Hydrophobic interaction chromatography is employed to determine if calmodulin might associate with its target enzymes such as cyclic nucleotide phosphodiesterase and calcineurin through its Ca\(^{2+}\)-induced hydrophobic binding region. The majority of protein in a bovine brain extract that binds to a calmodulin-Sepharose affinity column also is observed to bind in a metal ion-independent manner to phenyl-Sepharose through hydrophobic interactions. Cyclic nucleotide phosphodiesterase activity that is bound to phenyl-Sepharose can be resolved into two activity peaks; one peak of activity is eluted with low ionic strength buffer, while the second peak eluted with an ethylene glycol gradient. Calcineurin bound tightly to the phenyl-Sepharose column and could only be eluted with 8 M urea. Increasing ethylene glycol concentrations in the reaction mixture selectively inhibited the ability of calmodulin to stimulate phosphodiesterase activity, suggesting that hydrophobic interaction is required for activation. Comparison of the proteins which are bound to and eluted from phenyl- and calmodulin-Sepharose affinity columns indicates that chromatography involving calmodulin-Sepharose resembles hydrophobic interaction chromatography with charged ligands. In this type of interaction, hydrophobic binding either is reinforced by electrostatic attractions or opposed by electrostatic repulsions to create a degree of specificity in the binding of calmodulin to certain proteins with accessible hydrophobic regions.

Calmodulin, a ubiquitous Ca\(^{2+}\)-binding protein, mediates numerous Ca\(^{2+}\)-regulated enzyme systems and cellular processes (see Refs. 1 and 2). The site(s) through which calmodulin interacts with its target enzymes and the nature of this interaction are not clearly understood. Studies conducted on selective binding of antipsychotic phenothiazine drugs and their ability to inhibit calmodulin-mediated activation of target enzymes led to the identification of a Ca\(^{2+}\)-induced hydrophobic region on calmodulin (3). This was confirmed by fluorescence probe analysis for hydrophobic sites (4). Recently, we demonstrated the strongly hydrophobic nature of a Ca\(^{2+}\)-induced site on calmodulin by using phenyl-Sepharose hydrophobic interaction chromatography (6). This information was utilized to develop a rapid purification procedure for calmodulin (6).

Several agents such as drugs (adriamycin, vincalkaloids), polypeptides (melittin, \(\beta\)-endorphin), and nonionic detergents (Triton X-100) which are of diverse structure but which have hydrophobic properties are known to interact with calmodulin to prevent its activation of target enzymes (7-11). The inhibitory action may be due to the binding of these agents to the hydrophobic region on calmodulin to convert it to an inactive conformation. Or, these agents may inhibit by binding to both calmodulin and to the target protein, thereby effectively preventing their association. If the latter is true, the calmodulin target proteins also must possess accessible hydrophobic regions. While this has been suggested previously (4, 5), it has not been effectively demonstrated.

To better understand the nature of the calmodulin binding site on target proteins, we have studied the calmodulin-dependent cyclic nucleotide phosphodiesterase and the calmodulin inhibitor protein calcineurin (12, 13). Recently, calcineurin was reported to be a Ca\(^{2+}\)-dependent phosphoprotein phosphatase (14). The use of fluorescent probes to study the hydrophobic region of proteins requires high concentrations (10 mg protein/ml) of a homogeneous protein preparation. In the case of the calmodulin binding proteins, sufficient amounts of purified preparation have proven difficult to obtain. This is particularly so for the calmodulin-activated cyclic nucleotide phosphodiesterase which is unstable and rapidly loses its dependence on calmodulin with purification.

Hydrophobic interaction chromatography, however, can be used to detect and characterize hydrophobic regions even on partially purified proteins (15, 16). In this study, we present evidence for the presence of a metal ion-independent hydrophobic binding region on both cyclic nucleotide phosphodiesterase and calcineurin through which calmodulin can bind. The importance of hydrophobic interaction with calmodulin to stimulate phosphodiesterase activity is also established.

