Demonstration of the Hydrophilic Character of Adenylate Cyclase following Hydrophobic Resolution on Immobilized Alkyl Residues

CRITICAL ROLE OF ALKYL CHAIN LENGTH*

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The hydrophobic character of the membrane-bound enzyme, adenylate cyclase, was investigated by examining its interaction with a variety of noncharged hydrophobic resins consisting of long chain alkyl groups linked to Sepharose via ether bonds. Either dodecyl or cetyl resins effected the separation of cyclase from more hydrophobic proteins while octyl-, hexyl-, and butyl-Sepharose did not prevent protein aggregation. A critical factor in the resolving capacity of these long chain alkyl resins was their low degree of substitution. Following hydrophobic resolution, gel filtration with Sepharose 6B in the absence of detergent produced a single symmetrical peak of activity in the included volume of the gel duplicating elution patterns with detergent of the unresolved enzyme. Following this step, several fractionation procedures that had previously been ineffective proved successful. Ion exchange chromatography resulted in a single symmetrical peak of activity eluting over a discrete portion of the gradient in contrast to the broad, trailing pattern obtained prior to hydrophobic chromatography. Adenylate cyclase, which previously behaved as a hydrophobic protein, could now be manipulated as a conventional hydrophobic protein. The enzyme now also adhered to a benzyl-Sepharose resin in 1 M NaCl and was eluted with a reverse salt gradient. Resins of similar hydrophobicity such as hexyl- or butyl-Sepharose, bound the enzyme weakly or not at all, which suggested that II-II interactions played a significant role in cyclase adsorption to the benzyl resin. These results suggest that alkyl residues immobilized on a noncharged matrix behave as solid phase detergents by preventing protein aggregation and simultaneously promoting separation of membrane proteins of varying hydrophobicity. They are likely to provide new and powerful tools in the fractionation and isolation of membrane-bound proteins.

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Adenylate cyclase appears to fit into the latter of these categories (8). In a systematic attempt to resolve solubilized membrane proteins on the basis of their intrinsic hydrophobicity, a series of noncharged hydrophobic resins was synthesized. Following its separation from detergent and other membrane proteins on such a resin, the hydrophobic character of adenylate cyclase was apparent as seen by its altered behavior during various chromatographic procedures. Furthermore, the hydrophobic properties of this membrane-bound enzyme could now be more clearly characterized by its binding to both alkyl and aryl resins which previously had not prevented its aggregation.

EXPERIMENTAL PROCEDURES

Materials

1-Butanol, 1-hexanol, 1-octanol, 1-dodecanol, n-cetyl alcohol, benzyl alcohol, epichlorhydrin, and boron trifluoride etherate were purchased from Aldrich Chemical Co. Phosphocreatine, creatine phosphokinase, cyclic adenosine monophosphate, adenosine triphosphate, the sodium or Tris salt, were obtained from Sigma. Dowex AG 50W-X4 was from Bio-Rad and Alumina, Brockman Activity grade I, was from Merck or Fisher. Adenosine [3H]triphosphate was either from ICN (5 to 10 Ci/mmol) or from New England Nuclear (6 to 13 Ci/mmol) or from New England Nuclear. Sepharose 4B and 6B and Sephadex G-25 were purchased from Pharmacia. Whatman DE52 was from Metropolitan Supply, Boston, Mass. The scintillation fluids, Bray's and 3a70B, were, respectively, from New England Nuclear and Research Products International, Elk Grove Village, Ill. Deuterated dimethylsulfoxide was from Merck and D2O was purchased from Bio-Rad. Brij 56 and 58 were gifts of ICI. Triton X-100 was purchased from Packard. NMR spectra were performed on a Varian T-60. Infrared spectra were run on a Perkin-Elmer, model 237B.

