Structure of Human Erythrocyte Acetylcholinesterase
CHARACTERIZATION OF INTERSUBUNIT DISULFIDE BONDING AND DETERGENT INTERACTION*

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A large scale purification procedure for human erythrocyte acetylcholinesterase that involved affinity chromatography on an acridinium resin permitted the routine isolation of about 5 mg of enzyme from 10 liters of outdated erythrocytes. The purified enzyme had a specific activity of 5000–5800 units/mg of protein and was free of polypeptide contaminants by gel electrophoresis criteria. In detergents, the isolated enzyme corresponded in part by the payment of enzyme from 10 liters of outdated erythrocytes. The purified enzyme had a specific activity of 5000–5800 units/mg of protein and was free of polypeptide contaminants by gel electrophoresis criteria. In detergents, the isolated enzyme corresponded in part.

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The abbreviations used are: AChE, acetylcholinesterase; BCGH, bovine catalase and Escherichia coli β-galactosidase (Sigma) were the highest purity available. Polypeptide standards for SDS-PAGE gels were from Sigma.

Affinity Resin—The acridinium ligand 9-(N7-ε-aminocaproyl-γ-amino propylamino)acridine dihydrobromide (II) was coupled to Sepharose CL-4B (Pharmacia Fine Chemicals) according to Method.

Materials and Methods

Proteins—RBC AChE was extracted and purified as outlined below. Bovine catalase and Escherichia coli β-galactosidase (Sigma) were the highest purity available. Polypeptide standards for SDS-PAGE gels were from Sigma.

Affinity Resin—The acridinium ligand 9-(N7-ε-aminocaproyl-γ-amino propylamino)acridine dihydrobromide (II) was coupled to Sepharose CL-4B (Pharmacia Fine Chemicals) according to Method.

3 M. C. Sheldon and T. L. Rosenberry; unpublished observations.
was mixed in the same proportions with 15% Triton X-100 in 5 mM buffer and added to the sedimented resin. After another 1 h of swirling, the resin was sedimented, the supernatant discarded, and the resin (1 volume) resuspended in 5–8 volumes of 5 mM buffered 1% Triton X-100 wash. The washed resin was sedimented (3000 x g for 10 min), resuspended in 0.5–3 volumes of 5 mM buffered 1% Triton X-100 containing 50 mM NaCl and 0.1 M NaOAc. The enzyme was eluted with 5 mM buffered 1% Triton X-100 containing 50 mM NaCl and 5 mM decamethonium bromide (Sigma) at a flow rate of 0.05 volumes/h. Peak fractions of eluted enzyme (50–100 ml) were diluted to 20 mM NaCl with buffer, adjusted to pH 6, and depleted of detergent and concentrated by chromatography on hydroxyapatite (Ott et al., 1975) at 4°C. Recoveries of AChE (Table I) from hydroxyapatite (Bio-Rad Bio-Gel HTP, regular grade) were much higher than from alternative resins investigated for detergent depletion (DEAP-Sephadex, QAE (Quaternary aminomethyl) Sephadex, Amberlite XAD-2 beads). The affinity resin was regenerated for subsequent reuse by an overnight wash with 6 M guanidine hydrochloride (Chen et al., 1974) and re-equilibrated in 5 mM buffer.

### Enzyme Assays

The activity of RBC AChE during purification was re-assayed by a modified Filtered Film assay (Rosenberry and Richardson, 1977) that included 1% Triton X-100 to avoid partial losses of activity (Wiedmer et al., 1979). A 30% adjustment to approximate standard pH-stat assay conditions (Rosenberry, 1976) was calculated assuming a $k_{\text{cat}}$ of 100 $\mu$M and an $a_{\text{cat}}$ of 6.3. Thus, $V_{\text{cat}} = 14.15$ pmol acetylthiocholine hydrolysed per min (at 25°C) was estimated to correspond to 3.67 A$_{411}$/min. Catalase was monitored by its Amn$_{240}$, and g-glucosidase by the method of Craven et al. (1985).

### Protein Determinations

An extinction coefficient of 1.70 ± 0.05 (n = 10 determinations from six different preparations) was calculated for purified RBC AChE from absolute amino acid recoveries. Protein in impure fractions or in the presence of Triton X-100 (<0.06% final) was estimated according to Lowry et al. (1951) after assay clarification by centrifugation, with bovine serum albumin as a standard. The Lowry estimate of the extinction coefficient for purified RBC AChE (1.77 ± 0.08, n = 11) agreed with that from amino acid analysis, in contrast to the report by Niday et al. (1977).