**EXPERIMENTAL PROCEDURES**

**Materials**

Phenylo-Sepharose and Sepharose 4B were obtained from Pharmacia. Calmodulin was purified from bovine brain by phenyl-Sepharose hydrophobic interaction chromatography as described previously (6). Calmodulin-Sepharose (~0.05 \(\mu\)mol of calmodulin/g of gel, wet weight) was prepared by coupling calmodulin to cyanogen bromide-activated Sepharose (17).

**Methods**

**Preparation of Calmodulin Binding Protein Fractions**—A calmodulin depleted bovine brain (Pel-Freeze Biologicals, Rogers, AR) protein fraction containing cyclic nucleotide phosphodiesterase and calcineurin was prepared as described by Klee and Krinks (18) and...
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modified to include Nε-p-tosyl-L-lysine chloromethyl ketone in all buffers. Briefly, bovine brain (600 g) was homogenized in 4 volumes of 0.05 M Tris-HCl, pH 7.5, containing 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 5 mM 2-mercaptoethanol. The 13,000 × g supernatant protein fraction was precipitated with a 30 to 60% ammonium sulfate cut, dialyzed, and then applied to a DEAE-cellulose column. The protein fraction which eluted with 0.15 M ammonium sulfate was used for calmodulin-Sepharose affinity chromatography. This protein fraction was dialyzed versus Buffer A (0.04 M Tris-HCl, pH 7.4, 0.05 M NaCl, 2 mM MgCl₂, 0.2 mM dithiothreitol) and applied to a 60-ml bed volume calmodulin-Sepharose affinity column. After washing successively with 3 bed volumes of Buffer A and 3 bed volumes of Buffer B containing 0.2 M NaCl, bound protein was eluted with Buffer A containing 2 mM EGTA in place of 0.2 mM CaCl₂. Protein eluted with the EGTA-Buffer A was dialyzed versus Buffer A. Protein not adsorbed initially to the calmodulin-Sepharose affinity column was reapplied to a second calmodulin-Sepharose column to ensure complete removal of calmodulin binding proteins from this fraction. This unbound protein fraction which does not exhibit any affinity toward calmodulin also was subjected to phenyl-Sepharose chromatography as described below to further characterize the hydrophobic nature of these proteins.

Characterization of Calmodulin Binding Proteins by Hydrophobic Interaction Chromatography—Half of the dialyzed protein fraction obtained by calmodulin-Sepharose affinity chromatography was subjected to phenyl-Sepharose (40 µmol of ligands/ml) hydrophobic interaction chromatography (10-ml bed volume), while the other half was rechromatographed on calmodulin-Sepharose to characterize the type of binding these proteins exhibit toward calmodulin.

Assays—Calmodulin-dependent cyclic nucleotide phosphodiesterase activity was assayed at 30 °C in the presence of 0.2 mM ammonium chloride as described by Klee et al. (19). Calcineurin was determined by its ability to inhibit calmodulin stimulation of phosphodiesterase activity (18) or by identification by characteristic migration of its two subunits on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12). Protein concentration was estimated by 280-nm absorbance or was determined by the method of Bradford (20).

RESULTS AND DISCUSSION

Phenyl-Sepharose Hydrophobic Interaction Chromatography—The protein fraction which bound to and was eluted from a calmodulin-Sepharose column was applied to a hydrophobic phenyl-Sepharose column. The majority of the calmodulin binding protein fraction applied to the hydrophobic phenyl-Sepharose column was adsorbed, including calmodulin-stimulated cyclic nucleotide phosphodiesterase (Fig. 1A) and calcineurin (Fig. 2, lane 5). Protein which did not bind during initial application also did not bind with reapplication to a second phenyl-Sepharose column. The calmodulin binding proteins which bound to the phenyl-Sepharose column did not elute with a high salt (1 M NaCl) wash, suggesting that the binding of these proteins to the phenyl affinity column is not mediated through ionic or hydrogen bonding. Rather, adsorption must be due to hydrophobic forces. Even when the calmodulin binding protein fraction was applied in low ionic strength buffer (with 0.05 M NaCl) and at low temperature (4 °C) where hydrophobic interactions are weak, all of the protein bound to phenyl-Sepharose. Such strong binding under suboptimal conditions indicates that these proteins are clearly capable of binding to other hydrophobic ligands in vivo.