Methods

Synthesis of Alkyl and Aryl Glycidyl Ethers—Benzyl, butyl, hexyl, octyl, and dodecyl glycidyl ethers were synthesized according to the method of Ulbrich et al. (13). In the case of cetyl glycidyl ether, the cyclization with NaOH was followed by transfer of the reactants to diethyl ether which was then washed twice with water and dried over MgSO4. Each glycidyl ether was purified by fractional distillation under vacuum when necessary. In particular, cetyl glycidyl ether was distilled at 6 mm Hg at a temperature of 235°. The fractions were identified and their purity was assessed by infrared and nuclear magnetic resonance (NMR) spectroscopy. All fractions distilled within 2-3° of the expected temperature. Synthesis of the hydrophobic gels was performed according to the method of Hjerten et al. (14). Degree of substitution was determined according to the method of Rosengren et al. (15). Multiple determinations with the same resin indicated that there was a 10% average error inherent in this procedure. It should be noted that in resins with low degrees of substitution, the signal from the terminal CH3 protons was diminutive and in certain cases it could not be integrated. The degree of substitution could then be computed from CH3 protons which are present in a constant proportion to the terminal-CH3 protons for any given alkyl residue. Certain gels were so lightly substituted that the alkyl chain proton signal could not be detected by this technique. Therefore, comparison of the hydrophobicity of the various gels synthesized from different chain length glycidyl ethers was also made by determining the salt concentrations at which a particular gel bound and released a protein standard, myoglobin.

Adenylate Cyclase Assay—The assay is a modification of the method of Salomon et al. (16). A 60-μl assay mixture contains 20 mm creatine phosphate, 6 units/ml of creatine phosphokinase, 1 mm cyclic AMP with approximately 2,000 cpm of cyclic [3H]AMP, 25 mm Tris, pH 7.5, 3.5 mm MgCl2, 0.5 mm (α,β-ATP)2 (10 to 20 Ci/mmol) in addition to the enzyme preparation. After a 10- or 15-min incubation, 50 μl of the stopping solution was added which contained 40 mm ATP, 10 mm cyclic AMP, and 2% sodium dodecyl sulfate. The sample was then heated in a 100° dry bath for 2.5 min. Then 0.9 ml of H2O was added to each tube and this was applied to a 1-ml Dowex AG 50W-X8 column (200 to 400 mesh, 15 × 0.6 cm) equilibrated in H2O. The columns were then washed with 2 ml of H2O and were eluted with 3 ml of H2O directly onto 0.6-ml alumina columns, Brockman Activity Grade I, equilibrated in H2O. These were then washed with 3.0 ml of a 50 mm Tris solution, pH 7.4, directly into scintillation vials containing 7.5 ml of a 3a70B counting mixture and 10-min counts were obtained. Tritium efficiency was 30%; 3P efficiency was 75 to 80%. All counts were then corrected for recovery based on cyclic [3H]AMP. In general, cyclic AMP recovery ranged from 50 to 90%.

This assay produced blanks that approached background.

Preparation of Soluble Adenylate Cyclase—Canine left ventricle was obtained from freshly exsanguinated animals previously anesthetized with sodium pentobarbital. The endocardium and epicardium were excised and the myocardium was homogenized in a Waring Blender followed by a 10-s homogenization in a Polytron at high speed in 1 mm KHCO3 buffer, pH 7.4. The homogenate was then spun in a Sorvall centrifuge at 20,000 × g, and the supernatant fluid was discarded. The pellet was washed twice with the same buffer. Thereafter, 5 times the volume of a 1% Brij 56 solution in 100 ml of Tris buffer was added. The mixture was sonicated with a Toshiba motor-driven homogenizer. The homogenate was then spun at 20,000 × g; the supernatant fluid was decanted and centrifuged at 100,000 × g for 1 h in a Beckman ultracentrifuge model L28B. This procedure resulted in solubilization of approximately 60% of the total cyclase activity present in the cardiac preparation. The supernatant fluid was either used immediately or frozen in a dry ice-acetone bath and stored at −80°. Concentration of samples was achieved by addition of dry Sephadex G-25 with subsequent filtering of the supernatant under vacuum.

