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Rapid purification of cotton seed membrane-bound N-acylphosphatidylethanolamine synthase by immobilized artificial membrane chromatography

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Abstract

N-Acylphosphatidylethanolamine synthase (NAPES) is a membrane-bound enzyme present in cotton seedlings at a concentration of ≤0.02% of the total protein. NAPES was purified to electrophoretic homogeneity in a single chromatographic step using immobilized artificial membrane (IAM) chromatography. The IAM column used for NAPES purification was ^{ether}IAM.PE$^{C10/C3}$ and this surface contains a monolayer of immobilized phosphatidylethanolamine (PE). Since PE is an analogue of the natural substrate for NAPES, ^{ether}IAM.PE$^{C10/C3}$ columns function as an affinity column for this enzyme. Detergent-solubilized microsomal proteins from cotton were loaded on to the ^{ether}IAM.PE$^{C10/C3}$ column and eluted with buffered mobile phases containing 0.2 m*M* dimyristoylphosphatidylethanolamine (DMPE) and 2 m*M* dodecylmaltoside. Little NAPES functional activity eluted if DMPE was removed from the mobile phase. Mobile phase DMPE is also a substrate for NAPES, and therefore both the mobile phase and IAM surface contains NAPES substrates. Mobile phase DMPE may function as both a surfactant-type affinity displacing ligand effecting protein elution and also a stabilizing factor of NAPES functional activity. The loading capacity on semi-preparative ^{ether}IAM.PE$^{C10/C3}$ (6.5 × 1.0 cm) columns was ca. 5 mg of total detergent solubilized microsomal proteins, and protein recovery was quantitative. This one-step IAM purification of NAPES resulted in a single band on silver-stained polyacrylamide gels, and 3940 fold increase in NAPES specific activity. The molecular mass of the purified NAPES protein is 64 000. ^{125}I labeled [12-(4-azidosalicyl)amino]dodecanoic acid is a photoreactive fatty acid substrate of NAPES that was used to confirm protein purity.

1. Introduction

During the postgerminative growth phase of plants, the primary amine of membrane-associated phosphatidylethanolamine (PE) may be acylated with palmitic acid and perhaps other free fatty acids. N-Acylphosphatidylethanolamine (NAPE) was initially found in cotyledons of cotton seedlings but appears to be a phospholipid found in several other plants including

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soybean cotyledons, castor bean endosperm, okra cotyledons and rice cell suspensions [1]. In addition to plant cells, NAPE has also been found in animal cells. However, the presence of NAPE analogues in animal cells has always been associated with cell-stress or cell damage. For instance, NAPE has been found in degenerating epidermal cells, infarcted heart tissue, ischemic brain tissue and a few tumor cell lines (reviewed in Ref. [2]). During cell stress or tissue damage, free fatty acids are enzymatically released from membrane phospholipids and the free fatty acids destabilize healthy cell membranes [3–5]. Thus to minimize fatty acid induced cellular toxicity, an efficient fatty acid scavenger mechanisms likely exists that involves the formation of NAPE.

NAPE is enzymatically synthesized from PE and free fatty acids by NAPE synthase (NAPES) in a time-, temperature-, pH- and protein concentration-dependent manner [6,7]. NAPES was found in cotton seed microsomes [1] and partially purified by isoelectric focusing [7]. In this report, we describe the purification of this enzyme to homogeneity in one step using immobilized artificial membrane (IAM) chromatography. In addition, the enzyme was unambiguously identified using a photoreactive affinity cross-linking ligand [8].

IAM surfaces have been used for (i) enzyme immobilization [9], (ii) facilitating the coupling of polar and non polar molecules [10], (iii) predicting drug transport across human skin and other biological barriers [11–13], and (iv) predicting the pathophysiological effects of bile salts [14]. IAMs are also chromatography surfaces designed to emulate the membrane surfaces found in liposomes [15–17] and several membrane proteins have been purified using IAM chromatography: (i) cytochrome P450 [18], (ii) cholesterol transfer protein [19], (iii) phospholipase A_2 [20], and an intestinal peptide transporter protein [21]. Typically, IAM chromatography results in $\geq$70% of the contaminating proteins being removed from the target protein in one step. In this report we extend the chromatographic applications of IAM chromatography to the purification of NAPES.

2. Experimental

2.1. Chemicals

Sodium dodecyl sulfate (SDS), N,N'-methylene-bis-acrylamide (BIS), acrylamide, ammonium persulfate (APS), N,N,N',N'-tetramethylethylenediamine (TEMED) and molecular mass markers for gel electrophoresis were purchased from Bio-Rad (Hercules, CA, USA). 3-[3-(Chloroamidopropyl)dimethylammonio] - 1 - propane - sulfonate (CHAPS) was from Aldrich (Milwaukee, WI, USA). Glacial acetic acid, hydrochloric acid (concentrated), sodium phosphate (monobasic), ethylene glycol (EG), silver nitrate, 37% formaldehyde solution were obtained from Fisher Scientific. Sodium carbonate, sodium thiosulfate and sodium chloride were from J.T. Baker (Philipsburg, NJ, USA). Glycine, dodecylmaltoside (DDM), dimyristoyl phosphatidylethanolamine (DMPE), sodium deoxycholate (DOC), trichloroacetic acid (TCA), phenylmethyl sulfonyl fluoride (PMSF), pepstatin A, leupeptin, benzamidin and Tris base were ordered from Sigma (St. Louis, MO, USA). Micro bicinchoninic acid (BCA) protein assay reagent kit was obtained from Pierce (Rockford, IL, USA). Methanol was obtained from Mallinckrodt (Paris, KY, USA). Absolute ethanol was obtained from McCormick Distilling Co. (Pekin, IL, USA). Centricon and Centriprep devices were purchased from Amicon (Beverly, MA, USA). [1-^{14}C] palmitic acid (57 mCi/mmol) was obtained from DuPont NEN (Wilmington, DE, USA).

2.2. Membrane preparation and solubilization

Microsomal proteins from 1-day old cotton seedlings were prepared according to Chapman and Moore [7]. Briefly, cotyledons were homogenized in a solution containing 100 m*M* K-PO_4 (pH 7.2), 10 m*M* KCl, 1 m*M* EDTA, 1 m*M* $MgCl_2$, 400 m*M* sucrose plus a cocktail of protease inhibitors (1 m*M* PMSF, 1 m*M* benzamidin, 1 m*M* ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 1 m*M* pepstatin A, 1 m*M* leupeptin). The

homogenate was filtered through four layers of cheesecloth and the filtrate centrifuged at 10 000 *g* for 20 min at 3°C. The resulting supernatant was ultracentrifuged at 150 000 *g* for 1 h at 3°C. The resulting microsomal pellet was resuspended in 400 m*M* sucrose plus protease inhibitors to a concentration of 10 mg protein/ml buffered with 20 m*M* Na-PO_4 (pH 7.2). After washing with 1 *M* NaCl, the microsomes were resuspended in buffer containing 20% glycerol, the cocktail of protease inhibitors, 0.2 m*M* DDM, 20 m*M* Na-phosphate (pH 8.0) and 1 m*M* EDTA. The critical micelle concentration of DDM is 0.14 m*M*. Detergent-extracted microsomes were subjected to centrifugation at 150 000 *g* for 1 h; the supernatant contained the target enzyme.

