Purification and Properties of an Ethanolamine-Serine Base Exchange Enzyme of Rat Brain Microsomes*

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The activity of an ethanolamine and serine base exchange enzyme of rat brain microsomes was copurified to near homogeneity. The purification sequence involved detergent solubilization, Sepharose 4B column chromatography, phenyl-Sepharose 4B column chromatography, glycerol gradient sedimentation, and agarose-polyacrylamide gel electrophoresis under non-denaturing conditions. The ratio of the ethanolamine and serine base exchange activities remained almost constant during purification, and both enzyme activities were enriched 25-fold over the initial microsomal suspension. The final enzyme preparation which contained both enzyme activities showed a single protein band on sodium dodecyl sulfate-polyacrylamide gel, having an apparent molecular mass of about 100 kDa.

Serine inhibited the ethanolamine incorporation by this preparation and ethanolamine inhibited the serine incorporation. The competitive nature of this inhibition was apparent from Lineweaver-Burk plots, suggesting that the enzyme catalyzes the incorporation of both ethanolamine and serine into their corresponding phospholipids. The $K_m$ and $K_i$ values for ethanolamine were quite similar, being 0.02 and 0.025 mM, respectively. The $K_m$ and $K_i$ values for serine were also quite similar being 0.11 and 0.12 mM, respectively. The pH optimum was the same at 7.6 with both substrates. The optimal Ca$^{2+}$ concentration was 8 mM for serine incorporation.

The base exchange enzymes catalyze the incorporation of L-serine, ethanolamine, monomethylethanolamine, and choline into their corresponding phospholipid. These reactions are energy-independent, require Ca$^{2+}$, and have a slightly alkaline pH optimum (1-6). It was suggested that each of the base exchange reactions was catalyzed by separate enzymes, based upon observations of variable sensitivity (7), Ca$^{2+}$ dependency (6), kinetic properties (4, 8), and the asymmetric distribution on membranes (9, 10). To establish the existence of separate enzymes, attempts at purification were carried out, utilizing the microdispersion technique (9, 10). The standard incubation medium contained 100 pmol of HEPES, pH 7.23, under nitrogen flow using a Heat Systems-Ultra Sonic Inc. probe-type sonicator set at 50 watts, followed by dialysis overnight against approximately 200 volumes of the same buffer at 4°C (12). The dialyzed solution was centrifuged at 100,000 X g for 30 min at 3°C, and the supernatant was used in these experiments. The phospholipid concentration of the microdispersion was determined according to the method of Bartlett (13).

Base Exchange Enzyme Assay—The assay followed the method of Miura and Kanfer (10). The standard incubation medium contained 10 μmol of HEPES, pH 7.23, 2 μmol of CaCl₂, asolectin microdispersion (25 μg of phospholipid phosphorus), the particular radioactive substrate, and enzyme preparation in a total volume of 0.24 ml. The specific activity of the labeled precursor used in the purification steps was about 30 nmol/μCi for serine incorporation, 40 nmol/μCi for ethanolamine incorporation, or about 50 nmol/μCi for choline incorporation. The amount of base for characterization of the purified enzyme was 76.8 nmol/μCi for serine, 19.2 nmol/μCi for ethanolamine, or 50 nmol/μCi for choline incorporation. The reaction was started by substrate addition, incubated at 37°C for 18 min, and stopped by the addition of 1 ml of ice-cold 1% trichloroacetic acid. The mixture was transferred onto a nitrocellulose membrane filter (HA 0.45 μm, 2.5 cm; Millipore Co., Bedford, MA) and washed successively with 25 ml of ice-cold 5% trichloroacetic acid. The filter was dried, and radioactivity was determined by scintillation counting. An incubation time of 60 min and the addition of bovine serum albumin (0.6 mg/0.6 ml) prior to the addition of trichloroacetic acid was used for the enzyme characterization. The precipitates were collected by centrifugation, and the phospholipids present in the pellet were extracted by the method of Folch et al. (14). The extract was dried with N₂ gas, and radioactivity was determined by scintillation counting. Where required, the nature of the radioactive phospholipid present in the extract was provisionally identified by two-dimensional thin layer chromatography with chloroform/methanol/ammonia (65:35:5, v/v) for the first dimension and chloroform/acetone/acetic acid/methanol/water (50:20:10:10:5, v/v) for the second dimension.