Gel Filtration—All gel filtration experiments were performed on the same Kontes column (98 × 2.5 cm) packed with Sepharose 4B equilibrated in 10 mm Tris, 5 mm MgCl2, 1 mm EDTA, and 1 mm diithiothreitol. Higher ionic strength buffers did not alter the elution pattern of the enzyme. A constant 1.5 ml pressure head was employed which resulted in a flow rate of 37 ml/h; 5-ml fractions were collected.

Ion Exchange Chromatography—All experiments employed a Kontes column (15 × 1.2 cm) packed with Whatman DE52 equilibrated with the same buffer. After application of the sample, the resin was washed with 1 to 2 column volumes. The column was then eluted with a continuous Tris gradient. The total volume of the gradient was 8 to 10 column volumes in each experiment. All buffers consisted of Tris/IC1, pH 7.5 (4°), 5 mm MgCl2, 1 mm EDTA, and 1 mm diethiothreitol. All columns were run at 4°. Recovery routinely was in the range of 50% in the presence or absence of detergent.

Protein determinations were performed according to the method of Lowry et al. (17) with desiccated (4°) defatted bovine serum albumin as the standard. In samples with high detergent concentrations, a precipitate was formed and subsequently removed with centrifugation without affecting the validity of the procedure.

RESULTS

Effect of Alkyl Chain Length on Resin-Cyclase Interaction—Adenylate cyclase typically aggregates on removal from detergent during filtration on Sepharose 4B and appears in the excluded volume as seen in Fig. 1A. In this set of experiments, 10-ml aliquots of Brij 56-solubilized enzyme were passed over columns of identical size containing Sepharose 4B and each of the derivatized hydrophobic resins. Following this procedure, each column was washed with 10 mm Tris buffer without
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Fig. 1. Chromatography of 10 ml of crude solubilized enzyme on unsubstituted Sepharose 4B and a variety of alkyl- and aryl-substituted resins. Approximately 1.0- to 1.5-ml fractions were collected. Column dimensions were 15 x 1.2 cm with a bed volume of 15 ml. The columns were initially equilibrated in 10 mM Tris, 5 mM MgCl₂, 1 mM EDTA, and 1 mM dithiothreitol. After sample application, each was washed with 1 to 2 column volumes and then with 0.2% and 2.0% Triton in the identical buffer as indicated. 30-µl aliquots were assayed for adenylate cyclase as indicated in the text. The degree of substitution (millimoles of glycidyl ether per mol of galactose) of each resin was as follows: benzyl and butyl, 40 to 60 mmol/mol; octyl, 47 mmol/mol; dodecyl I, 10 mmol/mol; dodecyl II, 25 mmol/mol; and cetyl, 5 mmol/mol. Recovery, respectively, was 80%, 75%, 55%, 75%, and 60%.

Detergent followed by a 0.2% Triton wash. The elution pattern of the enzyme was not altered on passage through butyl-, benzyl-, or hexyl-Sepharose (Fig. 1B). In each case, the activity appeared as a single peak fronting in the void volume, and on ultracentrifugation it could be pelleted. However, with octyl-Sepharose (Fig. 1C), a different pattern was evident. Approximately 10% of the activity remained bound to the column with approximately 90% eluting in the void volume. Although the lightly substituted dodecyl resin (Fig. 1D) was substituted to a significantly less degree than the octyl resin, it produced a marked effect on the elution profile with 60% of the total activity appearing as a discrete single
peak within the included volume of the gel (Fig. 1D). The significance of this result is underscored by the fact that the octyl resin was substituted at 47 mmol of glycidyl ether/mol of galactose while the more lightly substituted dodecyl resin was substituted at only 10 mmol/mol. Unlike the enzyme appearing in the void volume, the peak of activity in the included volume of the gel remains in solution following centrifugation at 100,000 \( \times g \) for 1 h. If the more highly substituted dodecyl (25 mmol/mol) resin was employed under identical conditions, then the same fraction of total activity remained bound to the resin and could be eluted with a 0.2% Triton wash (Fig. 1D). Finally, with cetyl-Sepharose, still another elution pattern was observed (Fig. 1E). Again, an initial peak of aggregated material appeared followed by a second diminutive peak eluting at a position identical with that noted with the lightly substituted dodecyl resin (Fig. 1D). Approximately 50% of the enzymatic activity remained bound to the resin but could be eluted with a 0.2% Triton wash. The degree of substitution on the cetyl resin was approximately 5 mmol/mol at the limit of detection by NMR.