The supernatant containing the DDM-solubilized microsomal proteins were concentrated on Centricon-30 or Centriprep-10 device depending on the sample volume. Centricon-30 or Centriprep-10 were centrifuged at 6000 rpm (5000 *g*) for 30 min at 4°C on Sorvall RC2-B with Sorvall Type SS-34 rotor to concentrate the proteins prior to injection on IAM columns. The solubilized microsomal protein samples were concentrated as follows: 2 ml of sample to 40 μl for pilot columns, 5 ml to 900 μl for analytical columns, and 45 ml to ca. 2 ml for semi-preparative IAM columns.

2.3. IAM chromatography

Several phospholipids have been immobilized at monolayer densities to prepare IAM chromatography packing materials suitable for purifying membrane proteins [10,22]. The membrane lipids that have been immobilized include phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidic acid (PA). Immobilized PE surfaces were used for the purification of NAPES and this IAM surface is denoted as ^{ether}IAM.PE$^{C10/C3}$ (Fig. 1). ^{ether}IAM.PE$^{C10/C3}$ was synthesized in our laboratory [10,22] and the superscript "ether" denotes an ether linkage between the alkyl chain and the glycerol backbone of the phospholipid. IAMs are routinely prepared by a three-step bonding pro-

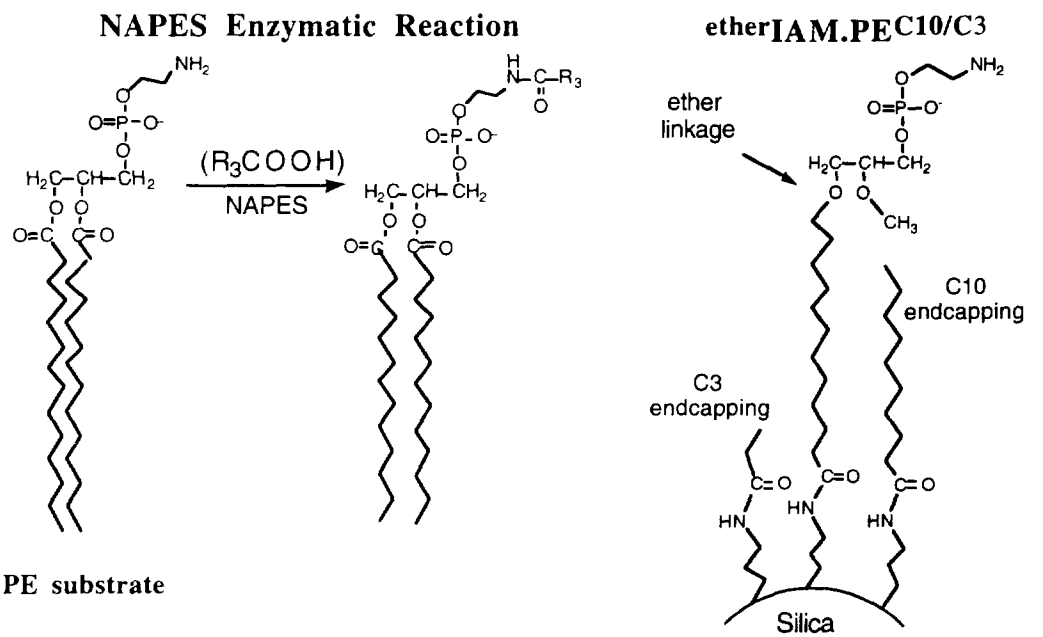


Fig. 1. Comparison of the PE lipid substrate for the NAPES enzymatic reaction to the PE lipid substrate immobilized on the IAM surface. R_3COOH is free fatty acid, for example, palmitic acid.

cess: step (i) involves phospholipid immobilization, step (ii) involves end capping with decanoic (C10) anhydrides, and then propionic (C3) anhydrides until the IAM surface is ninhydrin negative, step (iii) involves removing phospholipid protecting groups, e.g., for the ^{ether}IAM.PE$^{C10/C3}$ column, *tert.*-butoxycarbonyl from the amino group of PE. The C10 and C3 alkyl chains from the end capping reactions are shown in Fig. 1. PE was bonded to chromatographically efficient 12-μm silica propylamine particles containing 300-Å pores. The surface coverage of PE was 83 mg PE/g silica propylamine which corresponds to a surface density of ca. 66 Å^2/PE molecule on the IAM surface [10]. HPLC columns were packed at Regis (Skokie, IL, USA). Pilot size columns were 3 × 0.46 cm, analytical size columns were 15 × 0.46 cm, and semi-preparative columns were 6.5 × 1.0 cm. The IAM surface shown in Fig. 1 is very stable from pH 2 to pH 8.

Our HPLC system employs a Rainin Rabbit-HP pump and a Milton Roy Variable Wavelength UV Monitor set at 254 nm for detection. All chromatography experiments were performed at room temperature. Typically, the detector was set to a range of 0.05 and a response time of 0.05 s. The flow-rate was 1 ml/min for analytical size and semi-preparative size IAM columns and 0.2 ml/min for the pilot size column. Prior to sample loading, the IAM column was always equilibrated with at least 30 column volumes of mobile phase A. Mobile phase A contained 20 m*M* sodium phosphate (pH 8.0), 20% EG and 1 m*M* EDTA. EG was included in the mobile phase as a precautionary measure to preserve NAPES activity during protein purification [23] and EDTA was used to inhibit the inactivation of NAPES caused by divalent metal ions [7]. Mobile phase B was comprised of mobile phase A plus 0.2 m*M* DMPE and 2 m*M* DDM. To prepare mobile phase B, approximately 100 mg of DMPE powder was added to mobile phase A containing DDM and stirred for 2–3 h at room temperature. Filtration of the mobile phase through 0.2-μm nylon-66 filters (Rainin) resulted in several milligrams of non solubilized DMPE remaining on the filter. Gravimetric analysis of the non solubilized DMPE remaining on the filter was used to calculate the final concentration of DMPE in mobile phase B.

