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AFFINITY CHROMATOGRAPHY OF MEMBRANE FRAGMENTS

SEPARATION OF CARBONIC ANHYDRASE-ENRICHED AND -DEPLETED BRAIN MEMBRANES

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SUMMARY

Chick brain membranes were fractionated by affinity chromatography on AH-Sepharose 6MB linked to *p*-sulfamylbenzoic acid, an inhibitor of carbonic anhydrase. Two major fractions were obtained. One, designated unbound fraction, representing 42% of the total membrane protein, eluted freely from the affinity adsorbent. A second fraction, designated specifically bound fraction, contained 36% of the total membrane proteins. In addition, 11% of the total membranes bound non-specifically and could be eluted only by the use of shearing forces. Various lines of evidence indicated that the sulfonamide binding site of membrane-bound carbonic anhydrase, in addition to the carbonic anhydrase inhibitor immobilized to AH-Sepharose 6MB, was responsible for the observed specific binding. The specifically bound fraction was highly enriched in carbonic anhydrase while the unbound fraction was completely devoid of this enzyme activity.

INTRODUCTION

In the brain, as in other tissues, carbonic anhydrase occurs both in the soluble and membrane-bound forms[1]. Carbonic anhydrase in the brain is thought to be localized in glial cells[2,3] although Ghandour *et al.*[4] have proposed that carbonic anhydrase is localized in oligodendrocytes and does not occur is astroglial cells. Recently we found substantial amounts of carbonic anhydrase activity in our crude synaptic plasma membranes (SPM), prepared from seven-day-old chickens by Ficoll gradient centrifugation[5]. This suggested that carbonic anhydrase activity in these synaptic membrane fractions was due to contamination with carbonic anhydrase-containing membranes, presumably glial membranes. Because synaptosomes from which these membrane subfractions are prepared are known to be contaminated with gliasomes[6]. In the present work affinity chromatography of chick brain SPM on

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immobilized PSBA*, an inhibitor of carbonic anhydrase,[6] has demonstrated the feasibility of removing carbonic anhydrase-containing membranes from SPM. A preliminary account of this work has been presented[7].

MATERIALS AND METHODS

Cyanogen bromide activated Sepharose 6MB, ouabain, ATP, NADP, acetylthiocholine chloride, butrylthiocholine chloride, carbonic anhydrase, 1-ethyl-3-(3-dimethylaminopropyl) carbodimide and dithionitrobenzene were all from Sigma (St. Louis, MO, U.S.A.). The carbonic anhydrase inhibitors, PSBA and PAMBS were obtained from Aldrich (Milwaukee, WI, U.S.A.). Acetazolamide was supplied by Pfaltz and Bauer (Stamford, CO, U.S.A.). 1,6-Diaminohexane was from Matheson, Coleman and Bell.

Preparation of synaptic plasma membranes

Synaptic plasma membranes were prepared from seven-day-old male white leghorn chicks by the method of Babitch *et al.*[5].

Membrane protein determination

Protein determination of membrane fractions was carried out according to Bradford[8] using bovine serum albumin as the standard[9].

Enzyme assays

Ouabain-sensitive (Na^+–K^+)-activated ATPase (E.C. 3.6.1.4) was assayed by a method combined from those of Whittaker and Baker[10], Kimelberg and Papahadjopoulis[11] and Cotman *et al.*[12] as described in Babitch *et al.*[5]. Acetylcholine esterase (E.C. 3.1.1.7) was measured spectrophotometrically at room temperature by following the method of Steck and Kant[13]. Butrylcholine esterase activity was also determined in an exactly similar manner. 2′,3′-CNP activity was assayed according to Sogin[14].

Carbonic anhydrase (E.C. 4.2.1.1.) activity** was determined with respect to hydrolysis of an ester[15], namely *p*-nitrophenyl acetate. Small aliquots of samples were pre-equilibrated with acetazolamide inhibitor to give a final concentration of 10–50 μM in the curette. After an incubation period of 10 min at 25°C with continual shaking, the acetazolamide-insensitive activity was determined. Carbonic anhydrase activity was calculated as the difference between total esterase activity and inhibitor-insensitiitive activity[17]. An extinction coefficient of $18 \cdot 10^3\ M^{-1}\ cm^{-1}$ is used for *p*-nitrophenol[18]. The enzyme activity is expressed as nmoles product per mg protein.