It should be emphasized that the effect achieved with dodecyl-Sepharose under these conditions could not be duplicated with more highly substituted octyl resins, which suggests that the length of the alkyl substituent on the resin was the critical determinant rather than the degree of substitution. On the other hand, still more highly substituted dodecyl and cetyl resins bound all of the applied enzymatic activity as well as most of the protein; subsequent desorption with detergent resulted in poor recovery.

To define more clearly the role of alkyl chain length, a high ratio of resin to detergent-solubilized cyclase in a moderately high ionic strength buffer was employed to effect a maximal interaction of cyclase with resin alkyl groups. For these experiments, 0.5 ml of the cyclase preparation was applied to a 5-ml resin bed volume, equilibrated in 10 mm Tris and 0.5 M NaCl. This contrasted with a ratio of 10 ml of cyclase solution to 10 ml bed volume in the prior experiments. Each of the resins in this experiment was lightly substituted as judged by NMR analysis. Neither the dodecyl nor cetyl resin bound a protein standard, myoglobin, in 4 M NaCl. Both the dodecyl and cetyl gels were substituted to such a low degree that quantitation with NMR was not possible, while the octyl resin had approximately 15 mmol of octyl glycidyl ether/mol of galactose. As evident in Fig. 2, octyl-Sepharose bound approximately 45% of the activity applied while both the dodecyl and cetyl resins bind almost 95%. With a subsequent decrease in ionic strength, 15% of the activity was released from the octyl resin. However, a detergent wash was required to release the bulk of the activity from both of the longer chain resins. In additional experiments not shown, maximal ratios of resin to cyclase preparations (v/v) utilizing benzyl- and butyl-Sepharose were ineffective in preventing protein aggregation, all of the cyclase activity appearing in the void volume. Each of these resins had 40 to 60 mmol of glycidyl ether/mol of galactose.

**Gel Filtration Prior to and Following Hydrophobic Resolution** —The altered pattern of cyclase elution on dodecyl-Sepharose (Fig. 1D) could be explained by either of two mechanisms. First, long chain alkyl residues might dissociate the enzyme by binding to an intrinsic hydrophobic subunit and releasing an active catalytic fragment. An alternative explanation is based on the possibility that cyclase is an intrinsically hydrophilic enzyme with only a small portion of its surface providing the hydrophobic character necessary for anchorage to the lipid bilayer. Chromatography on dodecyl-Sepharose might have removed more hydrophobic proteins by competing with detergent for their hydrophobic binding sites as well as preventing their interaction with cyclase on removal from the detergent environment. Gel filtration before and after dodecyl-Sepharose chromatography was therefore performed to determine whether the elution profile of cyclase had been altered. 1% Brij 56 or 1% Triton X-100 solubilized cyclase preparations were run on the same Sepharose 6B column (98 x 2.5 cm) equilibrated in either 0.1% Brij 58 or 0.1% Triton X-100, respectively (Fig. 3, A and B). Although both types of detergent were equally effective in solubilizing membrane protein, considerably less cyclase activity was preserved with Triton X-100, approximately 20% of that obtained with Brij 56 or 58. For comparison, a 1% Brij 56-solubilized preparation was first subjected to dodecyl-Sepharose chromatography. By employing a high ratio of lightly substituted (15 mmol/mol of galactose) resin/enzyme preparation (v/v), conditions were obtained that allowed 80% of the cyclase activity to elute within the included volume of the gel. No activity appeared in the void volume. This peak was pooled, concentrated, and applied to the same Sepharose 6B column (98 x 2.5 cm) equilibrated in the identical buffer without detergent (Fig. 3C). For comparison, each column run was carefully monitored so that pressure head and buffers were identical. Fraction volumes were carefully monitored. The protein concentration of the applied sample in each run ranged from 7.5 to 12.5 mg/ml and the total volume was 11 ml. Recovery in each case was in the range of 75%. As shown in Fig. 3, there was no difference in the apparent Stokes radius of cyclase as determined under any of the above conditions. In contrast, when the crude solubilized preparation was directly subjected to gel filtration, then all of the activity eluted as an aggregate in the void volume (Fig. 3C). If detergent was removed by first adsorbing cyclase to an ion exchange resin, subsequent gel filtration produced multiple small peaks of activity. Approximately 50% of the total activity fronts in the void volume, and only 20 to 30% appeared in those fractions where the unaggregated enzyme would be