2.4. NAPES activity assay

NAPES activity was monitored by the method of Chapman and Moore [7]. Briefly, on ice, a stock solution of Na-phosphate buffer, DDM and dioleoyl phosphatidylethanolamine (DOPE) were mixed with each chromatographic fraction (usually 100 μl) to a final volume of 0.495 ml (final concentration: 20 m*M* Na-PO_4, pH 8.0, 0.2 m*M* DDM, 40 μM DOPE). Then 2.5 μl of [1-^{14}C]palmitic acid (50 μ*M* final; 10 mCi/nmol in ethanol) was added, vortexed briefly and sonicated for 2 s. The solution was placed in a 45°C water bath for 10 min with shaking at 120 rpm. The reaction was terminated by the addition of 2 ml of boiling 2-propanol followed by incubation at 70°C for 30 min. The reaction tubes were cooled in ice bath and 1 ml of chloroform was added. After intermittent vortexing for 30 min, 1 ml of chloroform and 2 ml of 1 *M* KCl were added to separate the mixture into 2 phases. The resulting mixtures were centrifuged for 10 min at 2000 rpm (325 *g*) to complete phase separation. The upper aqueous phase was aspirated and discarded and the chloroform phase containing extracted phospholipids was washed twice with 2 ml of 1 *M* KCl. The residual chloroform phase was dried under N_2 and the residue suspended in 50 μl chloroform–methanol (2:1, v/v) and separated by TLC on silica gel G-60 plates. TLC plates were developed first with hexane–ethyl ether (8:2, v/v) for 45 min, and then with chloroform–methanol–water (80:35:1, v/v/v) for 60 min in the same direction. The lipids and standards were visualized by I_2 vapor, and then radiolabeled lipids were scanned and quantified for radioactivity using a Bioscan System 200 radiometric scanner [7]. The activity was expressed as nmol of NAPE produced/h/ml.

2.5. SDS-PAGE

Chromatography fractions were subjected to TCA precipitation to concentrate the proteins prior to gel electrophoresis. The TCA precipi-

tation method involved adding 0.1 ml of 0.15% DOC to the ca. 1-ml chromatography fractions, incubating at room temperature for 10 min, and then adding 0.1 ml of 72% TCA [24]. The sample was incubated on ice for 1 h and then centrifuged at 14 000 rpm (16 000 *g*) using an Eppendorf Centrifuge (Model 5415) for 15 min. After centrifugation, the supernatant was decanted and the pellet washed with 1 *M* NaCl. The protein pellet was solubilized with 20 μl tank buffer (25 m*M* Tris, 0.2 *M* glycine, 0.1% SDS, pH 8.3) and if necessary, adjusted to pH ≈ 8 by a trace amount of dry Tris base powder. The 20 μl solubilized sample was mixed with 20 μl of twice concentrated gel electrophoresis sample treatment buffer (2×) that contained trace amounts of bromophenol blue indicator-dye, and then heated at 75°C for 15 min. The samples were sonicated for a few seconds, briefly vortexed and ca. 10 μl loaded onto 12% polyacrylamide gel. Gels were run at constant voltage (200 V) with cold water circulation, until the dye reached the gel front. The gels were stained with silver using the method of Merril [25].

In addition, photoreactive affinity cross-linking ligand was utilized to identify the target protein on the gel. The radiolabeled photoaffinity ligand is [12-(4-azidosalicyl)amino]dodecanoic acid (^{125}I-ASD), which is a fatty acid substrate of NAPES [8].

2.6. Protein content measurement

Protein content was measured based on the BCA method of Cu^{2+} chelation. Chromatography fractions contain EG, EDTA, lipids and detergents that may interfere with BCA protein assays. Consequently, TCA precipitation was performed according to the manufacturer's (Pierce) suggestion as follows. Each chromatography fraction (1 ml) was mixed with 200 μl of 100% TCA and incubated on ice for 1 h. Precipitated proteins were pelleted by centrifugation at 14 000 rpm for 8 min (16 000 *g*). The supernatant was decanted and the pellet was washed gently with 1 *M* NaCl solution. After the pellet was solubilized with 1 ml of 0.3 *M* phosphate buffer (pH 8.0), 1 ml of newly prepared BCA microassay working reagent was added to each sample and the samples were then incubated at 60°C for 60 min. The absorbance at 562 nm was measured on a Beckman-7 spectrophotometer against the blank. The protein concentration was calculated from a standard curve using bovine serum albumin (BSA) as the protein standard, in which, known concentrations of BSA solutions were subjected to the same TCA precipitation.

3. Results and discussion

Free fatty acids and PE are substrates for NAPES (Fig. 1). The purification strategy for NAPES was developed based on the concept that PE analogues might function as affinity surfaces for the purification of this enzyme. The IAM surface prepared from PE is denoted as ^{ether}IAM.PE$^{C10/C3}$ and the structure is depicted in Fig. 1. NAPES binding to the ^{ether}IAM.PE$^{C10/C3}$ surface requires elution with mobile phase modifiers that selectively bind and elute the enzyme from the column. Since natural diacylated phospholipids, such as DMPE, are substrates for NAPES, these compounds may function as "affinity displacing ligands" for NAPES bound to ^{ether}IAM.PE$^{C10/C3}$. The purification strategy for NAPES is thus based on both affinity ligands tethered to the chromatographic surface (e.g. PE analogues) and "affinity displacing ligands" (e.g. DMPE) in the mobile phase.

DMPE is insoluble in aqueous buffers, and therefore detergents were required to assure solubilization. For IAM chromatography, DDM was used as the mobile phase detergent for DMPE solubilization because DDM maintains NAPES activity at a weight ratio of detergent:protein of 2:1 [7]. Fig. 2 shows the chromatographic profile eluting from an ^{ether}IAM.PE$^{C10/C3}$ pilot column after injecting DDM-solubilized microsomal proteins and eluting with a DDM–DMPE detergent gradient. A 40 μl volume of the microsomal proteins (ca. 200 μg total protein) was loaded at 0.2 ml/min and after 10 min, a shallow detergent gradient from 10 to 30 min was applied to elute the proteins. Prior to the detergent gradient, two peaks eluted from the column: peak 1 at 3 min, and peak 2 at