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‡ The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate.
ond dimension (16). Individual lipids present on the thin layer chromatographic plates were visualized by iodine vapor.

Preparation of Microsomes and Solubilization—Preparation and solubilization of rat brain microsomes was by the procedure of Miura and Kanfer (10), with some modifications. Brains from 25 1-month-old rats were homogenized in 200 ml of cold 2 mM HEPES buffer, pH 7.23, containing 0.32 M sucrose and 1 mM EDTA, using a Polytron instrument (kinematica, CMH, Luzern, Switzerland) at maximum speed for 30 s. The homogenate was centrifuged at 7,000 × g for 10 min, followed by centrifugation of the supernatant at 12,000 × g for 10 min. The resulting supernatant was centrifuged at 56,000 × g for 60 min, and the pellet designated as the microsomal fraction was suspended in 25 ml of homogenization medium with a Dounce homogenizer. The microsomal suspension (25 ml) was mixed with 130 ml of a solution composed of 0.5% Miranol H2M (w/v), 0.3% sodium cholate (w/v), 5 mM HEPES, pH 7.5, 20% glycerol, 1 mM mercaptoethanol, and 1 for 10 min in an ice bath and centrifuged at 165,000 × g for 60 min. The resulting supernatant was designated as the solubilized fraction and processed for enzyme purification.

**Sepharose CL-4B Column Chromatography**—The solubilized enzyme containing extracts was applied on a Sepharose CL-4B column (10 × 100 cm) which was pre-equilibrated with 5 mM HEPES buffer, pH 7.23, containing 20% glycerol, 1 mM EDTA, and 1 mM mercaptoethanol (GHME buffer). The column was eluted with the GHME buffer. Approximately 10-ml samples were collected, and the fractions from 61 to 71 (Fig. 1) containing high base exchange enzyme activity were pooled.

**Phenyl-Sepharose 4B Column Chromatography**—The pooled fraction (120 ml) from the Sepharose CL-4B column was adjusted to 1 M NaCl by the addition of solid NaCl and applied on a 25-ml bed volume phenyl-Sepharose 4B column which had been equilibrated with GHME buffer containing 1 M NaCl. The column was washed successively with 3-bed volumes of GHME buffer, 2.5-bed volumes of GHME buffer containing 0.1% Triton X-100, and 3-bed volumes of GHME buffer containing 0.4% Triton X-100. The base exchange activities were present in both the 0.1% Triton X-100 and 0.4% GHME buffer containing 0.1% Triton X-100 and 0.4% Triton X-100 eluates (Fig. 2). Approximately 50 ml of the 0.4% Triton X-100 fractions were pooled and concentrated to 15 ml by ultrafiltration through an Amicon PM 30 membrane.

**Glycerol Gradient Sedimentation**—Sufficient 10% Triton X-100 was added to the concentrated phenyl-Sepharose 4B fraction to yield a final concentration of 0.05%. A 2-ml sample was gently layered onto a continuous glycerol gradient which was prepared according to the procedure of Miyaura et al. (17). The tube contents were withdrawn from the bottom to the top and the glycerol solutions consisted of 2 ml of 36%, 3.3 ml of 34%, 2.2 ml of 30%, 1.4 ml of 26%, 1.3 ml of 21%, and 0.8 ml of 15% into a centrifuge tube and allowing it to remain at 4 °C overnight. The glycerol solutions contained 5 mM HEPES, pH 7.23, 1 mM mercaptoethanol. Centrifugation was carried out with a Beckman SW 41 Ti rotor at 30,000 rpm for 72 h at 3 °C. The tube contents were withdrawn from the bottom to give 24 aliquots of 0.6 ml each. Fractions 16–19 of each gradient were pooled and concentrated to 3 ml by ultrafiltration through an Amicon PM30 membrane.

**Nondenaturing Gel Electrophoresis**—Attempts to purify the en-

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**Fig. 1.** Gel filtration of a solubilized microsomal extract containing the base exchange enzymes on a Sepharose CL 4B column. Aliquots of 40 μl of each fraction were used for detecting serine (A—A) and ethanolamine (O—O) base exchange activities as described under "Materials and Methods." Protein was measured by absorbance at 280 nm (0—0). Enzyme active fractions (62–72) were combined and used for next purification step. Activity is expressed as counts incorporated per 18 min/40-μl sample into phospholipids from the radioactive substrates.