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* Abbreviations: *p*-sulfamylbenzoic acid, PSBA; *p*-aminomethylbenzenesulfonamide, PAMBS; 2′,3′-cyclicnucleotide phosphohydrolase, 2′,3′-CNP; aminohexyl-sepharose 6MB, AH-sepharose 6MB; synaptic plasma membranes, SPM.

** Determination of carbonic anhydrase activity is commonly performed by the CO_2 hydration method[16]. We attempted to use this method for measuring carbonic anhydrase activity presumed to be present in our chick brain synaptic plasma membrane preparations. Poor sensitivity precluded the use of this procedure for the synaptic plasma membrane fractions. Because we required a minimum of 100 μg of membrane protein (5 mg/ml) per assay to produce detectable activity. Initially we attempted to measure acetazolamide-insensitive esterase activity by including 1 mM acetazolamide in 1 mM phosphate buffer, pH 7.2. But under these conditions and using chick brain extracts we found that the substrate became more labile for some unexplained reason(s).

Preparation of the affinity-matrix

The gel, AH-Sepharose 6MB, was prepared by coupling 1,6-diaminohexane to cyanogen bromide activated sepharose 6MB. The spacer arm, 1,6-diaminohexane (500 mg) was dissolved in 50 ml of coupling buffer (0.1 *M* sodium hydrogen carbonate, 0.5 *M* sodium chloride, pH 8.3) and mixed with the swollen Sepharose 6MB beads in an end-over-end rotation. The mixture is allowed to stand for 2 h at room temperature with occasional stirring. Immobilization of *p*-sulfamylbenzoic acid was then performed by the carbodiimide coupling procedure[19].

Batchwise adsorption procedure

Affinity chromatography was carried out by using a batchwise technique instead of the usual column chromatographic method[20]. All experimental procedures were performed at 4°C unless otherwise stated. Typically, 2–10 mg of SPM was applied to 20 ml the PSBA-Sepharose 6MB beads. The mixture was allowed to stir gently for 3–6 min on ice, and allowed to settle on ice for 50 min. After incubation, the unbound membranes were removed with a Pasteur pipette. The gel is washed twice with 30 ml of 0.01 *M* HEPES buffer, (0.14 *M* potassium chloride, pH 7). All the supernatants were pooled together and spun down at 106,500 *g* for 60 min in a Sorval Ti 50.2 rotor. In order to release the bound membranes, the beads were incubated with 30 ml of the eluting buffer (0.01 *M* Hepes, 0.14 *M* potassium chloride, 50 m*M* *p*-aminomethylbenzenesulfonamide or acetazolamide, pH 6.5) for 4–6 h. The supernatant was taken out, and the above incubation was repeated twice. All the supernatants were collected and spun down as described above. The tightly bound membranes (11%) were released by employing mechanical forces. A small stirring bar is added to the beads equilibrated with 0.01 *M* HEPES, 0.14 *M* potassium chloride, pH 7 and placed on a Nuova 7 stir plate and the speed knob was set at 4. After stirring for 30 min the beads were allowed to settle for 3 min on ice. The eluate is taken out and spun at 106,500 *g* for 45 min at 4°C.

RESULTS AND DISCUSSION

The ligand, *p*-sulfamylbenzoic acid, was selected as an ideal derivative for coupling to AH-Sepharose 6MB for the present work, since it is an inhibitor of the carbonic anhydrase enzyme[21]. When SPM prepared from seven-day-old chicks, were subjected to affinity chromatography on immobilized PSBA, two major subfractions were obtained. One membrane subfraction, designated as unbound membrane, was not retained on the affinity beads and eluted freely. It represented 42 ± 3% of the total synaptic membrane protein. Membrane fragments which bound specifically to the affinity adsorbent could be eluted with a competitive counter ligand, PAMBS or acetazolamide. This subfraction is designated specifically bound membranes. It represented 36 ± 3% of total synaptic plasma membrane protein applied to the affinity beads. About 11 ± 3% of the synaptic membrane protein bound non-specifically and could be eluted only by the use of shearing forces.