Several of the calmodulin-dependent enzymes bind metal ions. Phosphodiesterase activity is stimulated by divalent cations such as Mg²⁺ and Mn²⁺ (21), while calcineurin B is a Ca²⁺-binding protein. To determine if metal ions can influence or expose the hydrophobic region of these proteins, preparations of calmodulin binding proteins were chromatographed over phenyl-Sepharose in the presence of different metal ions or in the presence of 1 mM EDTA. The results obtained indicate that metal ions do not alter either the binding to or elution of these proteins from the hydrophobic column. Thus, the calmodulin target proteins apparently have metal ion-independent hydrophobic sites. This is opposed to calmodulin itself where Ca²⁺ binding is required to expose a hydrophobic site (6).

Protein binding to hydrophobic adsorbents is due to multiple contact interactions (15). The strength of this interaction is proportional to the surface area of the hydrophobic region exposed and to the hydrophobicity of the amino acid side chains that are involved (22). Thus, to determine the degree of hydrophobic interactions between the calmodulin binding proteins and the phenyl-Sepharose affinity column, various agents and buffer systems were used to elute the bound proteins from this column.

As presented in Fig. 1A, buffer without salt eluted protein peak I which contained 30 to 50% of the initially applied cyclic nucleotide phosphodiesterase activity. A more hydrophobic second protein fraction (peak II) which contained the remaining phosphodiesterase activity eluted with a 0 to 50% ethylene glycol gradient. If this column is eluted directly with an ethylene glycol gradient without the prior low ionic strength buffer wash, then peak I phosphodiesterase elutes in the first fractions of the ethylene glycol gradient, while peak II enzyme

1 The abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid.


Fig. 1. Phenyl-Sepharose hydrophobic interaction chromatography of calmodulin binding and nonbinding protein fractions from bovine brain. A, the protein fraction which bound to and was eluted from a calmodulin-Sepharose affinity column was applied to a phenyl-Sepharose column (1.5 × 6 cm) equilibrated with Buffer B (0.04 M Tris-HCl, pH 7.5, 1 mM EDTA, 0.2 mM dithiothreitol) and applied to a 60-ml bed volume calmodulin-Sepharose affinity column. After washing successively with 3 bed volumes of buffer A and 3 bed volumes of Buffer B containing 0.2 M NaCl, bound protein was eluted with Buffer A containing 2 mM EGTA in place of 0.2 mM CaCl₂. Protein eluted with the EGTA-Buffer A was dialyzed versus Buffer A. Protein not adsorbed initially to the calmodulin-Sepharose affinity column was reapplied to a second calmodulin-Sepharose column to ensure complete removal of calmodulin binding proteins from this fraction. This unbound protein fraction which does not exhibit any affinity toward calmodulin also was subjected to phenyl-Sepharose chromatography as described below to further characterize the hydrophobic nature of these proteins.

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is eluted in later fractions. This indicates the hydrophobic nature of the peak I enzyme. However, peak I and peak II enzymes are not completely separated using ethylene glycol gradient elution, while low ionic strength buffer elution of peak I polyphosphodiesterase followed by ethylene glycol elution of peak II phosphodiesterase does give complete separation. The sum of the phosphodiesterase activities recovered in these two fractions were slightly higher than that initially applied to the column, suggesting an underestimate of phosphodiesterase activity in the initially applied sample. Highly hydrophobic protein, which included calcineurin, finally eluted with 8 M urea. Bound cyclic nucleotide phosphodiesterase could not be eluted with Buffer A containing 1 mM cyclic GMP or cyclic AMP, suggesting that this enzyme binds to phenyl residues through a site other than its substrate binding site. The two peaks of phosphodiesterase activity were found to be not interconvertible by rechromatography of the separated fractions, either after storage for 1 week at 4°C or after further purification by affinity chromatography using Cibacron blue F3GA-coupled Sepharose (23). Of interest is the finding that peak I phosphodiesterase activity is stimulated 60- to 100-fold by calmodulin, whereas peak II activity is stimulated by only 6- to 9-fold (see Fig. 4A). While these two forms of phosphodiesterase activity could be detected at all levels of purification, as well as in the crude homogenate, they do not appear to be two separate gene products. If pepstatin is omitted from the homogenization, buffer peak I activity is low or nonexistent, suggesting that endogenous proteases may convert peak I to the peak II form by limited proteolysis. If so, the peptide fragment cleaved off must be small since both phosphodiesterase forms exhibit similar size by gel filtration.