**Fig. 2.** Phenyl-Sepharose 4B column chromatography. The pooled Sepharose CL 4B fraction was adjusted to 1 M NaCl and then placed on the column, and elution was carried out with 20% (w/v) glycerol, 1 mM EDTA, 1 mM mercaptoethanol, 5 mM HEPES, pH 7.23 (buffer A), 0.1% (w/v) and 0.4% Triton X-100 in buffer A. The arrows indicate change of solutions. Protein was measured by absorbance at 595 nm using a Coomassie Blue-staining assay (0—0) and 40 μl aliquots of each fraction was assayed for serine (A—A) and ethanolamine (O—O) exchange enzyme activities.

**Fig. 3.** Glycerol gradient sedimentation. Two-ml aliquots of the concentrated phenyl-Sepharose 4B fraction were layered onto the glycerol gradient and centrifuged at 30,000 rpm for 72 h at 3 °C. Protein was measured by absorbance at 585 nm using a Coomassie Blue-staining assay (0—0). The incorporations of serine (A—A) and ethanolamine (O—O) into phospholipids were measured.
enzymatic activity appeared in a very turbid void volume fraction, and the remainder was in a broad second peak. It appeared that the aggregated state of the enzymes, after ammonium sulfate treatment, resisted solubilization. This was also suggestive from the previous study (11). Therefore, the detergent extract of the microsomes was applied directly to a CL-Sepharose 4B column without ammonium sulfate precipitation. A typical elution profile is shown in Fig. 1. Most of the proteins appeared in the very turbid breakthrough fraction which had low base exchange activities. This was followed by a second small protein peak containing 80% of the base exchange enzyme activity. The activities of this fraction are stable for several months. There was no visible aggregation at -20°C storage, even though a considerable quantity of the detergents employed for solubilization had been removed by the gel filtration.

The contents of tubes 67–71 were pooled and applied to a phenyl-Sepharose 4B column, and a typical elution profile is shown in Fig. 2. Miranol H2M, sodium cholate, octyl-glucoside, and Triton X-100 were tested for elution of the enzyme activity from this column; however, except for Triton X-100, all destroyed the applied enzyme. Enzymatic activity was recovered in the 0.1 and 0.4% Triton X-100 eluates. The 0.1% concentration of Miranol H2M and sodium cholate were decreased from 0.8 and 0.5% to 0.5 and 0.3%, respectively. Only the ethanolamine and serine base exchange enzyme activities were present in the solubilized preparation with negligible detectable choline base exchange activity.

Initially, a 35–55% ammonium sulfate saturation precipitate from the solubilized extract was applied to the Sepharose 4B column. About one-half of the total base exchange enzyme activity appeared in a very turbid void volume fraction, and the remainder was in a broad second peak. It appeared that the aggregated state of the enzymes, after ammonium sulfate treatment, resisted solubilization. This was also suggestive from the previous study (11). Therefore, the detergent extract of the microsomes was applied directly on a CL-Sepharose 4B column without ammonium sulfate precipitation. A typical elution profile is shown in Fig. 1. Most of the proteins appeared in the very turbid breakthrough fraction which had low base exchange activities. This was followed by a second small protein peak containing 80% of the base exchange enzyme activity. The activities of this fraction are stable for several months. There was no visible aggregation at −20°C storage, even though a considerable quantity of the detergents employed for solubilization had been removed by the gel filtration.

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INCUBATION TIME (MIN.)  PROTEIN CONCENTRATION (µg)

**FIG. 5.** a, time dependency of ethanolamine base exchange enzyme (○-○) and serine base exchange enzyme (■-■) activities. The amount of protein present in the reaction mixture was 0.6 µg. The incubations were terminated at the time intervals indicated. b, dependence of base incorporation on enzyme concentration. Incubations for ethanolamine (○-○) or serine (■-■) base exchange enzyme activity were for 60 min with varying the concentration of enzyme.