When increasing amounts of synaptosomal protein was applied to PSBA-Sepharose 6MB, the distribution between the two fractions (unbound and total-bound membranes) was constant up to an amount of membranes, which was defined by the capacity of the affinity system (Fig. 1). Above this (10 mg input), the yield of bound

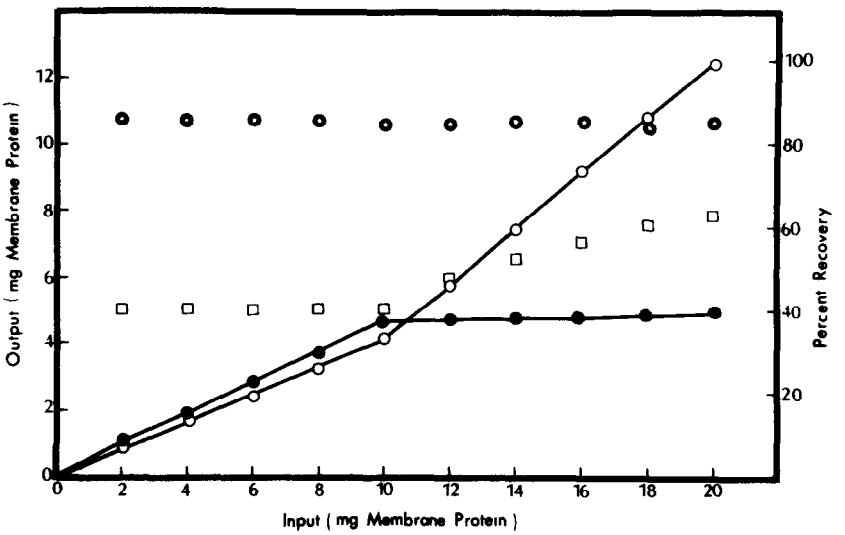

Fig. 1. Affinity chromatography of crude chick brain membranes on PSBA-AH-Sepharose 6MB. Membranes were fractionated with 20 ml Sepharose beads. Membrane protein (2–20 mg) was mixed with the beads. Binding and elution was performed at 4°C. Open circles represent unbound membranes; solid circles, total bound membranes. Open squares represent percent unbound membranes; stars indicate total percent recovery.

membranes was constant for a given amount (20 ml settled beads) of PSBA-Sepharose beads. Within the maximum capacity of the beads (up to 10 mg membrane protein) it was found that total-bound membranes represent 47% of the total membranes while unbound membranes comprise about 42% of total membranes.

Notably, in all separation experiments some loss (11–15%) of membrane protein occurred. In all subsequent experiments, bound and unbound membranes were resolved under conditions where the capacity of the PSBA-Sepharose beads was not exhausted. Various lines of evidence indicate that the sulfonamide binding site of membrane-bound carbonic anhydrase, in addition to the carbonic anhydrase inhibitor (PSBA) coupled to AH-Sepharose 6MB, was responsible for observed specific binding (36 ± 3%). As shown in Table I, the evidence includes:

(a) Specifically bound membrane fraction (36 ± 3%) did not adhere to the ligand-less beads, namely AH-Sepharose 6MB.

(b) The strong and specific binding of membranes to beads was abolished when the membranes were preptreated with an inhibitor of carbonic anhydrase, PAMBS.

(c) Likewise, when the immobilized ligand, PSBA, was saturated with soluble carbonic anhydrase enzyme, the specifically bound membranes showed no retardation. However, when carbonic anhydrase was completely washed off from the beads, the specifically bound membranes were again retained by the beads to the same extent (36 ± 3%).

