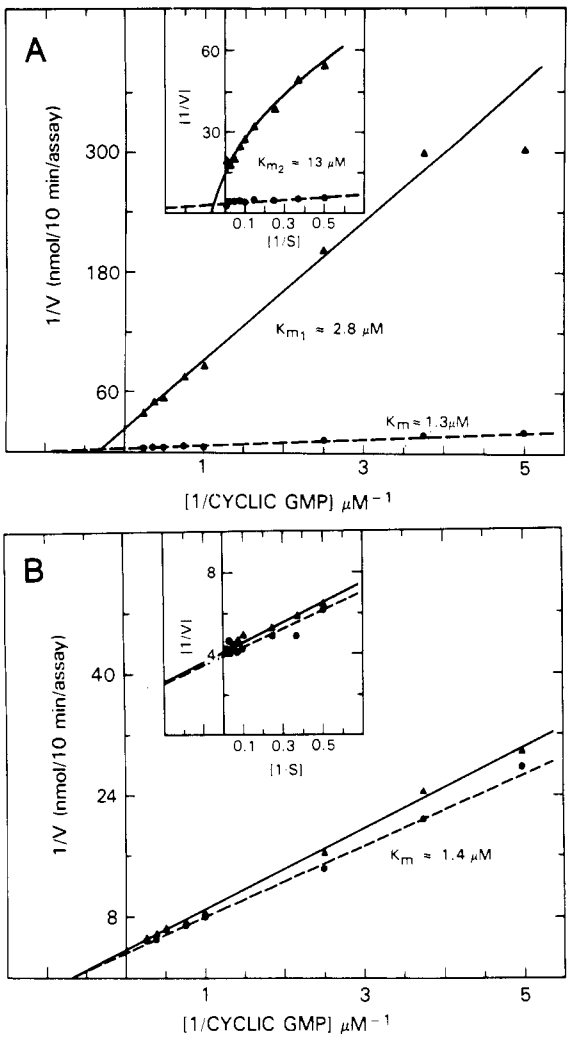

FIGURE 3: (A) Hydrolysis of cGMP by PDP-CaM-linked phosphodiesterase and control enzyme in the presence of metal chelator. Fractions of peak enzyme activity from chromatography of control (▲) and PDP-CaM-linked (●) phosphodiesterase were pooled and frozen after dilution as described in Figure 2. Samples (~1 ng) were later assayed for basal activity with the indicated concentrations of cGMP. Maximal velocity of the enzyme linked to PDP-CaM in this and other experiments was ~30–40 μmol min^{-1} (mg of protein)$^{-1}$ (with 100 μM EGTA, without added Ca^{2+} and CaM). (B) Hydrolysis of cGMP by PDP-CaM-linked phosphodiesterase and control enzyme in the presence of Ca^{2+} and CaM. All procedures were as indicated in (A) except that assays were carried out in the presence of 330 μM $CaCl_2$ and 50 nM CaM. The insets represent data from the same experiment for substrate concentrations greater than 2 μM.

(Table I). The activity of the cross-linked complex was increased by CaM and calcium only slightly (20%) above that observed in the presence of 100 μM EGTA, whereas activity of the control enzyme was increased 7-fold. Ca^{2+} alone had a small effect on the activity of the complex and stimulated the control enzyme ~120%. The effect of Ca^{2+} on the control enzyme presumably resulted from the presence of a small amount of the reduced CaM derivative in the pooled fraction, since the native enzyme was not significantly activated by Ca^{2+}. The naphthalenesulfonamide CaM antagonist, W-7, which dramatically inhibited the CaM-stimulated activity of control enzyme, did not significantly reduce activity of the cross-linked complex, suggesting that a reversible association of calmodulin and phosphodiesterase is required for inhibition (Table I). Similar data were obtained with the phenothiazine trifluoperazine.

Data for hydrolysis of cGMP by the control enzyme in the absence of Ca^{2+} and CaM yielded nonlinear Lineweaver–Burk plots with apparent Michaelis constants of 2.8 and 13 μM (Figure 3A). In contrast, a linear Lineweaver–Burk plot (K_m ~1.3 μM) was obtained for the isolated cross-linked complex (Figure 3A) that was virtually identical with that for the control enzyme assayed in the presence of 200 μM Ca^{2+} and 50 nM CaM (Figure 3B).