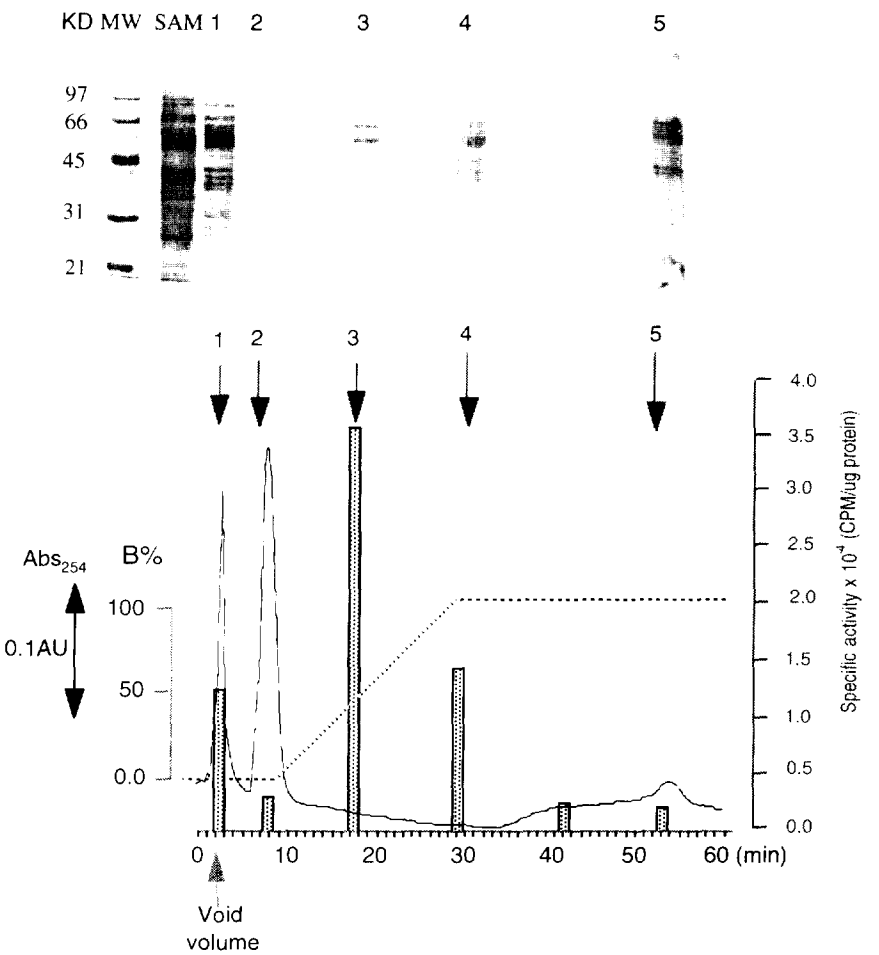


Fig. 2. ^{ether}IAM.PE$^{C10\ C3}$ Chromatography on a pilot size column (3 × 0.46 cm). The flow-rate was 0.2 ml/min, and detection was at 254 nm (solid line). Prior to injecting 40 μl (0.2 mg microsomal protein), the column was equilibrated with 30 ml of mobile phase A which contained 20 m*M* sodium phosphate (pH 8.0), 20% EG and 1 m*M* EDTA. Mobile phase B was mobile phase A containing 0.2 m*M* DMPE and 2 m*M* DDM. The broken line is the detergent gradient used in the chromatography. A 20-min detergent gradient was used for this experiment. The histogram heights represent NAPES specific activity for particular chromatography fractions.

7 min. Peak 1 and peak 2 can be considered as pass-through peaks that represents either (i) column overloading, or (ii) molecules that do not have high affinity for the ^{ether}IAM.PE$^{C10/C3}$ surface.

Significant amount of proteins were only found in peak 1, but both peak 1 and peak 2 contained small amounts of NAPES activity (compare gel lanes 1 and 2 in Fig. 2). Since peak 1 contained NAPES activity, albeit very low amounts, the ^{ether}IAM.PE$^{C10/C3}$ pilot column was overloaded for this target protein under these experimental conditions. Furthermore, since there are virtually no proteins in peak 2 which eluted at 7 min, the strong absorbance at 254 nm must represent the elution of small molecular mass UV absorbing compounds that are present in the DDM solubilized sample; most likely these UV absorbing species are protease inhibitors (pepstatin, benzamidin, etc.).

Most of the NAPES activity eluted immediately after the initiation of the DDM–DMPE

detergent gradient (gel lane 3, Fig. 2). However, the NAPES activity continued to elute during the entire gradient and most of the contaminating proteins were removed from the target protein (gel lane 3–5). Thus the shallow detergent gradient used for this initial chromatography experiment removed most of the contaminating proteins in the pass-through peak, but the NAPES target protein was not purified to homogeneity. Nevertheless, we estimate that there is ca. 2.5–3 fold increased specific activity of NAPES.

Since pilot size columns could not resolve NAPES activity from contaminating proteins, larger analytical size columns were used. In addition, slightly larger amounts of protein were loaded onto the ^{ether}IAM.PE$^{C10/C3}$ column. Fig. 3 shows the elution profile after loading a 900 μl