(d) The specifically bound membranes can be distinguished from the non-specifically bound membranes (11%) by assaying them for cabonic anhydrase activity. As expected, the non-specifically bound membranes were devoid of carbonic anhydrase activity like the unbound membranes.

TABLE I

AFFINITY CHROMATOGRAPHY OF CHICK BRAIN MEMBRANE FRAGMENTS ON IMMOBILIZED INHIBITOR OF CARBONIC ANHYDRASE *p*-SULFAMYLBENZOIC ACID

Solid support	*Membrane protein ± S.D. (%)*			*Total recovery (%)*
	Unbound membranes	*Specifically-bound membranes***	*Non-specifically-bound membranes***	
AH-Sepharose 6MB	82 ± 3	0	10 ± 2	92 ± 3
PSBA-AH-Sepharose 6MB****	80 ± 3	0	11 ± 3	91 ± 3
PSBA-AH-Sepharose 6MB§	79 ± 4	0	9 ± 2	88 ± 3
PSBA-AH-Sepharose 6MB	42 ± 3	36 ± 3	11 ± 3	89 ± 3

* Bound membranes eluted in the presence of PAMBS.

** Bound membranes eluted by shearing forces alone.

*** Membranes were pretreated with PAMBS and then treated with the beads.

§ The beads were first saturated with soluble carbonic anhydrase. After washing off carbonic anhydrase with PAMBS, the affinity matrix regained its full capacity of binding membranes specifically (data not shown).

The above findings were further substantiated by re-chromatography experiments (data not shown). Re-chromatography of the isolated membrane fraction on PSBA-Sepharose 6MB showed that the previously bound (specifically) membrane fraction could be completely rebound, while the unbound fraction remained unbound. However, we consistently observed an 11–15% loss in membrane protein during each time the re-chromatography experiment was performed.

The most interesting aspect of the affinity chromatography of these membranes is the dependence of the binding and dissociation of these membranes on shearing forces. When synaptic plasma membranes (2–10 mg) were allowed to mix with the beads with continuous stirring, the percentage of membrane protein appeared in the unbound fraction increased from 42% (Fig. 1) to 53% (Fig. 2) irrespective of the amount of membrane protein applied. The binding of an additional 11% of the membrane protein is prevented if the mixture is stirred throughout the binding phase of the separation process. The remaining adherent membranes were removed by biospecific elution with a carbonic anhydrase inhibitor, PAMBS. The specifically bound membranes represented 34% of the total membrane protein applied. Nevertheless, the total membrane protein recovered in this experiment (Fig. 2) is essentially the same as in the earlier experiment (Fig. 1).

When binding of the SPM (2–10 mg) was carried out in the absence of any stirring, the unbound fraction contained only 42% of the total synaptic membranes applied. An additional 11% of the membrane protein was eluted by stirring the incutation mixture. Up to this point the total recovery was 53%. Thisv alue is indistinguishable from the value obtained in Fig. 2 in which binding of 11% membrane protein was abolished by shearing forces. Finally, the specifically adsorbed membrane fragments (36%) were eltued by using PAMBS.

Mechanical forces (stirring) are known to play an important role in the affinity chromatography of membrane vesicles from calf thymocytes[22]. The binding of membrane vesicles to Con A-Sepharose was prevented if the mixture was stirred through-