### Polyacrylamide Gel Electrophoresis and (H)IDFP Labeling

Cylindrical polyacrylamide electrophoresis gels (3.5%) were run in 1.0% SDS (Fairbanks et al., 1971). Gel slicing followed Barnett and Rosenberry (1979). Gradient slab polyacrylamide electrophoresis gels (separating gel 5–13%) were prepared and run according to Rudolph and Krueger (1979) with the following modifications. TEMED and ammonium persulfate concentrations were one-eighth of those indicated, and the acrylamide polymerization (ACN) was initiated with 1% acrylamide stock solutions to minimize gel tearing (Zinker and Warner, 1976), and electrophoresis was conducted at 60 V for 16 h at 4°C. Cylindrical gels were stained for protein with Coomassie brilliant blue R and slab gels, with a silver staining procedure (Morrissey, 1981). Molecular weights of RBC AChE polypeptides were estimated from standard curves of log (kilodaltons) versus relative migration of the polyacrylamide standards. Labeling of RBC AChE with (H)IDFP (6.5 Ci/mmole, Amersham Corp.; Barnett and Rosenberry, 1979) was followed by dialysis for 48–72 h at 4°C to remove residual 13H radioactivity.

### Disulfide Reduction of RBC AChE under Nondenaturing Conditions

Purified RBC AChE in detergent-free buffer (5–200 mM) was diluted in Tris chloride (20–200 mM) containing dithiorthietol (5–20 mM) at pH 8–8.5 for 30 min at 25°C. In some experiments, edrophonium chloride (1 mM; gift of Dr. William Scott, Hoffman-La Roche) was present during the reduction. Reduction was terminated by the addition of N-ethylmaleimide or iodoacetamide (4–5 mol/mol of dithiorthietol) for 30 min at 25°C.

### Akylation of Reduced RBC AChE with [14C]N-Ethylmaleimide

*Error estimates correspond to standard errors of the mean unless followed by the designation standard deviation (S.D.), in which case they refer to the standard deviation of the population.

*The extinction coefficient does not refer to the enol form, which may contribute up to an additional 15% of the enzyme mass (Bon et al., 1976; Niday et al., 1977). Given the apparent catalytic subunit mass of 75 kDa on PAGE gels in SDS (Fig. 1) and 80–85 kDa from hydrodynamic data (Table IV), we assume that the protein contribution to this subunit mass is 70 kDa.

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3 “Buffer” in this paper refers to a mixture of mono- and dabisac sodium phosphate, pH 7, at the phosphate concentration indicated, unless otherwise noted.
large aliquot of purified RBC AChE (1.53 mg in 2.0 ml) was dialyzed at 4 °C in two steps against 20 mm buffer (16 h) and 5 mm buffer (4 h) and then concentrated in a Speedvac concentrator (Savant Instruments Inc.) to 25 μl. Additions (25 μl) were made to final concentrations of 200 mm Tris chloride, 1.0 mm edrophonium chloride, and 5 mm diithiothreitol, pH 8.2, and the mixture was incubated for 30 min at 25 °C. During this time no decrease in specific activity determined from the counts/min recovery and the ninhydrin reaction was detected. After incubation, the sample was diluted 100-fold with 20 mM Tris and 0.1 mM HCl, and a further 30-min incubation at 25 °C, the sample was diluted to 1.5 ml with 0.1 M sodium phosphate, pH 7, and dialyzed in three steps against this same buffer (40 h at 4 °C). Recovered labeled enzyme corresponded to 0.27% of the total activity and 67% of the initial protein.

The specific activity of the [14C]ethylenemalimide stock was checked both by the A_{280} of the residue in 0.1 M HCl (100° = 620; Benesch and Benesch, 1982) and by alkylation of a known amount of glutathione (reduced, Sigma; 0.10 μM) with [14C]ethylenemalimide, 0.12 μM (of stock above) in 100 μl of 0.1 M sodium phosphate, pH 6. The glutathione adduct was identified by acid hydrolysis and amino acid analysis in a four-buffer system (Haas and Rosenberry, 1984). Acid hydrolysates of labeled glutathione and RBC AChE gave identical radioactive profiles, confirming that only cysteine residues were labeled in the protein. A quantitative amino acid analysis following 32-h hydrolyses was performed on aliquots eluting at 36-38 min (just before aspartic acid) and a single peak at 59 min (near glutamic acid), but an increased hydrolysis time (32 h) resulted in considerable conversion of the 59-min product to the 36-38-mn peaks. The specific activity determined from the counts/min recovery and the ninhydrin reaction was similar to that of other unhydrolyzed labeled glutathione. AChE hydrolysis product was 94 ± 7% of the specific activity determined spectrophotometrically (following corrections for 10% scintillation count quenching in the analyzer effluent and 13% initial oxidized glutathione).