About 15% of the cyclic nucleotide phosphodiesterase activity present in the calmodulin-free bovine brain protein extract did not bind to the calmodulin affinity column even with a second passage over such a column. This protein fraction, which does not exhibit any calmodulin binding capacity, was applied to and eluted from a phenyl-Sepharose column as described above (Fig. 1B). Cyclic nucleotide phosphodiesterase activity present in this protein fraction did bind to the phenyl-Sepharose hydrophobic column and was eluted with the ethylene glycol gradient. This form of phosphodiesterase activity did not exhibit any stimulation by calmodulin. It cannot be ruled out that this modulin-independent form of the enzyme might be a result of proteolytic modification of the modulin-dependent form of phosphodiesterase. The presence of a hydrophobic site on a calmodulin-independent, cyclic GMP-activated form of phosphodiesterase from liver has been reported (24).

Proteins present in the various fractions obtained with phenyl-Sepharose chromatography also were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2, lanes 2 to 5). The A subunit of calcineurin migrates as a Mr = 60,000 species, while the Ca2+-sensitive B subunit exhibits a mobility corresponding to Mr = 15,000 in the presence of 0.1 mM Ca2+ and Mr = 18,000 in the presence of 1 mM EDTA (22). Some calcineurin is noted in fractions eluted from phenyl-Sepharose with the ethylene glycol gradient (Fig. 2, lane 4). However, the majority of calcineurin is tightly bound to this hydrophobic column and eluted only with 8 M urea (Fig. 2, lane 5).

Calmodulin-Sepharose Hydrophobic Interaction Chromatography—It is apparent from these phenyl-Sepharose hydrophobic interaction chromatography studies that the calmodulin binding proteins, as well as calmodulin itself, possess hydrophobic regions. Thus, we wanted to establish if these hydrophobic regions might be involved in target protein-calmodulin interaction. The relative strength of such hydrophobic binding might relate to the relative affinity of these proteins for calmodulin.

With the use of a limited size calmodulin-Sepharose column, it was possible to separate out proteins which exhibit low affinity calmodulin binding as these proteins are displaced from calmodulin by proteins which bind strongly and with high affinity. The protein fraction which initially bound to and was eluted from the preparative calmodulin affinity column was reapplied to a limited size calmodulin-Sepharose column (Fig. 3). Protein which did not bind to this second, limited size calmodulin affinity column did not possess either phosphodiesterase activity (Fig. 3) or calcineurin (see Fig. 2, lane 6). Tightly bound protein could not be eluted with 1 mM NaCl, again indicating that these proteins are not bound to calmodulin solely by ionic interactions or hydrogen bonding. At the same time, one cannot rule out the participation of such binding to complement hydrophobic interactions. Ethylene glycol (50%) does elute some protein which apparently associates with calmodulin through hydrophobic binding alone (Fig. 3). This fraction eluted with 50% ethylene glycol did not contain either phosphodiesterase activity or calcineurin (see Fig. 2, lane 7). Washing with 50% ethylene glycol in the presence of 0.2 or 0.5 mM NaCl did not elute additional protein. Finally, washing with 2 mM EGTA eluted a protein fraction which contained about 80% of the phosphodiesterase activity and calcineurin initially applied to the column (Fig. 3, Fig. 2, lane 8). The combination of 50% ethylene glycol along with 0.5 mM NaCl was used in an attempt to decrease both hydrophobic and ionic interactions simultaneously. However, the combination of these two reagents has certain limitations due to the opposing action of one reagent toward the other. Ethylene glycol lowers the dielectric constant of the medium and promotes ionic interaction, while the presence of salt would...
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Enhance hydrophobic interaction as it decreases ionic binding. Inspite of these opposite effects, it is possible to elute proteins from the affinity column with a combination of these two reagents provided the protein-ligand interaction exhibits weak hydrophobic and ionic characteristics. The failure to elute phosphodiesterase from the calmodulin-Sepharose column with a combination of ethylene glycol and NaCl indicates this enzyme binds to calmodulin with strongly hydrophobic and ionic interactions. In a related study, it was noted that troponin-I also binds to calmodulin-Sepharose in a Ca\textsuperscript{2+} -dependent manner.\textsuperscript{4} The bound troponin-I is not eluted with either 50% ethylene glycol or with 0.4 M NaCl alone, but it is eluted with a combination of the two reagents.