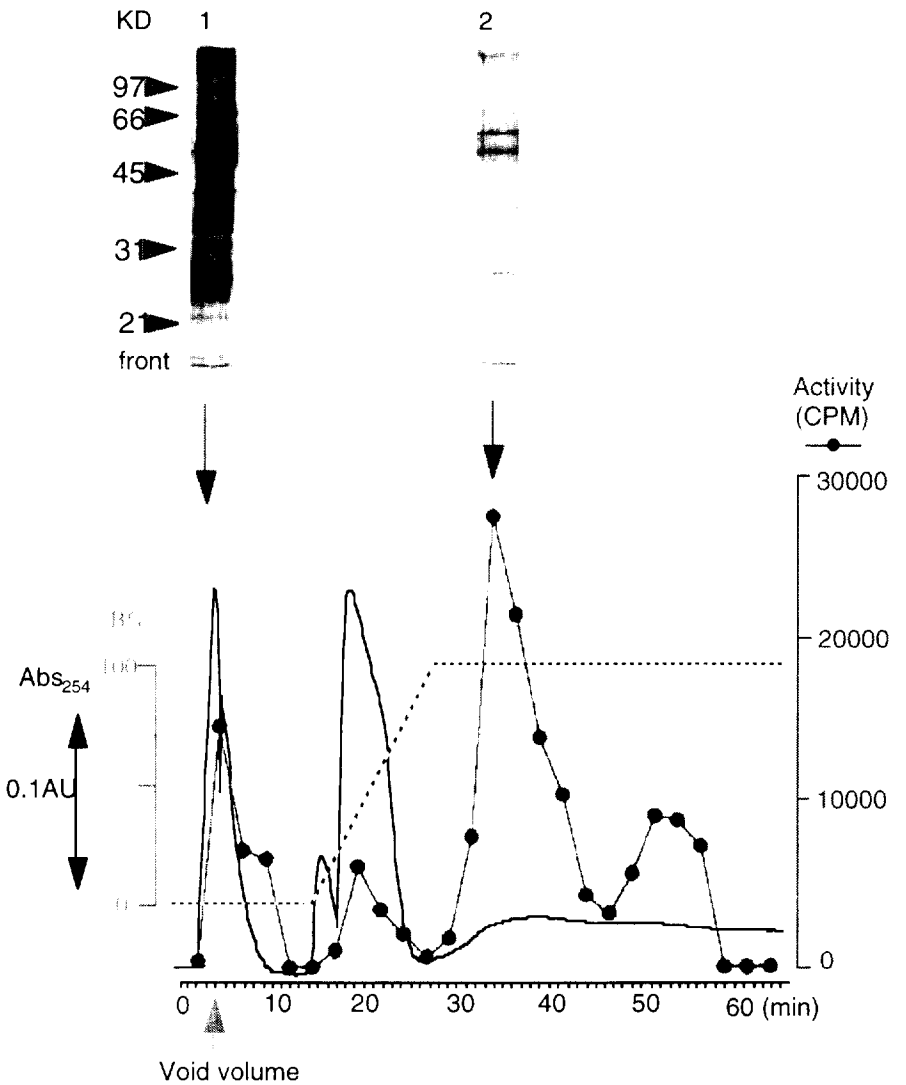


Fig. 3. ^{ether}IAM.PE$^{C10/C3}$ Chromatography on an analytical size column (15 × 0.46 cm). Mobile phases A and B are given in the legend to Fig. 2. Prior to injecting 900 μl (0.5 mg microsomal protein), the column was equilibrated with 30 ml of mobile phase A. The flow-rate was 0.4 ml/min and detection was at 254 nm. The solid line is absorbance at 254 nm eluting from the column and the broken line is the detergent gradient. The filled circles represent NAPES activity for particular chromatography fractions.

volume of the microsomal proteins (ca. 500 μg total protein) at 0.4 ml/min on a 10 × 0.46 cm analytical size ^{ether}IAM.PE$^{C10/C3}$ column. Similar to results obtained using the smaller pilot column, two peaks eluted during the loading step; however, these two peaks were better resolved because the analytical column is longer than the pilot column. Very little NAPES activity eluted prior to the DDM–DMPE detergent gradient. A steep 10-min DDM–DMPE detergent gradient resulted in the elution of NAPES activity in primarily one peak, and most importantly, very few contaminating proteins were in the sample (Fig. 3, gel lane 2).

Both Fig. 2 and Fig. 3 demonstrate that highly purified NAPES resulted from small IAM columns, but some NAPES activity eluted in the pass-through peak(s) from both experiments. A major objective during protein purification is to obtain high recovery of functional protein, and therefore, loss of the target protein in the pass-through peak should be minimized. To eliminate the NAPES activity eluting in the pass-through peak, a semi-preparative IAM column was used with elution conditions similar to that used to generate the data in Fig. 2 and Fig. 3. NAPES purification on a semi-preparative 6.5 × 1.0 cm ^{ether}IAM.PE$^{C10/C3}$ column was tested. Fig. 4 shows the elution profile after loading 2 ml of the microsomal proteins (ca. 4.5 mg total protein) at 1 ml/min. As expected, NAPE synthase activity did not elute in the pass-through peak in spite of the higher protein loading (5 mg of total protein). After 18 min of sample loading and column equilibration, a 15-min linear gradient from 0 to 100% B (containing buffered 0.2 m*M* DMPE and 2 m*M* DDM) was used to elute the proteins. Several small UV absorbing peaks were detected after elution with mobile phase B. However, the maximum NAPES activity eluted in a region of the chromatogram that had little or no UV absorbance. The key finding from Fig. 4 is that larger amounts of NAPES can be loaded on the ^{ether}IAM.PE$^{C10/C3}$ column. We estimate

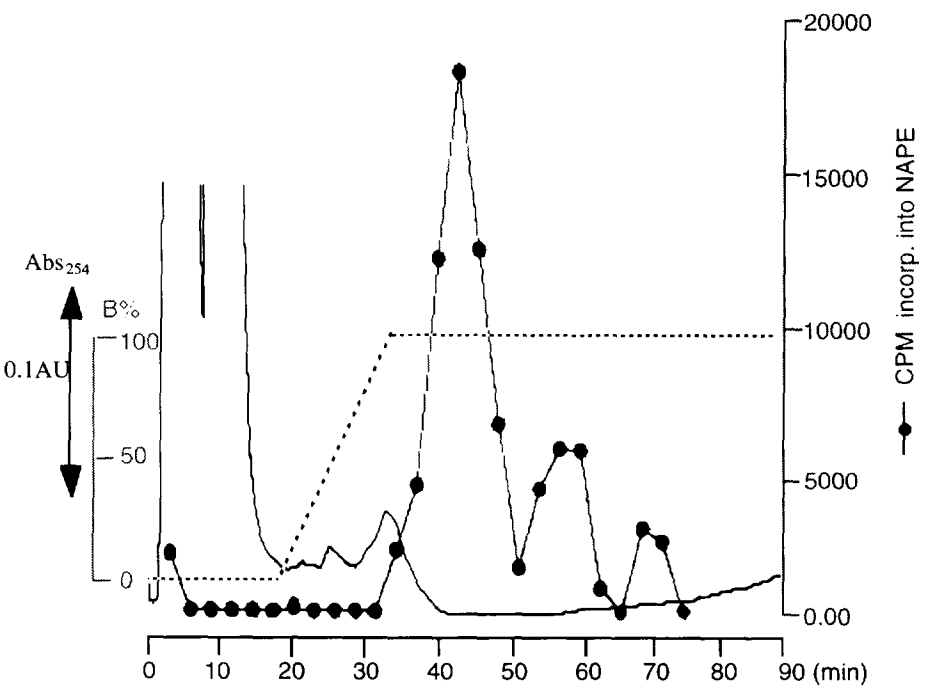


Fig. 4. ^{ether}IAM.PE$^{C10/C3}$ Chromatography on a semi-preparative column (6.5 × 1.0 cm). Mobile phases A and B are given in the legend to Fig. 2. Prior to injecting 2 ml (ca. 5 mg microsomal protein), the column was equilibrated with 30 ml of mobile phase A. The flow-rate was 1 ml/min, detection was at 254 nm (solid line), and the gradient (broken line) are shown. The solid circles represent NAPES activity for particular chromatography fractions.

that the capacity of semi-preparative ^{ether}IAM.PE$^{C10/C3}$ columns is at least 10 times the capacity of pilot columns. Although protein loading on semi-preparative columns was good (i.e., ca. 5 mg total protein loaded and no NAPES activity in the pass-through peaks), NAPES was not purified to homogeneity and other elution conditions were tested with the intent of obtaining pure NAPES.

Since shallow detergent gradients were unsuccessful at purifying NAPES, a two-step detergent gradient was tested. As shown in Fig. 5, immediately after the 7-min peak eluted, the IAM column was perfused with the first DDM–DMPE detergent gradient, a steep 10-min gradient from 0 to 0.8 m*M* DDM. After a 10-min plateau elution, a second steep DDM–DMPE detergent gradient from 0.8 m*M* to 2.0 m*M* DDM detergent was applied over 5 min. As shown in Fig. 5, this two-step detergent gradient increased the spreading of the NAPES activity eluting from the ^{ether}IAM.PE$^{C10/C3}$ column. Thus this two-step gradient did not improve the purity of NAPES and two-step gradients were not further pursued.