Amino Acid Compositions—Analyses were conducted as outlined previously (Mays and Rosenberry, 1981) with minor changes in the pH of buffers. Raw mole percentages were corrected for decomposition or other changes during hydrolysis by assessing three controls 1) RBC AChE sample compositions following 8-, 16-, 32-, and 48-h hydrolyses at 115 °C were extrapolated to zero time; 2) the changes in amino acid standards following 48-h hydrolysis were evaluated; and 3) the mole percentage compositions of three standard proteins (egg lysozyme, bovine serum albumin, and bovine o-chymotrypsin) were compared to compositions calculated from their published sequences. Raw mole percentages were multiplied by significant correction factors (>1.0 or <0.98) for five amino acids: threonine, 0.943 ± 0.013 (from controls 2 and 3); serine, 1.090 ± 0.012 (from all three controls); proline, 0.896 ± 0.037 (from all three controls); methionine, 1.217 ± 0.019 (from controls 2 and 3); and isoleucine, 1.120 ± 0.015 (from control 3 only). The absolute recoveries for the other 11 amino acids decreased 3.0 ± 2.2% (average of all three controls) after 16-h hydrolyses, and protein determinations based on amino acid recoveries were adjusted by this factor.

Estimates of RBC AChE protein content in Coomassie-stained polyacrylamide gel bands followed Mays and Rosenberry (1981) but involved hydrolysis in 3 ml of acid solution. Total protein was calculated from the content of amino acids whose mole percentages corresponded to those in Table III, following correction for background levels in blank gels (generally all but three or four amino acids).

**Seedimentation Coefficients—**Samples were applied to isokinetic sucrose gradients that contained 10 mm sodium phosphate, pH 7, and sedimented as described previously (Younkin et al., 1982). Some gradients also contained 0.1% Triton X-100. gradients were sedimented for 18 h by mixing 5 and 26.2% (w/w) sucrose stocks according to a protocol for SW 41 rotors at 4 °C (McCarty et al., 1974) to give a 20.0% sucrose concentration at the bottom of the gradient. Apparent sedimentation coefficients (S) were obtained from the migration relative to catalase (S_{280}) = 11.4). Estimates of S_{280} values corrected for partial specific volume (v) were made from the raw sedimentation data with the tables of integrals provided by McEwen (1967) for linear sucrose gradients. The approximation of these tables to our isokinetic gradients was justified by noting that for runs with defined w, the value of S_{280} calculated from the tables for catalase by Method A (which assumed a 5-20% linear gradient) was 8% low and by Method B (in which actual gradient calculations were performed (McCarty et al., 1968) and matched to the McEwen integrals) was 11% high. The partial specific volumes of the RBC AChE-Triton X-100 complexes (c) were calculated as weighted averages of the contributions from protein and detergent (v = (v_p + av_d)/1 + a); Clarke, 1975), with v_d of Triton X-100 = 0.506 ml/g (Tanford et al., 1974), v_p of RBC AChE = 0.715 ml/g (Bon et al., 1976, 1979), and (a gram of Triton X-100 bound per g of protein was experimentally determined. The actual corrections due to relative differences in calculated S_{280} values between u = 0.715 and 0.851% was large enough to increase the measured sedimentation coefficients.

**Quality of Hydrodynamic Properties—**Molecular weights were calculated from the Svedberg equation m = (1.432 × 10^{-7} × M_w)(D/L1−c), where L = 6.87, a = 0.348, and c = a, the viscosity of water and N is Avogadro's number. For S_{280}, in Svedberg units and Rs, in nanometers, L = 114. For detergent-protein complexes, v was determined as indicated above and M, for the protein was obtained by dividing M by the complex by (1 + a).