Incubation of the cross-linked phosphodiesterase–CaM complex with dithiothreitol partially reversed the "activated" state and increased stimulation by Ca^{2+} and CaM (Figure 4). In this experiment, the cross-linked enzyme incubated in the absence of reductant was activated only 18% by saturating Ca^{2+} and CaM. After incubation with 20 mM dithiothreitol for 5 min at 30 °C, stimulation was 225% while total activity decreased ~15%. Thus, the increase in CaM activation resulted from a reduction in basal activity (i.e., assayed with EGTA). Calmodulin activation (8–10-fold) of the control enzyme was not greatly altered by incubation with 20 mM dithiothreitol for 5 min at 30 °C, and a somewhat greater loss

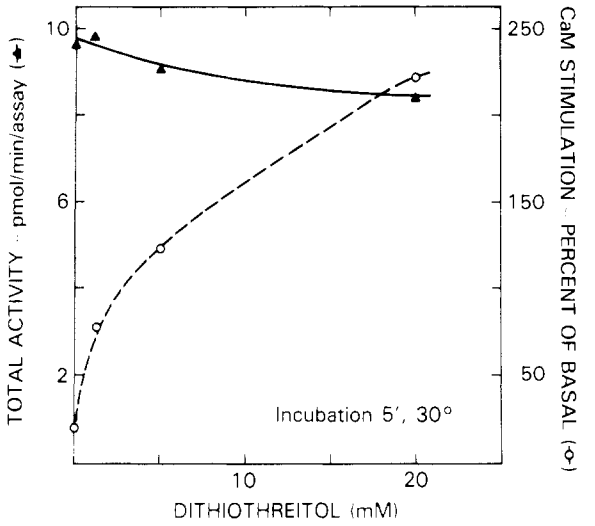

FIGURE 4: Effec incubation with dithiothreitol on activity of the cross-linked phosphodiesterase–CaM complex. Samples (~4 ng) of pooled peak fractions from chromatography of cross-linked phosphodiesterase (see Figure 2) were incubated in 10 mM BES, pH 7.2, containing 0.2 mM EDTA, bovine serum albumin (0.5 mg/mL), and the indicated concentration of dithiothreitol (total volume 120 μL) for 5 min at 30 °C. Samples were placed on ice, and 30-μL portions were immediately assayed for basal (with 0.3 mM EGTA) and total (with 0.5 mM $CaCl_2$ and 50 nM CaM) enzyme activity. Total activity (▲) and percentage increase in activity produced by saturating Ca^{2+} plus CaM (○) are plotted as a function of dithiothreitol concentration.

in total activity (36%) was observed (data not shown). Incubation of the cross-linked enzyme with dithiothreitol at lower temperatures also reduced basal activity but more slowly than at 30 °C. At temperatures above 30 °C, substantial loss of total enzyme activity occurred (data not shown).

Discussion

The data presented here provide the first description of an enzymatically active, cross-linked complex between CaM and one of its binding proteins, cyclic nucleotide phosphodiesterase. Since most cross-linking procedures used to investigate protein–protein interactions employ bifunctional cross-linking agents of relatively broad specificity (e.g., reactivity toward primary amino groups) with empirically determined ratios of cross-linking agent and protein(s), characterization and quantitation of cross-linked species are difficult to achieve, and preservation of enzymatic activity is seldom described. Other problems often encountered include difficulty in obtaining quantitative conversion of the desired protein to a cross-linked form and/or formation of large cross-linked aggregates of homologous protein species. The approach described obviates many of these problems. By use of an activated form of one protein, which itself provides the source of cross-linking potential, no additional cross-linking agent is required. Other advantages include the ability to characterize the number and topology of substituent groups and define rigorously the requirements for optimal interaction and cross-linking. Many of these considerations have been discussed (Kincaid & Vaughan, 1983).