Although both the phenyl group and calmodulin represent hydrophobic ligands attached to Sepharose, the phenyl residue is uncharged, while calmodulin is a negatively charged protein (pI 4.3). This difference in charge probably accounts for some of the differences noted in the binding pattern of applied proteins. For example, a \( M \text{r} = 43,000 \) protein exhibits tight binding to phenyl-Sepharose (elutes with 8 M urea) (Fig. 2, lane 5), while this \( M \text{r} = 43,000 \) protein exhibits low affinity binding properties to calmodulin (does not bind to the limited size column (Fig. 2, lane 6). As a second example, phosphodiesterase activity bound to phenyl-Sepharose can be eluted with low ionic strength buffer (peak I) or with 30% ethylene glycol (peak II), while phosphodiesterase activity bound to calmodulin-Sepharose can not be eluted with 50% ethylene glycol. This suggests that phosphodiesterase binds to calmodulin through other types of interactions such as ionic and/or hydrogen bonding in addition to hydrophobic forces. It also was observed that some protein which did not bind to calmodulin-Sepharose nonetheless bound to phenyl-Sepharose, and the total amount of protein that bound to phenyl-Sepharose was several fold greater than the amount which bound to calmodulin-Sepharose (Fig. 1).

In other studies, it was noted that Ca\textsuperscript{2+} binding to troponin-C from skeletal muscle and to S-100 protein from bovine brain exposes a hydrophobic region to promote tight binding to phenyl-Sepharose.\textsuperscript{5} Yet, neither of these proteins exhibited binding to calmodulin-Sepharose, apparently because of electrostatic repulsions. While both calmodulin and troponin-C bind tightly to charge-free hydrophobic ligands, neither protein binds to negatively charged hydrophobic ligands such as 3-carboxypropionylaminodecane-Sepharose.\textsuperscript{6} These results further indicate that electrostatic repulsions can regulate hydrophobic interactions.

It is thus apparent that the presence of exposed hydrophobic regions on certain proteins is not the sole criteria for determining specific binding to calmodulin. Rather, the isoelectric point of the protein, as well as the ionic environment surrounding the hydrophobic region, apparently play a critical role in modulating such interactions. Furthermore, the disposition and availability of amino acid side chains, which will determine the complementarity of the protein surfaces at the point of protein-protein interaction, will play a crucial role in determining the degree of affinity. Calmodulin affinity chromatography seems to resemble hydrophobic chromatography with charged ligands where hydrophobic interactions can be reinforced by electrostatic attractive forces or opposed by electrostatic repulsions (25). Such considerations are probably responsible for the varying degree of affinity shown by different proteins in binding to calmodulin.

The presence of hydrophobic regions on the calmodulin target proteins might be responsible for the association of significant amounts of these proteins with particulate subcellular fractions. If so, alterations in hydrophobicity may be responsible for altered localization (translocation) of these proteins between particulate and cytosolic fractions. Furthermore, the presence of hydrophobic regions could be responsible for the varying degree of affinity shown by different proteins in binding to calmodulin.

Effect of Ethylene Glycol on Calmodulin Activation of Phosphodiesterase—To determine if hydrophobic interactions play a role in calmodulin activation of cyclic nucleotide phosphodiesterase activity, increasing concentrations of ethylene glycol were included in the reaction mixture. Ethylene glycol at concentrations between 7 and 50% progressively inhibited both peak I and peak II calmodulin-stimulated phosphodiesterase activities (Fig. 4A). Peak II basal phosphodiesterase activity was not appreciably altered by increasing ethylene glycol, while peak I basal activity was enhanced in the presence of 30 to 50% ethylene glycol. As presented in Fig. 4B, ethylene glycol markedly decreases the ability of calmodulin to activate this enzymatic activity as shown by the decrease in fold stimulation observed with calmodulin.