**Binding of [14C]Triton X-100 by RBC AChE—**[14C]Triton X-100 (New England Nuclear, 1.58 mCi/mg) was diluted to 0.30 μCi/ml in 1.0% Triton X-100, 10 mM buffer and dialyzed against 10 mM buffer for 24-40 h. The radioactivity partitioned completely with the Triton X-100 absorbance (98.4 ± 7.7% [S.D.]; see Clarke, 1975). Triton X-100 concentrations were determined using E = 2.02 (Clarke, 1975). [14C]Triton X-100 binding estimates were obtained by affinity chromatography at 4 °C either with the above resin (A) or with a new resin B with less attached ligand (0.2 μmol of acridinium/ml of packed resin) that was prepared to allow elution either with 1 mM decamethonium bromide or with tetraproplammonium iodide. Dialedyl 1.0% [14C]Triton X-100 stock solutions were added to a sample of purified and dialyzed stock RBC AChE (0.8-1.5 mg) to final concentrations of 0.10% [14C]Triton X-100, 10 mM buffer, and 0.08-0.15 mg/ml of AChE. This mixture was largely adsorbed (>96% on resin A, >70% on resin B) by passage through 4-8 ml columns (0.8 × 6-12 cm) of affinity resin. The column was washed with 10-15 ml of the same buffered [14C]triton X-100 concentration and eluted by the addition of the eluting ligand to this solvent. When tetraproplammonium iodide was the eluting ligand, an equivalent concentration of sodium iodide was added to the wash buffer to compensate for slight scintillation count quenching by iodides. Some RBC AChE samples (1.0% Trition X-100 stock solution was added to a sample of purified and dialyzed stock RBC AChE (0.8-1.5 mg) to final concentrations of 0.10% [14C]Triton X-100, 10 mM buffer, and 0.08-0.15 mg/ml of AChE. This mixture was largely adsorbed (>96% on resin A, >70% on resin B) by passage through 4-8 ml columns (0.8 × 6-12 cm) of affinity resin. The column was washed with 10-15 ml of the same buffered [14C]triton X-100 concentration and eluted by the addition of the eluting ligand to this solvent. When tetraproplammonium iodide was the eluting ligand, an equivalent concentration of sodium iodide was added to the wash buffer to compensate for slight scintillation count quenching by iodides. Some RBC AChE samples (1.0% Trition X-100 stock solution was added to a sample of purified and dialyzed stock RBC AChE (0.8-1.5 mg) to final concentrations of 0.10% [14C]Triton X-100, 10 mM buffer, and 0.08-0.15 mg/ml of AChE. This mixture was largely adsorbed (>96% on resin A, >70% on resin B) by passage through 4-8 ml columns (0.8 × 6-12 cm) of affinity resin. The column was washed with 10-15 ml of the same buffered [14C]triton X-100 concentration and eluted by the addition of the eluting ligand to this solvent.
TABLE I
Summary of affinity chromatography purification of RBC AcChE

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific activity</th>
<th>Activity recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial erythrocyte membrane</td>
<td></td>
<td></td>
</tr>
<tr>
<td>suspension in 1% Triton X-100</td>
<td>1.1</td>
<td>100%</td>
</tr>
<tr>
<td>Peak fractions eluted from</td>
<td>5500</td>
<td>71 ± 20</td>
</tr>
<tr>
<td>the affinity resin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak fractions recovered from</td>
<td>5060 ± 590°</td>
<td>48 ± 16</td>
</tr>
<tr>
<td>the hydroxyapatite column</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The total activity in the initial erythrocyte membrane suspension averaged 47,900 ± 10,000 units (S.D.). The percent recovery refers to the overall yield for all purification steps to that point.

A second cycle of affinity chromatography, conducted in the detergent binding experiments in Table IV, resulted in a specific activity of 5850 ± 960 (S.D.) for eight preparations (see "Discussion").

yapatite chromatography step (Ott et al., 1975) serves primarily to remove Triton X-100 and to concentrate the recovered RBC AcChE. No significant additional purification was apparent following this step. Hydroxyapatite removal of 0.1% [3H]Triton X-100 from purified RBC AcChE reduced the bound detergent to 0.02 g of [3H]Triton X-100/g of protein.