All of the observations reported here are consistent with the conclusion that PDP-CaM and the phosphodiesterase interact to form an activated disulfide-linked complex. Although the activation constants for native and PDP-CaM were comparable, after incubation of phosphodiesterase and PDP-CaM at concentrations 10 times their interaction constant, activation of the enzyme by roughly stoichiometric amounts of PDP-CaM was demonstrated when assayed under dilute conditions (one-tenth the interaction constant). Prior incubation with native CaM did not alter its apparent activation constant as would be expected for a reversibly associating system.

On high-performance gel-permeation chromatography, the phosphodiesterase–PDP-CaM complex behaved as a larger molecule than the native enzyme. Estimates of molecular weight of the complex yielded values of ~200 000, somewhat greater than expected for a tetrameric structure ($\alpha_2\beta_2$). However, the shape of the complex may not be globular, and further characterization of its physical properties is in progress. Conversion of phosphodiesterase to a cross-linked form appears to have been essentially complete, since enzyme activity eluted in a symmetrical peak with an equivalent degree of activation in all fractions and addition of native CaM increased activity only 10% above that observed with Ca^{2+} alone.

Reduction of the reactive sulfhydryl groups of PDP-CaM before incubation with phosphodiesterase prevented the changes in physical and functional properties of the enzyme, suggesting that disulfide bonding is involved in complex formation. This view is supported by the observation that incubation of the isolated PDP-CaM–phosphodiesterase complex with dithiothreitol resulted in a reversal of the activated state in a fashion dependent on both reductant concentration (Figure 4) and incubation temperature (unpublished data). It may be inferred that a free sulfhydryl group(s) is (are) present at or near the site of interaction with CaM in this and perhaps other[2] CaM-binding proteins. Localization of the PDP substituents on CaM may facilitate identification of the domains involved in its interaction with binding proteins.

The observed changes in enzymatic properties are consistent with irreversible binding of PDP-CaM to the phosphodiesterase. The change in the pattern of substrate hydrolysis in the presence of metal chelators to one characteristic of a CaM-activated state and the inability of CaM antagonists (trifluoperazine and W-7) to inhibit the activity measured in the presence of calcium strongly support the formation of a stable, cross-linked complex. Furthermore, since full activity is preserved without a discernible change in substrate affinity, it appears that the fundamental enzymatic properties of phosphodiesterase have not been greatly altered.

The observation that activity of the phosphodiesterase–PDP-CaM complex assayed in the presence of EGTA is 70–90% of the maximal activity could be explained in several ways, which are being investigated. Covalent complex formation might greatly increase calcium sensitivity such that even in the presence of excess EGTA there is sufficient free calcium to activate. (Calculated free calcium concentrations in the assays with EGTA were in the nanomolar range.) Alternatively, Ca^{2+} bound to PDP-CaM at the time of its interaction with the enzyme may be sequestered in the complex, i.e., not freely dissociable and available for chelation. A third, perhaps most interesting, possibility is that formation of the covalent complex fixes the phosphodiesterase in an activated conformation, maintenance of which no longer requires Ca^{2+} binding to CaM.

Because of the covalent but reversible nature of the disulfide linkage, CaM-binding protein complexes like that described here may be particularly useful for investigation of the mechanisms of CaM activation and the action of drugs or other effectors on enzyme activity. Used in conjunction with selective proteolytic cleavage, they may also facilitate identification of specific binding protein domains with which CaM interacts.

[2] Apparent formation of complexes of PDP-CaM with calcineurin and myosin light chain kinase have been observed as well (data to be published).

Acknowledgments

The assistance and valuable advice of Dr. Martha Vaughan in preparation of the manuscript is gratefully acknowledged. I also thank Dr. Hideo Hidaka for kindly providing a gift of W-7, D. Marie Sherwood and Barbara Mihalko for expert secretarial help, and Carol Coulson for excellent technical assistance in some of the experiments.

Registry No. cGMP, 7665-99-8; W-7, 65595-90-6; Ca, 7440-70-2; trifluoperazine, 117-89-5; dithiothreitol, 3483-12-3.