To validate the necessity of DMPE for NAPES elution, the chromatography in the presence and absence of DMPE was performed. Fig. 6 compares the elution of proteins from the ^{ether}IAM.PE$^{C10/C3}$ column with and without DMPE present in the DDM detergent gradient. For this comparison, a steep 15-min DDM detergent gradient was used and the flow-rate was 0.2 ml/min. This increased flow-rate and steep DDM detergent gradient was chosen because the 20-min gradient at 0.2 ml/min spread NAPES activity that eluted from the column as shown in Fig. 2. When DMPE was omitted from the detergent gradient, very little NAPES activity eluted from the ^{ether}IAM.PE$^{C10/C3}$ column (Fig. 6, open circles). However, elution with mobile phases containing both DDM and DMPE resulted in very high amounts of NAPES activity eluting from the column (Fig. 6, closed circles). This demonstrates that mobile phase DMPE increases the NAPES activity eluting from the IAM column.

All of the above chromatography experiments utilized linear detergent gradients that were maintained at a plateau detergent concentration

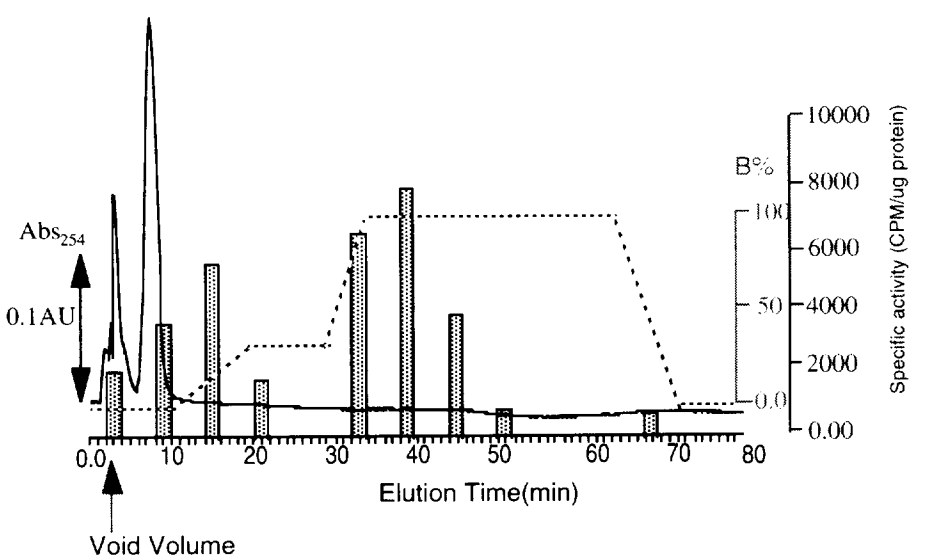


Fig. 5. Two-step gradient chromatography on an ^{ether}IAM.PE$^{C10/C3}$ pilot size column (3 × 0.46 cm). Mobile phases A and B are given in the legend to Fig. 2. Prior to injecting 40 μl (0.2 mg microsomal protein), the column was equilibrated with 30 ml of mobile phase A. The flow-rate was 0.2 ml/min, detection at 254 nm (solid line) and the detergent gradient (broken line) are shown. The solid circles represent NAPES activity for particular chromatography fractions.

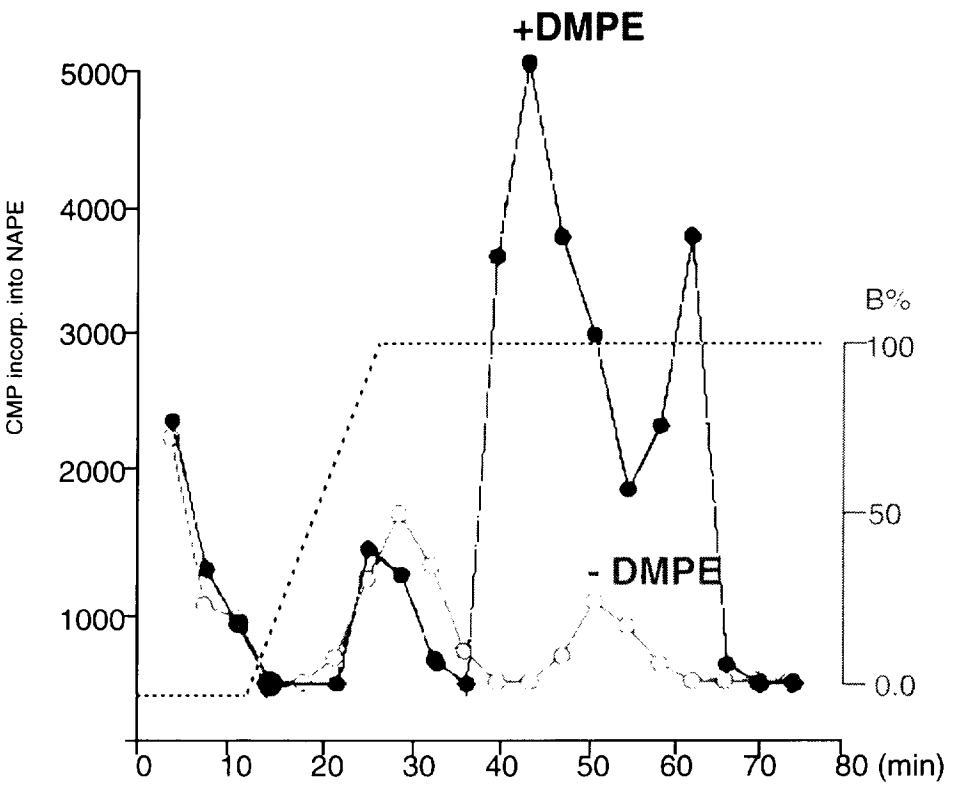
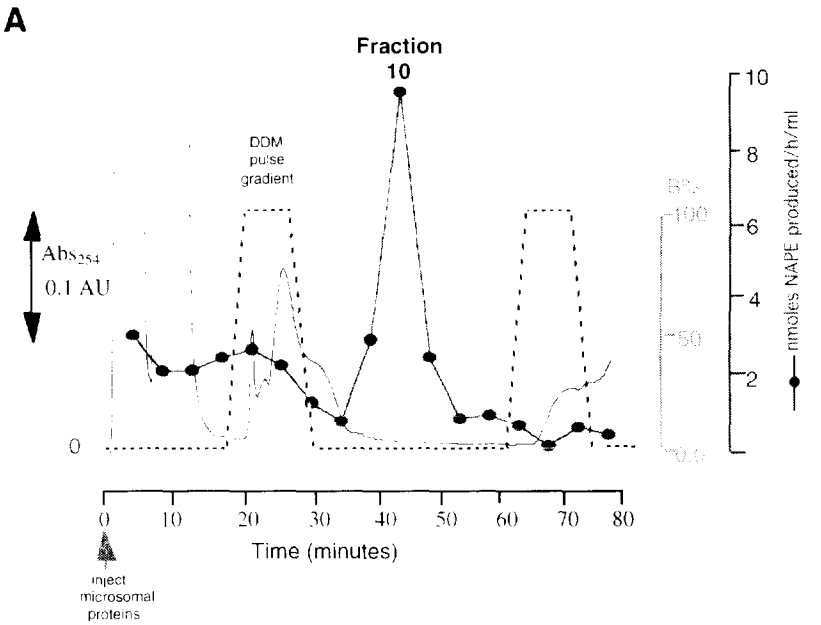