Triton X-100 fraction was discarded because of its low specific activity, and the 0.4% Triton X-100 fraction was further processed. The specific activities of both base exchange enzymes of this fraction were decreased, compared to the Sepharose 4B fraction; however, they were increased 1.2-fold after the removal of Triton X-100 by a Sephadex G-25 column. An advantage of this hydrophobic column was the removal of considerable amounts of phospholipids. The content of tubes 47-55 were pooled as the active fraction.

A typical glycerol sedimentation profile is shown in Fig. 3. Initially, a glycerol gradient containing 0.05% Triton X-100 was used for enzyme separation, but little of the enzyme activity was recovered after the centrifugation. Therefore, the gradient centrifugation was carried out in the absence of detergent. The recombined active fractions 16-18 appeared to be about 80% homogeneous on 7.5% polyacrylamide-SDS slab gel electrophoresis and is stable for several months at -20 °C.

The active sample from the sucrose gradient purification was examined by disc gel electrophoresis on either 5% polyacrylamide gel or 2% polyacrylamide, 0.5% agarose gel or 1.5% polyacrylamide, 0.5% agarose gel. The enzymes remained on top of gel and did not migrate into the 5% polyacrylamide gel and 2% polyacrylamide, 0.5% agarose gel. Ethanolamine and serine base exchange activities co-migrated in the 1.5% polyacrylamide, 0.5% agarose gel, suggesting that both enzymes had similar charge and possessed a molecular weight over 10^6, compared to the molecular weight estimation of several RNA species according to Dingman and Peacock (17, 18). The enzyme recovery from the gel electrophoresis procedure was only about 30%. Other established methods were examined to improve the enzyme recovery; however, they resulted in greater losses of enzyme activities (21). The simple extraction procedure described under "Materials and Methods" was less destructive and provided an enzyme that was stable for about 2 months.

The enzyme preparation was examined by SDS-polyacrylamide gel electrophoresis (Fig. 4) and found to exhibit a single protein band suggesting that it was homogeneous. The molecular mass of the enzyme was approximately 100 kDa.

The results for a typical enzyme purification from about 25 g of fresh rat brain are summarized in Table I. The procedure seems reproducible and has been employed for three separate preparations. The ratio of the ethanolamine and serine base exchange activities remained nearly constant during the various stages of purification. They had similar stability, and both specific activities were enriched 25-fold compared to that of microsomes. However, compared to the solubilized microsomal extract, the serine exchange activity was purified 43-fold and the ethanolamine exchange activity 76-fold. Negligible choline base exchange enzyme activity was present in the solubilized enzyme fraction and negligible phospholipase D activity was found in the final preparation. A proportional increase of serine and ethanolamine incorporation was observed as a function of time of incubation (Fig. 5a) and quantity of enzyme protein present (Fig. 5b). Therefore, a 1-
TABLE II

| Substrate | $K_m$ (mM) | $V_{max}$ (nmol/mg/h) | $K_i$ (mM) |
|-----------|------------|------------------------|------------|
| Serine, $^{14}$C | 0.11 | 330 | 0.123 |
| Ethanolamine, $^{14}$C | 0.02 | 40 | 0.025 |

**Fig. 8.** Effect of varying the pH of the incubations on ethanolamine (O—O) and serine (△—△) incorporation. Both enzyme activities were measured with HEPES/NaOH buffer over the range of pH values examined. Reactions were carried out for 60 min at 37°C with 0.6 μg of purified protein.

**Fig. 9.** Effect of varying potential phospholipid acceptors on the incorporation of ethanolamine (A) or serine (B) asolectin (O—O), phosphatidylethanolamine (△—△), phosphatidylcholine (●—●), phosphatidylserine (■—■), and phosphatidylinositol (□—□). Maximal incorporation of both ethanolamine and serine occurred at pH 7.0 (Fig. 8). The asolectins employed as a source of acceptor is a heterogeneous mixture of phospholipids. It was of interest to compare the capacity of several microdispersions of individual phospholipids for ethanolamine and serine incorporation. Phosphatidylserine, phosphatidylinositol, and phosphatidylcholine were inactive (Fig. 9, A and B) as were lysophosphatidylcholine and phosphatidylglycerol (results not shown).