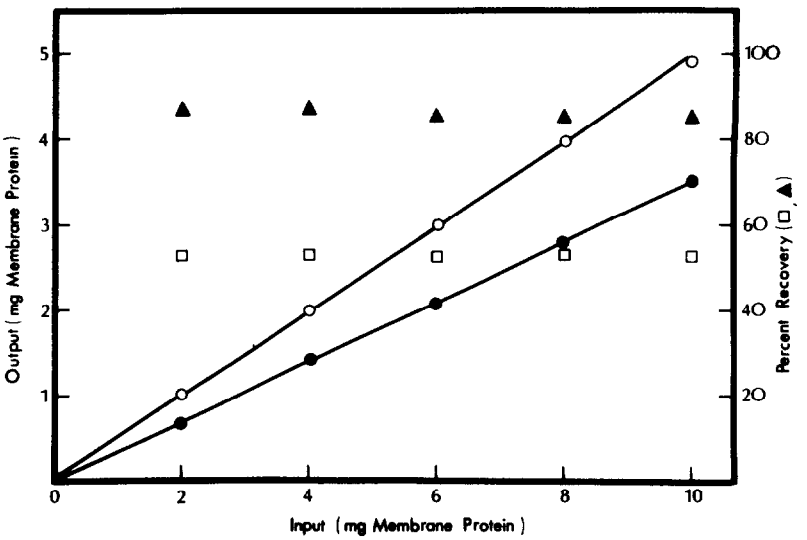

Fig. 2. Effect of stirring on the affinity chromatography of membranes on PSBA-AH-Sepharose 6MB. Membranes and beads were allowed to mix together by magnetic stirring. O–O, unbound membranes; ●–●, specifically bound membranes; □, percent total unbound membranes. The bound membranes were eluted by bioelution with PAMBS. ▲, total membranes recovered.

out the whole separation procedure. Elution of the bound membranes with the competitive inhibition alone was not possible[22,23]. Likewise, the binding of mouse bone marrow cells to wheat germ agglutinin (WGA)-Sepharose 6MB was reduced by mechanical agitation[24]. About 75% of the bone marrow cells were bound where the cells and beads were mixed with gentle stirring, compared with 90% binding where the cells were incubated in a stationary column. Our present findings are in agreement with these results.

Several plasma membrane-located enzymes were measured for their distribution in the membrane subfractions (Table II). The non-specifically bound membranes (11%) were not assayed for any of these enzymes except for carbonic anhydrase. For comparison purposes, data for the control membranes are also included. As shown in Table II, acetylcholine esterse and butrylcholine esterase showed no preferential location, ouabain-sensitives Na^+–K^+-ATPase was highly enriched in the unbound membranes. In contrast, 2',3'-CNP was moderately increased in the bound membranes. The unbound membranes, which had no bioaffinity for the immobilized PSBA, apparently were devoid of carbonic anhydrase activity (Table II). As expected, the bound membranes were found to be highly enriched in carbonic anhydrase activity. Total recoveries for the unbound and bound membranes were comparable to the enzymes tested. The data suggest that carbonic anhydrase enzyme is localized exclusively in the bound membranes.

To date, reports of the isolation of cells or membrane fragments using an affinity ligand for a single membrane component are as rare[25] as isolations of such membranes by other techniques and the great potential of affinity chromatography remains unexploited. The original stimulas for the present work was our desire to eliminate glial membranes from neuronal membranes. A wide variety of data[1,26–28]

TABLE II

ENZYME ACTIVITIES IN SPECIFICALLY BOUND AND UNBOUND MEMBRANE FRACTIONS

Details of each enzyme assay are described in the material and methods section. Each value is the mean of three experiments. Specific activities are all expressed in μmoles or nmoles per min per mg protein.

Enzyme	*Specific activity*			*Total enzyme activity (%)*
	Control membranes	*Unbound membranes**	*Specifically-bound membranes***	
Na^+-K^+-ATPase	83	132	69	81
Acetylcholine esterase	12.1	9.1	9.4	77
Butrylcholine esterase	0.56	0.4	0.47	74
2',3'-CNP	4.8	3.8	6.4	77
Carbonic anhydrase	4.1	0	13	82

* Like unbound membranes, the non-specifically bound membranes were totally inactive when tested against carbonic anhydrase substrate (data not whon).

** These membranes were eluted with 1 m*M* PAMBS.

have demonstrated the localization of carbonic anhydrase in both oligodendricytes and astrocytes, and as a consequence carbonic anhydrase is generally considered to be reliable glial marker during the purification and isolation of neuronal cells[29]. However, carbonic anhydrase has also been found in the choroid plexux and in epithelial cells and capillary walls of the intestine[30]. Thus, the removal of carbonic anhydrase containing membranes from a preparation of synaptic plasma membranes may effectively remove a wide variety of other carbonic anhydrase containing membrane fragments.

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