Fig. 6. Effect of DMPE on the elution of NAPES from ^{ether}IAM.PE$^{C10/C3}$. The flow-rate was 0.2 ml/min, detection at 254 nm, and a pilot size column (3 × 0.46 cm) was used. Prior to injecting 40 μl (0.2 mg microsomal protein), the column was equilibrated with 30 ml of mobile phase A. The composition of mobile phase A and B are given in the legend to Fig. 2. However, DMPE was omitted from mobile phase B in one experiment.

for ca. 30–60 min to facilitate the elution of proteins that exhibit high affinity for the ^{ether}IAM.PE$^{C10/C3}$ surface. However, polyacrylamide gel electrophoresis always showed contaminating proteins in chromatography fractions containing the NAPES activity. These contaminating proteins could not be resolved using conventional elution strategies shown in Figs. 2–6. We therefore attempted unconventional elution conditions. Preliminary studies demonstrated that bolus injections of mobile phase B (i.e., the detergent mobile phase containing DDM–DMPE) resulted in protein elution. In other words, instead of using a 10-min gradient of mobile phase B, a 1-ml pulse injection of mobile phase B resulted in the elution of a few proteins. Consequently, a "pulse gradient" was tested for eluting NAPES activity from the ^{ether}IAM.PE$^{C10/C3}$ column. A "pulse gradient" refers to a steep detergent gradient that plateaus for only a few minutes before the mobile phase concentration is reduced back to the equilibration buffer.

Using a DDM pulse gradient, Fig. 7 shows the elution profile after loading 1.6 ml of the microsomal proteins (ca. 5 mg total protein) at 1 ml/min on a 6.5 × 1.0 cm ^{ether}IAM.PE$^{C10/C3}$ column. The pulse gradient, applied 18 min after protein loading, was a steep 2-min linear gradient from 0–100%B, followed by a 9-min plateau, followed by a steep descending gradient back to the equilibration buffer A. Most interesting from this elution condition was that NAPES activity did not elute during the pulse gradient; NAPES activity eluted in fraction 10 which was collected ca. 15 min after the pulse gradient was



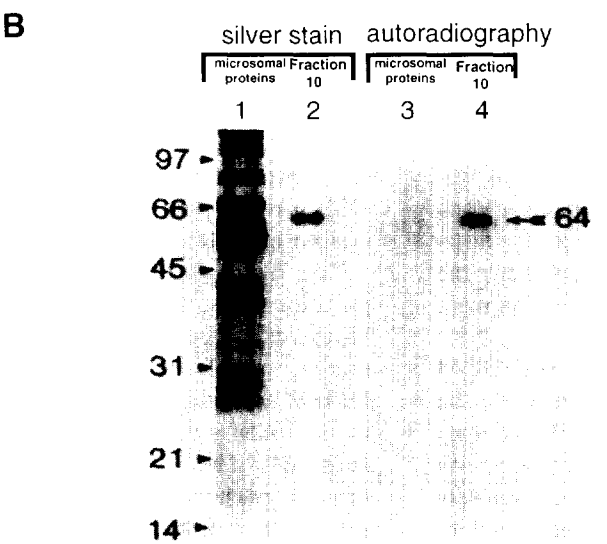


Fig. 7. (A) Purification of NAPES on a semi-preparative ^{ether}IAM.PE$^{C10/C3}$ (6.5 × 1.0 cm) column using a pulse detergent gradient system. The flow-rate was 1 ml/min, detection at 254 nm (solid line), and the two pulsed detergent gradients (broken lines) given. Prior to injecting 2 ml (5 mg microsomal protein), the column was equilibrated with 30 ml of mobile phase A. The solid circles represent NAPES activity. (B) SDS-PAGE analysis of NAPES purification on ^{ether}IAM.PE$^{C10/C3}$. Lane 1 shows the DDM-solubilized microsomal proteins (12 μg protein) mixture that was injected on to the column. Lane 2 (ca. 1 μg protein loaded on the gel) is from chromatography fraction 10. Lane 3 (DDM-solubilized microsomal proteins) and land 4 (chromatography fraction 10) are autoradiographs that contain ^{125}I-ASD (2.6 μ*M*, 2 μCi) labeling of NAPES prior to SDS-PAGE analysis.

completed. Furthermore the NAPES activity was concentrated in only 3 chromatography fractions (Fractions 9, 10, 11).

Fraction 10 eluting at ca. 42 min (Fig. 7A) was subjected to SDS-PAGE analysis (Fig. 7B). Protein visualization by silver staining showed a single intense band at 64 kilodalton indicating that NAPES was purified to homogeneity (lane 2). McAndrew et al. [8] prepared a ^{125}I photoreactive crosslinking substrate analogue of NAPES, and this photoaffinity ligand was used to confirm that the 64-kilodalton protein was NAPES (Fig. 7B, Lane 4). The single step purification of NAPES (Fig. 7A) from detergent solubilized microsomes is remarkable considering that NAPES is almost undetectable in the microsomal proteins (Fig. 7B); both autoradiographs and silver stain analysis show virtually no detectable NAPES in the detergent solubilized microsomes. The NAPES specific activity in detergent solubilized microsomes was 281 nmol/h/mg protein, and the specific activity in fraction 10 was 9890 nmol/h/mg protein. Thus NAPES was purified 3940 fold by using an ^{ether}IAM.PE$^{C10/C3}$ column.

As shown in Fig. 7A, a second DDM pulse gradient was applied to the ^{ether}IAM.PE$^{C10/C3}$ column to evaluate if additional proteins remained on the column. A few proteins eluted during the second DDM pulse gradient; however, since no NAPES activity eluted from the second DDM pulse gradient, these proteins were not further studied.

Novel elution conditions such as the DDM pulse gradient may leave residual proteins on the ^{ether}IAM.PE$^{C10/C3}$ column after chromatography. Thus the column should be washed with 1% CHAPS or other detergents to completely remove residual proteins. DDM is not used to wash the column because it is expensive relative to CHAPS. However, detergent washes leave residual detergents on ^{ether}IAM.PE$^{C10/C3}$ columns and these detergents must also be washed from the columns. Furthermore, DMPE phospholipids must also be removed from the ^{ether}IAM.PE$^{C10/C3}$ column. Thus ^{ether}IAM.PE$^{C10/C3}$ columns are routinely washed with MeOH (30–40 column volumes) after each experiment to remove residual DMPE and residual detergents.