**Fig. 10.** Effect of varying Ca$^{2+}$ concentration on the incorporation of ethanolamine (O—O) or serine (△—△). Maximal incorporation of both ethanolamine and serine occurred at pH 7.0 (Fig. 8). The asolectins employed as a source of acceptor is a heterogeneous mixture of phospholipids. The results of varying non-radioactive serine on labeled ethanolamine incorporation is presented in Fig. 6, and the effect of serine on ethanolamine incorporation is seen in Fig. 7. Lineweaver-Burk plots revealed that serine increased the $K_m$ for ethanolamine. Ethanolamine displayed the same effect on serine incorporation. The $K_m$ of 0.02 mM for ethanolamine calculated from the Lineweaver-Burk plots (Fig. 6) is similar to the $K_i$ of 0.025 mM for inhibition of serine incorporation (Fig. 7). The $K_m$ of 0.11 mM for serine incorporation calculated from Fig. 7 is similar to the $K_i$ of 0.117 for inhibition of ethanolamine incorporation. These results suggest that there may be an identical ethanolamine and serine binding site on the enzyme. The $K_m$ value for serine is four times greater than that for ethanolamine, indicating that there is a greater affinity for ethanolamine than serine (Table II).

**DISCUSSION**

The purification of the base exchange enzymes was hindered by the requirement for liberation from the microsomes and instability after detergent solubilization (10, 11). We now report the successful copurification apparently to near homogeneity of the ethanolamine and serine base exchange enzymes in a reasonably stable form.

Miura and Kanfer (10) investigated conditions for solubilization of the three base exchange enzymes from brain microsomes and found that a combination of 0.8% Miranol H2M and 0.5% sodium cholate seemed most suitable. We attempted
to reduce the presence of the choline base exchange enzyme in the microsomal extract. This was partially achieved by decreasing the concentrations of Miranol H2M and sodium cholate to 0.5 and 0.3%, respectively. Numerous column supports including ion-exchange and affinity supports and elution buffer were systematically examined in attempts to enrich the ethanolamine and serine exchange activities without success. This lack of success was attributed either to loss of enzyme activity, formation of intractable aggregate, or an inability to recover activity from columns. The activities were completely lost on DEAE-Sephadex column chromatography. Ethanolamine was linked to epoxy-agarose gel to provide an affinity column. The enzyme resisted elution with ethanolamine, but could be removed with 0.2 M NaCl without increasing its specific activity. In addition, both exchange activities were very unstable in the NaCl eluate. Therefore, we eliminated ion-exchange columns for purification.

The enzyme appears to diffuse at each phase of purification. This was especially apparent during the non-denaturing polyacrylamide-agarose gel electrophoresis where a single clear protein band was not visible; however, the enzyme preparation from the final electrophoretic purification step appeared as a nearly homogeneous protein band on SDS-polyacrylamide gel electrophoresis. These observations suggest that the enzyme tends to aggregate yielding heterogeneous multimers.

Taki and Kanfer (11) reported a partial purification of a serine base exchange enzyme devoid of choline and ethanolamine base exchange enzyme. The ethanolamine activity was removed from the serine activity only at the final steps of this purification procedure. Another portion of this particular separation on a DEAE-cellulose column contained both serine and ethanolamine incorporating activities. The current study shows that the ratio of the ethanolamine and serine base exchange activities remained relatively constant, and both activities in the final enzyme preparation had similar properties. In addition, the kinetics revealed that serine and ethanolamine were reciprocal competitive inhibitors for each other's incorporation. These results suggest that the same enzyme catalyzes the incorporation of both serine and ethanolamine into phospholipids.

The optimum Ca²⁺ concentration for ethanolamine and serine incorporation by the purified enzyme preparation, as well as for microsomes, were 8 and 10 mM, respectively. The minimum Ca²⁺ concentration to obtain detectable base exchange enzyme activity was around 500 μM. It is well known that physiological free Ca²⁺ concentration in cytosol is 10⁻⁷ to 10⁻⁶. M. Brattin et al. (22) investigated Ca²⁺ uptake into liver microsomes and showed that an intravesicular steady state concentrations of 7–8 mM Ca²⁺ had been achieved by activation of a Ca,Mg-ATPase. Similar information is not available about brain microsomes, but a similar concentration of Ca²⁺ might be sequestered. Therefore, it is possible that the base exchange enzymes could be activated after Ca²⁺ sequestration into microsome by a Ca,Mg-ATPase.

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