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**Fig. 2.** One-half-milliliter aliquots of a 2-fold concentrated preparation of crude solubilized adenylate cyclase were applied to columns (5 x 1.2 cm) (5 ml resin bed) equilibrated in 0.5 M NaCl plus buffer without detergent. Each column was then washed with 3 column volumes collected in 5-ml fractions of each of the solutions noted in the diagram. Thirty-microliter aliquots of each fraction were assayed for cyclase activity. The total activity eluted with each buffer wash was computed and is expressed as absolute activity as well as a percent of the original activity applied.
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Ion Exchange Chromatography Prior to and Following Hydrophobic Resolution—During attempts at purification of the enzyme by ion exchange chromatography, it was noted that cyclase eluted over a large portion of the gradient either in the presence or absence of detergent (0.1%) in the eluting buffers. When cyclase previously solubilized by detergent was chromatographed on DEAE-cellulose utilizing detergent-free eluting solutions, the pattern depicted in Fig. 4A was observed. The heterogeneous pattern observed could be caused either by multiple forms of cyclase or by varying amounts of bound detergent. To examine both possibilities, gel filtration in 0.1% Brij 58 was performed initially, and the fractions exhibiting enzymatic activity were applied to an ion exchange resin equilibrated in 0.1% of the same detergent. Fig. 4B again shows an heterogeneous pattern with activity eluting throughout most of the gradient. A possible explanation of this observation is the aggregation of cyclase with other solubilized proteins during the chromatographic procedure. Such an effect might have been predicted in that the procedure actually exposes the solubilized membrane proteins to an environment, the resin itself, with an intrinsic high charge density that would tend to promote hydrophobic interactions. Aggregation of the enzyme with different proteins would then explain the broad peaks of activity obtained during gradient elution (Fig. 4, A and B). Consequently, it was of particular interest to investigate the effects of hydrophobic chromatography when performed in conjunction with gel filtration prior to ion exchange chromatography. A crude solubilized preparation of the enzyme was therefore passed over a lightly substituted dodecyl resin (15 mmol of glycidyl ether/mol of galactose) and the activity peak was then pooled, concentrated, and applied to a Sepharose 6B column. The peak of cyclase activity was again pooled and applied to a DEAE-cellulose column under the conditions noted in Fig. 4C. The subsequent elution profile was clearly different from those previously described. All of the enzymatic activity eluted as a discrete peak over a narrow portion of the gradient at a conductivity of 4.5 mmho. Elution profiles developed without preceding hydrophobic chromatography (Fig. 4, A and B) showed a small peak of activity at a similar conductivity representing approximately 20 to 30% of the total. This peak was consistently present and may represent the fraction of the cyclase that had not aggregated.