The purification of NAPES using IAM chromatography was based on a membrane affinity concept. In our system, DMPE was intended to be used as the affinity displacing ligand to displace NAPES adsorbed to the IAM column. However, the elution of NAPES activity lagged behind the perfusion of DMPE, and in fact, when DDM pulse gradients containing DMPE were used, the NAPES activity did not elute during the detergent pulse gradient (e.g., Fig. 7). The explanation for NAPES activity lagging the DMPE mobile phase gradient may reside in the difference between small aqueous soluble displacing ligands compared to surfactant-type displacing ligands. Small aqueous soluble affinity displacing ligands are used in conventional affinity chromatography to elute target proteins that are bound to chromatography surfaces through bioaffinity interactions. In contrast, IAMs are a membrane affinity chromatography surface that requires surfactant-type displacing ligands for efficient displacement of proteins bound to the IAM surface. Water soluble displacing ligands have limited access to the protein–IAM binding site, but hydrophobic displacing ligands (e.g., DMPE) can partition into the IAM surface to facilitate protein displacement.

The hydrophobic tail of DMPE undoubtedly partitions into the immobilized PE monolayer. However, since DMPE is insoluble in aqueous media, mobile phase DDM was required for DMPE solubilization. Thus DMPE is delivered to the IAM surface via a DDM/DMPE micelle (Fig. 8). The short pulse DDM–DMPE detergent gradient contained a total of only ca. 1 mg of DMPE. The 6.5×1.0 cm ^{ether}IAM.PE$^{C10/C3}$ column (used for the purification shown in Fig. 6) contains ca. 4 g of packing material which corresponds to ca. 330 mg of immobilized PE. The 1 mg of DMPE equilibrates with the 330 mg of immobilized PE during the pulse gradient, but DMPE does not elute from the column during the pulse gradient. The key concept is that the equilibrium between detergent solubilized DMPE and immobilized

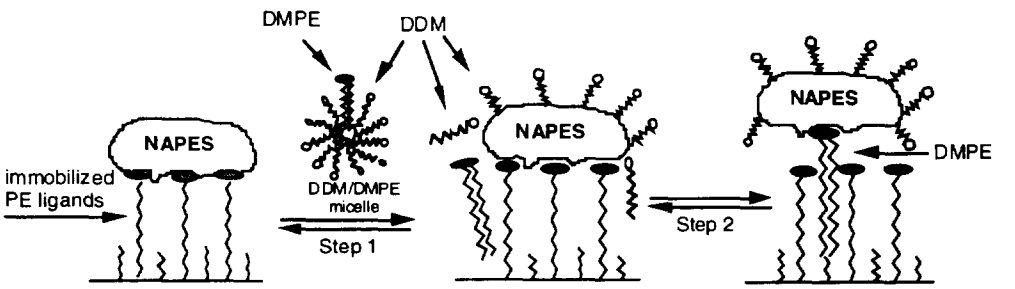


Fig. 8. NAPES purification by IAM chromatography using DDM–DMPE detergent mixtures.

PE makes the elution of DMPE lag behind the pulse gradient.

NAPES is a 64 kilodalton protein that chemically interacts with many immobilized PE ligands, but for clarity, only three immobilized PE molecules were used to depict the NAPES binding site on the ^{ether}IAM.PE$^{C10:C3}$ surface in Fig. 8. Both DDM gradients and DDM–DMPE gradients generate two NAPES peaks with little functional activity; however when DDM–DMPE detergent gradients are used, the late eluting NAPES peak has >5 fold increased functional activity (Fig. 6).

The explanation for the DMPE-induced increased NAPES activity (Fig. 6), and DMPE-induced protein purity (Fig. 7) most likely resides in the binding of DMPE with surface associated NAPES. As shown in Fig. 8, after NAPES is adsorbed to the IAM column, DDM–DMPE detergent gradients cause both DDM and DMPE to partition into the IAM surface. However, DDM is freely soluble in the mobile phase, whereas DMPE requires DDM for solubilization. Consequently, DMPE partitioning favors the IAM surface; lateral diffusion of DMPE allows DMPE to partition into the binding site of NAPES. Alternatively, DMPE in the mobile phase may partition into the NAPES active site through a micelle–protein interaction. Regardless of the DMPE diffusion path for entry into the NAPES active site, the NAPES–DMPE complex exhibits different elution and functional activity compared to the non complexed NAPES. Since no structural information is available regarding the NAPES binding site or the protein–membrane binding interactions, Fig. 8 should be considered as only a very preliminary model that is useful for discussing the chromatography of NAPES on IAM columns.

Regarding functional activity, it is known that substrates included in the mobile phase can stabilize membrane proteins and increase functional activity during the chromatographic process [26,27]. Since DMPE is a substrate of NAPES, the increased functional activity eluting from the column (Fig. 6) is caused, in part, by stabilization of NAPES–DMPE complex during IAM chromatography. Regarding protein elution, the NAPES–DMPE complex may have either higher or lower affinity for the IAM surface. Higher affinity would occur if DMPE functions as an affinity ligand as described [28], lower affinity would occur if DMPE facilitates displacement of NAPES from the IAM surface. The main peak of functional NAPES activity elutes ca. 10 min earlier when DMPE is included in the mobile phase (Fig. 6) and this suggests that the NAPES–DMPE complex increases the efficiency of DDM-induced elution of NAPES, i.e. DMPE is functioning as an affinity displacing ligand.

The utility of the pulse detergent gradient to purify NAPES to homogeneity involves the selective removal of only a few contaminating proteins. In other words, Figs. 2–4 demonstrate that steep (20 to 30 min) detergent gradients can purify NAPES to >90% purity in one step; only a few contaminating proteins are present based on SDS-PAGE analysis. The DDM–DMPE pulse gradient was able to selectively remove the few contaminating proteins from the target protein. As described above, small amounts of

DMPE in the mobile phase can initiate the formation of the NAPES–DMPE complexes, and DDM more efficiently elutes the complex from the surface. The slightly increased selectivity of mobile phase DMPE was sufficient to remove the contaminating proteins from NAPES in a single step. However, from a chromatographic point of view, the key concept is that NAPES exhibits high affinity for the IAM column; proteins that exhibit high affinity for IAM columns are usually purified >70–90% in one step as shown in Figs. 2–4.

DMPE is not an affinity surfactant as proposed by Torres et al. [28]. Affinity surfactants are custom synthesized using an analogue of the enzyme substrate linked to a surfactant, and the surfactant–substrate is non covalently coated onto C18 columns. The target enzyme exhibits increased affinity for the C18 surface by affinity interactions to the affinity surfactant. Since DMPE functioned as an affinity displacing ligand, the effect of DMPE on protein elution is distinct from affinity surfactants described by Torres et al. [28].

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