SDS-PAGE Analyses—Important criteria for the purity of our isolated RBC AcChE are provided by the SDS-polyacrylamide electrophoresis gels in Fig. 1A. Both cylindrical gels with heavily overloaded samples stained with Coomassie blue and silver-stained slab gels are presented. The cylindrical gel profiles establish that no contaminants detectable by this staining procedure are copurified with RBC AcChE. Three lanes corresponding to three states of protein disulfide reduction are displayed in each gel set. Prior to disulfide reduction, the enzyme existed as a disulfide-linked dimer (Fig. 1, lanes 1 and 5), and the dimer was converted to monomers of about 75 kDa by complete reduction in SDS (Fig. 1, lanes 3 and 7). These observations are consistent with previous reports for RBC AcChE (Bellborn et al., 1970; Ott et al., 1975; Grossmann and Leiflander, 1975; Niday et al., 1977). Conversion to monomers also occurred during reduction under nonnondenaturing conditions (Fig. 1, lanes 2 and 6), indicating that intersubunit disulfide(s) are relatively accessible to solvent. The subunits contained the catalytic site which could be labeled with [3H]DFP, as shown in Fig. 1B. No evidence of subunit fragmentation was observed in most cases, although some preparations showed small amounts (5–10%) of [3H]DFP-labeled fragments of about 40 kDa both before and after disulfide reduction (see Ott et al., 1975).

Disulfide Reduction and Alklylation with [14C]N-Ethylmaleimide under Nondenaturing Conditions—The dithiothreitol concentration required for generation of 75-kDa monomers under nondenaturing conditions was assessed by PAGE in SDS. After 30 min at pH 8.5, 0.3–1.0 mM dithiothreitol reduced one-half of the dimers to monomers (data not shown), and 5 mM was selected as the minimal concentration for quantitative conversion to monomers. The reduction procedure resulted in some loss of enzyme activity (50–70%); but this loss could be reduced to 40–50% by inclusion of edrophonium chloride (0.1–1 mM), a competitive inhibitor of RBC AcChE, in the reduction mixture.

Alklylation with [14C]N-ethylmaleimide was used to estimate the number of sulfhydryl groups generated by the reduction procedure (Table II). About 2 mol of [14C] label were associated with 70,000 g of protein immediately following dialysis of the labeled sample, and this stoichiometry decreased only slightly after subsequent gel exclusion chromatography (also see Fig. 5 below). PAGE in SDS in Fig. 2...
and extraction of the 75-kDa catalytic subunit band from gel slices. Chromatography of
three determinations. Values without errors were single measurements. Standard errors represent
was destroyed by amino acid analysis. Protein samples determined by amino acid analysis were 1.1
percentages exceeded 1% of the means only for methionine. Raw percentages were corrected to the tabulated values as outlined under "Materials
and Methods." The mole percentage sums total 97.1. Tryptophan was estimated spectrophotometrically (Beaven and Hoiday, 1952) to be 1.9 mol %.
Cysteine was assumed to be 1 mol % by analogy to Torpedo
acetylcholinesterase (Rosenberry, 1975) and from a previous report for RBC AChE (Ott and Brodbeck, 1978). Residues/catalytic subunit of 70,000 g of protein (see Footnote 5) were calculated from a mean residue weight of 109.57 determined from the mole percentages.

**Table II**
Quantitative analysis of RBC AChE reduced under nondenaturing conditions and labeled with [14C]N-ethylmaleimide in Fig. 2

<table>
<thead>
<tr>
<th>Protein determination</th>
<th>[14C]N-Ethylmaleimide/protein following Dialysis Chromatography PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>A&lt;sub&gt;280nm&lt;/sub&gt;</td>
<td>2.09 1.81</td>
</tr>
<tr>
<td>Amino acid analysis</td>
<td>1.86 1.73 ± 0.04 1.72 ± 0.04</td>
</tr>
</tbody>
</table>

**Figure 2.** PAGE analysis in SDS of purified RBC AChE that was reduced under nondenaturing conditions, alkylated with [14C]N-ethylmaleimide, and extensively dialyzed as described under "Materials and Methods" and in the legend to Table II. A, photograph of labeled sample (24 μg) run and stained with Coomassie blue under the conditions described for lane 2 in Fig. 1; B, counts/min profile of labeled sample (14 μg) run as in A and sliced as described in the legend to Fig. 1.

confirmed that the reduction resulted in >90% conversion to monomers, while gel slicing demonstrated that virtually all the recovered label was incorporated into the 75-kDa band. The ratio of 14 C label to protein (1.72 ± 0.04 mol/70,000 g of protein) was determined to be the same in isolated gel slices containing the 75-kDa band and in nondenatured samples following gel exclusion chromatography, as indicated in Table II. Control preparations taken through the same reaction procedure without dithiothreitol retained 0.32 ± 0.03 mol of 14 C label/70,000 g of protein (estimated from A<sub>280nm</sub> following dialysis, but little label (<0.05 mol/70,000 g of protein) remained associated with the 75-kDa band following PAGE in SDS. The importance of the association of the label only with the catalytic subunits is considered further under "Discussion."