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Binding of β-Scorpion Toxin: A Physicochemical Study†

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ABSTRACT: The binding to rat brain synaptosomes of a β-scorpion toxin, i.e., toxin II of *Centruroides suffusus suffusus* (Css II), was studied as a function of pH, temperature, and concentration of some monovalent and divalent cations. At 10 °C and pH 6.0, the specific binding of ^{125}I-labeled Css II corresponds to a single class of noninteracting high-affinity binding sites (K_D = 0.18 nM) with a capacity (4.2 pmol/mg of protein) that is almost identical with that generally accepted for saxitoxin. The equilibrium dissociation constant of β-scorpion toxin is pH independent, but the maximum binding capacity is reduced with increasing pH. Li^+, guanidinium, Ca^{2+}, Mg^{2+}, and Mn^{2+} modified the apparent K_D of the ^{125}I-labeled Css II toxin. The equilibrium dissociation constant varies markedly with the temperature. The van't Hoff plot of the data is curvilinear, corresponding to a standard free-energy change associated with an entropy-driven process. The association rate constant also varies considerably with the temperature whereas the Arrhenius plot is linear between 1 and 30 °C. The energy of activation determined from these data is 17.6 kcal/mol. These results support the hypothesis that a cluster of nonpolar amino acid residues present on one face of the molecule is involved in the toxin–receptor interaction.

A large number of neurotoxins of animal or plant origin modify the properties of the sodium channel involved in the action potential of excitable cells. Some have been radiolabeled and their binding properties compared to their pharmacological effect. These investigations lead Catterall (1980, 1982) to suggest the existence of three distinct neurotoxin binding sites associated with the sodium channel. Site "1" binds saxitoxin and tetrodotoxin, which are heterocyclic molecules with a guanidium group. Sodium transport is inhibited when this site, located on the outer side of the membrane, is occupied. Site "2" binds several liposoluble toxins, e.g., grayanotoxin and the alkaloids batrachotoxin, veratridine, and aconitine. These toxins cause persistent activation of sodium channels at the resting membrane potential by blocking channel inactivation and by shifting the voltage dependence of sodium-channel activation to more negative membrane potentials. Site "3" recognizes the scorpion neurotoxins that we have called α-scorpion toxins (Jover et al., 1980) and sea anemone neurotoxins. These neurotoxins slow or block the sodium-channel inactivation phase.

We have identified a fourth site linked to the sodium channel that recognizes neurotoxin II of *Centruroides suffusus suffusus* venom (Css II), a β-scorpion toxin (Jover et al., 1980). Contrary to α-scorpion toxins, the affinity of β-scorpion toxins for their receptor site does not vary with the membrane potential. Css II binding to site 4 modifies sodium-channel activation, as shown by the occurrence of an abnormal sodium current after repolarization of the myelinated frog nerve (Couraud et al., 1982) and by the blocking of sodium conductance activation in the frog skeletal muscle (Jaimovich et al., 1982). Css II toxin inhibits the uptake and stimulates the release of γ-aminobutyric acid by rat brain synaptosomes, an effect that is abolished by tetrodotoxin (Couraud et al., 1982). Two toxins from the venom of the scorpion *Centruroides sculpturatus* Ewing showed similar effects to those of Css II on the node of Ranvier of the myelinated frog nerve (Meves et al., 1982), and a toxin from *Tityus serrulatus* venom competes with Css II at the same binding site (Wheeler et al., 1982). The present paper describes a physicochemical study of a β-scorpion toxin (Css II) interaction with site 4.

The association and dissociation kinetic constants of this toxin have been determined and compared with those of saxitoxin as defined by Weigele & Barchi (1978a) and those of toxin II of *Androctonus australis* Hector, an α-scorpion toxin, which we have measured in the same biological system (Jover et al., 1978). In addition, the thermodynamic parameters in the ligand–receptor reaction were estimated. The results obtained suggest that the interaction of Css II with its receptor involves polar attraction, which may be followed by

† From the Laboratoire de Biochimie and U.172 de l'I.N.S.E.R.M., Faculté de Médecine, 13326 Marseille Cedex 15, France. *Received May 16, 1983.* This work was supported in part by the CNRS (ERA 070617, ATP Pharmacologie des Récepteurs Neuromédiateurs).

0006-2960/84/0423-1147$01.50/0