Conceivably, the presence of ethylene glycol could perturb protein conformations to elicit this decrease in calmodulin stimulation of phosphodiesterase activity. However, Tanford (28) has shown that the concentration of ethylene glycol must be well above 50% before the first indications of a conformational change can be observed. Water/ethylene glycol mixtures have very high viscosity and a dielectric constant considerably below that of water, so aqueous ethylene glycol solutions of proteins may be prepared without affecting the molecular properties of the protein (29). We have observed...
modulin to activate a given target enzyme because of its tendency to readily self-aggregate. Due to the acidic nature, the hydrophobic Ca\(^{2+}\)-calmodulin complex does not self-aggregate because of ionic repulsive forces and thus is able to function efficiently to interact with certain hydrophobic proteins.

Additional studies are required to determine if other calmodulin-dependent enzymes such as the myosin light chain kinase and (Ca\(^{2+}\), Mg\(^{2+}\))-ATPase possess similar accessible hydrophobic sites. This will shed additional light on the mechanism of calmodulin activation of various enzymes and might help explain the relative affinity exhibited by different proteins for calmodulin. Conceivably, the relative degree of affinity (dictated by a combination of hydrophobic and ionic interactions) might play a role in determining the sequential order of activation of these enzymes by the Ca\(^{2+}\)-calmodulin complex after the initiation of increased intracellular Ca\(^{2+}\) by a given physiological stimulus.

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**FIG. 4. Effect of increasing ethylene glycol concentration on calmodulin stimulation of cyclic nucleotide phosphodiesterase activity**

A, peak I (open circles) and peak II (closed circles) activities. A, activity of peak I and peak II cyclic nucleotide phosphodiesterases obtained with phenyl-Sepharose chromatography (Fig. 4A). Enzyme activity was determined at 30 °C in the presence (solid lines) and absence (dashed lines) of 1 μM calmodulin with 0.2 mM NH₄Cl and the indicated concentration of ethylene glycol. B, fold stimulation noted with calmodulin at the indicated concentrations of ethylene glycol was calculated from the results presented in A.

that phosphodiesterase activity is stable in the presence of 30 to 50% ethylene glycol at 4 °C for at least 1 week and at ~70 °C for at least 1 month. Thus, 50% ethylene glycol does not have an adverse effect on the stability of this enzyme. If ethylene glycol were producing drastic changes in phosphodiesterase conformation, it might be expected that basal activity (calmodulin-independent) would be altered. Since only the calmodulin-stimulated activity is decreased, it is likely that ethylene glycol prevents the activation of phosphodiesterase by decreasing hydrophobic interactions.

Yet, it is unlikely that ethylene glycol completely prevents the association of calmodulin with phosphodiesterase. Even though calmodulin-stimulated phosphodiesterase activity is inhibited completely with 50% ethylene glycol, this concentration of ethylene glycol alone is unable to elute phosphodiesterase from a calmodulin-Sepharose affinity column at 4 °C. Since hydrophobic interactions are weaker at 4 °C than at 30 °C, it seems likely that calmodulin does bind to phosphodiesterase even in the presence of ethylene glycol during assay at 30 °C, but that this binding is not sufficient to activate the enzyme due to weakening of hydrophobic interactions.

Certain calmodulin target enzymes have been shown to be activated by charged hydrophobic ligands such as lysophosphatidylcholine, phosphatidylserine, sodium dodecyl sulfate, and unsaturated fatty acids (4, 30, 31). However, significantly greater amounts of these agents are required relative to calmodulin to activate a given target enzyme because of their tendency to readily self-aggregate. Due to its acidic nature, the hydrophobic Ca\(^{2+}\)-calmodulin complex does not self-aggregate because of ionic repulsive forces and thus is able to function efficiently to interact with certain hydrophobic proteins.
Calmodulin interacts with cyclic nucleotide phosphodiesterase and calcineurin by binding to a metal ion-independent hydrophobic region on these proteins.

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