Hydrophobic Character of Adenylate Cyclase—Adenylate cyclase from crude, detergent-solubilized preparations would not bind to short chain butyl or benzyl resins, even in the presence of 1 M NaCl. Higher salt concentrations could not be used because of the lability of the enzyme on exposure to high ionic strength. At 1 M NaCl, cyclase activity was inhibited by 30 to 40%. It was of interest to determine whether this lack of

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**Fig. 3.** Gel filtration was performed as described in the text. The buffer consisted of 10 mM Tris, 5 mM MgCl₂, 1 mM EDTA, and 1 mM dithiothreitol. In those experiments where 0.1% detergent concentrations were maintained, the column was equilibrated with at least 3 column volumes. Each run was carefully monitored so that pressure head and fraction size were identical in all experiments. A

**Fig. 4.** Ion exchange chromatography was performed as described in the text. A is a characteristic chromatogram of crude solubilized cyclase in the absence of detergent. A 50-ml sample was initially applied. B represents chromatography of the enzyme in the presence of 0.1% Brij 58 following its initial fractionation on a Sepharose 6B gel filtration column in the same detergent. The entire 80-ml peak of activity was applied to the ion exchange resin. C is the chromatogram obtained subsequent to fractionation on a dodecyl-Sepharose hydrophobic resin followed by Sepharose 6B gel filtration in the absence of detergent.
interaction was related to limited hydrophobicity of cyclase or whether binding to the immobilized alkyl residues was hindered by the presence of detergent. Such competition may obscure the hydrophobic character of the native protein as assessed by its interaction with resins of varying hydrophobicity. Cyclase that had been partially purified and separated from detergent by passage over a dodecyl-Sepharose resin followed by gel filtration was then chromatographed on octyl-, hexyl-, butyl-, and benzyl-Sepharose equilibrated in either 0.75 M or 1.0 M NaCl. Elution was begun with 2 column volumes of the same buffer, followed by 0.1 M NaCl. Rechromatography on a lightly substituted octyl (10 mmol/mol of galactose) resin, which previously had adsorbed only a small fraction of the enzyme, now bound 100% of the activity, and a detergent wash was required for elution. Hexyl-Sepharose (40 to 60 mmol/mol of galactose) bound cyclase poorly while butyl-Sepharose did not bind at all (Fig. 5). Benzyl-Sepharose adsorbed almost 100% of the activity at either 0.75 M or 1.0 M NaCl, and 70% recovery could be effected by elution with 0.1 M NaCl (Fig. 5). Neither the hexyl nor benzyl resin bound myoglobin in 4 M NaCl. An approximate 10-fold purification of the enzyme resulted during benzyl-Sepharose chromatography.

**DISCUSSION**

Hydrophobic chromatography is a relatively new technique in protein purification. Shaltiel and Er (1) first described the use of alkylamine-Sepharose resins synthesized via cyanogen bromide activation and documented their effectiveness in certain model systems (18). Later the ionic character of such resins was emphasized and it was suggested that their resolving capacity was based on this property with hydrophobic interaction playing a secondary role (19). Recently Wilchek and Miron (20) have provided more evidence for this hypothesis in that acetylation of the isourea group formed during cyanogen bromide activation significantly alters the charge characteristics of the resin. This results in a major reduction in the capacity of the gel to bind protein. However, a different approach to the synthesis of hydrophobic resins was being developed simultaneously by other groups (14, 21). In particular, Hjerten et al. (14) linked alkyl groups to Sepharose in an aprotic solvent via an ether linkage, thus avoiding the introduction of charged groups as well as providing a more stable chemical bond than that resulting from cyanogen bromide activation. We chose to employ this resin in systematically examining the hydrophobic characteristics of adenylate cyclase. Such a resin displays all the characteristics of a hydrophobic matrix including adsorption of proteins at a high salt concentration as well as an increase in the strength of the interaction with an elevation in temperature (14, 15).

With the availability of such a resin, certain questions concerning the behavior of solubilized adenylate cyclase could be more decisively approached. Although detergent is required to liberate cyclase from the membrane, Levy (22) first demonstrated that the enzyme remained soluble following removal of detergent during ion exchange chromatography. This procedure also resulted in a 50 to 75% loss of activity. However, although solubility is preserved, additional data suggest that such a procedure promotes protein-protein interaction resulting in aberrant behavior of the enzyme during subsequent chromatography. Following such a protocol, Neer (9) demonstrated that adenylate cyclase eluted during gel filtration in an heterogeneous pattern with only 30 to 40% of the activity appearing in the position expected of the nonaggregated enzyme. Swislocki et al. (7) also utilized such a procedure to remove detergent from rat brain adenylate cyclase, but on subsequent gel filtration the enzymatic activity eluted at an apparent molecular weight of 800,000, clearly larger than has been reported for the nonaggregated form of the enzyme from a variety of other tissues (8, 22, 23). Since ion exchange resins simulate environments with high charge density, it is not unexpected that exposure of solubilized membrane proteins to such resins would promote hydrophobic interactions. This parallels the use of high salt concentrations to promote the adsorption of proteins to hydrophobic resins. It is thought that hydrophobic interactions are promoted under such conditions due to the favorable entropy resulting from a decrease in the ordering of water molecules around exposed nonpolar solutes (21, 24, 25). Such interactions may then explain the results obtained by several investigators in observing that ion exchange chromatography of adenylate cyclase results in either poor recovery with apparent strong adsorption to the resin or broad elution patterns with activity appearing throughout the gradient. Sutherland et al. (6) first emphasized that rapid and large changes in ionic strength were necessary to elute rat brain adenylate cyclase from DEAE-cellulose and that recovery was unpredictable. Later Swislocki and Tierney (7) noted an heterogeneous elution pattern of brain adenylate cyclase from an ion exchange resin during a gradient wash, requiring them to employ large stepwise changes in ionic strength to elute the enzyme. Our results both confirm and extend these observations. We have demonstrated that prior hydrophobic resolution of adenylate cyclase on a noncharged dodecyl resin allows subsequent gel filtration or ion exchange chromatography to proceed in the absence of detergent. More importantly, the enzyme, behaving as a homogenous species, demonstrates the same Stokes radius on gel filtration as does the unaggregated enzyme when detergent concentrations are maintained. The multiple peaks of activity appearing during gel filtration subsequent to removal of detergent on an ion exchange resin are not apparent. Similarly, if ion exchange chromatography is performed following both hydrophobic and gel filtration chromatography, a single peak of activity is eluted during gradient elution unlike the broad heterogeneous pattern occurring in the absence of preceding hydrophobic resolution. The ionic strength at which this peak elutes...
corresponds to that noted for a small peak of activity which predictably appeared during ion exchange chromatography of the crude solubilized preparation (Fig. 3A). This reproducible peak usually represented no more than 20% of the total activity, suggesting that 80% of the enzyme had aggregated with more hydrophobic proteins accounting for the early appearance of activity during the gradient wash. It might also be stressed that ion exchange chromatography subsequent to gel filtration in the absence of hydrophobic chromatography also produced a heterogeneous elution pattern, which indicates the critical role of hydrophobic resolution as an initial step.

These findings parallel the results of Garbers who has purified sea urchin sperm guanylate cyclase (26). It is of particular interest to compare the chromatographic characteristics of that enzyme with those of adenylate cyclase. First, after solubilization with detergent, the enzyme completely aggregates and appears in the void volume on gel filtration if eluted in the absence of detergent. Secondly, if the crude solubilized preparation is initially subjected to ion exchange chromatography, the activity elutes as a very broad peak. In contrast to these patterns, prior purification on a GTP affinity resin permits the enzyme to be gel-filtered in the absence of detergent with an elution profile expected of the nonaggregated form. Similarly, ion exchange chromatography subsequent to affinity resin purification also produces a single peak of activity eluting at a conductivity similar to that observed for adenylate cyclase following hydrophobic resolution. Other workers (4) have previously suggested that affinity separation may play a unique role in the purification of membrane-bound proteins, which reflects the effectiveness of this procedure despite the presence of detergents. These results additionally suggest that the success of the affinity adsorbent stems from its ability to separate guanylate cyclase from more hydrophobic proteins. Both gel filtration and ion exchange chromatography subsequent to affinity resin purification also produces a single peak of activity eluting at a conductivity similar to that observed for adenylate cyclase following hydrophobic resolution. Other workers (4) have previously suggested that affinity separation may play a unique role in the purification of membrane-bound proteins, which reflects the effectiveness of this procedure despite the presence of detergents. These results additionally suggest that the success of the affinity adsorbent stems from its ability to separate guanylate cyclase from more hydrophobic proteins. Both gel filtration and ion exchange chromatography could not effect a similar separation. In contrast, hydrophobic chromatography provides an alternative technique for separating solubilized membrane proteins at an initial step, avoiding aggregation and aberrant behavior during subsequent procedures.

Following separation of adenylate cyclase from detergent and other hydrophobic proteins on sequential dodecyl Sepharose hydrophobic chromatography and gel filtration, an attempt was made to investigate the hydrophobic character of the enzyme by examining its behavior on a variety of alkyl and aryl resins. Several features deserve consideration. First, all of the partially purified cyclase could be adsorbed to a lightly substituted octyl resin (10 mmol/mol of galactose) at low ionic strength (10 mM Tris). Previously, the same resin had been ineffective in preventing aggregation of the crude cyclase preparation. These results underscore the hydrophobic character of the enzyme when they are compared with the findings of Rosengren et al. (15) who examined the interaction of a typical globular protein, phycoerythrin, with a variety of alkyl resins. In their experiments, more highly substituted octyl resins (>20 mmol/mol of galactose) were required for protein binding and adsorption could only be effected at high salt concentrations (2 M NaCl). Secondly, attempts to use salt gradients to bind and elute the partially purified cyclase were successful only when less hydrophobic ligands were employed. Hexyl-Sepharose bound approximately 50% of the activity applied and this could not be increased by employing larger volumes of resin. Attempts to bind the enzyme to butyl-Sepharose were unsuccessful even with a highly substituted gel. Benzyl-Sepharose, however, was particularly effective with 100% of the activity binding at 0.75 to 1.0 M NaCl. The resin could bind large amounts of activity and an approximate 10-fold increase in specific activity could be achieved. These results are difficult to explain on the basis of simple hydrophobic interaction since benzyl groups should display an hydrophobicity less than that of alkyl butyl groups as determined by heats of transfer of the respective hydrocarbons from aqueous solvent to pure liquid hydrocarbon (25, 27). The affinity of the enzyme for benzyl-Sepharose suggests that II-Il interactions might be playing an important role and reflecting the capacity of the aromatic benzyl ligand to interact in a more specific manner with the phenyl and tyrosyl groups of proteins. Similarly, the interaction of human interferon with immobilized Cibacron blue dye has been attributed to II-II interactions (28).

In summary, the effectiveness of hydrophobic chromatography depended on two factors. First, the length of the alkyl side chain was critical in the ability of the resin to compete with detergent and other proteins for hydrophobic binding sites on adenylate cyclase. Octyl-Sepharose did not produce the effect achieved with dodecyl-Sepharose regardless of the degree of substitution of the gel. This is distinctly different from the behavior noted with hydrophilic globular proteins. The interactions of such proteins with hydrophobic resins can be altered by either varying the amount of residue per mol of gel or by elongating the alkyl side chain (15, 29). It appears that in these systems a critical hydrophobicity can be achieved utilizing either route in contrast to our results with solubilized adenylate proteins. The second important factor is the extremely low degree of substitution that was used in effecting the separation of adenylate cyclase from more hydrophobic proteins. Gels substituted 10-fold less were utilized in our experiments in contrast with those employed by Hjerten et al. (14) and Rosengren et al. (15) in the separation of serum proteins and a model protein, phycoerythrin. Such a low degree of substitution allowed a certain amount of specificity to be achieved in that the gel could still discriminate among the hydrophobic proteins in our preparation. Thus, by synthesizing gels that were lightly substituted with dodecyl or longer side chains, aggregation of the hydrophobic proteins was prevented while resolution was effected. These results suggest that alkyl residues immobilized on a noncharged matrix behave as solid phase detergents. Such resins effectively prevent hydrophobic protein-protein interactions and promote separation of membrane proteins of varying hydrophobicity. After such separation, membrane proteins with minimum hydrophobic mass such as adenylate cyclase may display a more typical hydrophilic character.

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