**Amino Acid Composition**—Table III shows the average amino acid compositions observed for several RBC AChE preparations corrected for amino acid degradation. The tabulated values show modest but significant differences from those reported by Ott and Brodbeck (1978) and large differences from those reported by Niday et al. (1977). Although the composition in Table III also varies significantly from that of the amphipathic torpedo G<sub>II</sub> form, the estimate of the hydrophobicity of RBC AChE of 0.34 from Table III provided by the discriminant function of Barrantes (1975) is the same as that calculated for the torpedo G<sub>II</sub> form (Lee et al., 1982). This value falls between the average values for several integral (0.52 ± 0.11) and peripheral (0.12 ± 0.16) membrane proteins and is somewhat larger than the discriminant functions for the apparently less hydrophobic torpedo asymmetric forms (Lee et al., 1982).

**Table III**
Amino acid composition of RBC AChE

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>mol %</th>
<th>Residues/70 kDa</th>
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</thead>
<tbody>
<tr>
<td>Asparagine</td>
<td>7.4</td>
<td>47</td>
</tr>
<tr>
<td>Threonine</td>
<td>4.5</td>
<td>29</td>
</tr>
<tr>
<td>Serine</td>
<td>5.8</td>
<td>37</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>9.7</td>
<td>62</td>
</tr>
<tr>
<td>Proline</td>
<td>7.8</td>
<td>50</td>
</tr>
<tr>
<td>Glycine</td>
<td>9.9</td>
<td>63</td>
</tr>
<tr>
<td>Alanine</td>
<td>9.7</td>
<td>62</td>
</tr>
<tr>
<td>Valine</td>
<td>8.6</td>
<td>55</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.2</td>
<td>14</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>2.1</td>
<td>13</td>
</tr>
<tr>
<td>Leucine</td>
<td>10.1</td>
<td>65</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.4</td>
<td>22</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.9</td>
<td>32</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.7</td>
<td>11</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.8</td>
<td>15</td>
</tr>
<tr>
<td>Arginine</td>
<td>6.8</td>
<td>44</td>
</tr>
</tbody>
</table>

Sedimentation Velocity Studies—The sucrose gradient sedimentation properties of RBC AChE vary with the presence of nonionic detergent. In the presence of 1% Triton X-100, the enzyme sedimented with an apparent sedimentation coefficient of 6 S (Fig. 3A), while in the absence of Triton X-100 the enzyme formed a mixture of aggregated oligomers (Fig. 3B). Similar observations have been reported for RBC AChE by Ott et al. (1975) and Ott and Brodbeck (1978) and for the amphipathic G<sub>II</sub> torpedo AChE (Lee et al., 1982). The distribution of oligomers in the aggregate was partially dependent on the ionic strength of the sucrose gradient, with lower ionic strengths favoring higher oligomers (also see Lee et al., 1982).<sup>7</sup> Intersubunit disulfide reduction under nondenaturing conditions shifted the peak in the presence of 1% Triton X-100 to an apparent 4 S value consistent with a G<sub>II</sub> form (Fig. 3A; Lee et al., 1982, Bon and Massouillé, 1980) but had little effect on the aggregated sedimentation pattern in the absence of detergent. Thus, the component responsible for aggregation is retained following intersubunit disulfide reduction. Precise determinations of the sedimentation coefficients required quantitative estimates of the amount of bound detergent, and these are presented in Table IV below.

The disaggregation of purified stock RBC AChE by Triton X-100 occurs when the Triton X-100 concentration exceeds its critical micelle concentration (see Clarke, 1975) of 0.016%. We observed aggregated RBC AChE sedimentation profiles similar to those in Fig. 3B at 0.01% Triton X-100, disaggregated profiles identical to those in Fig. 3A at 0.04% Triton X-100.

<sup>7</sup>T. A. Dutta-Choudhury and T. L. Rosenberry, unpublished observations.
Corresponding recoveries in containing no Triton X-100. To examine this possibility, AChE sedimented to a nonrecovered pellet in the absence of Triton. Dithiothreitol and alkylated with N-ethylmaleimide in the absence of for both the Gz and aggregates in B were about 80% of the corresponding recoveries in A, suggesting that about 20% of the RBC AChE sedimented to a nonrecovered pellet in the absence of Triton X-100.

X-100, and a broad peak centered at about 10 S at 0.02% Triton X-100 (data not shown). A similar pattern was reported by Wiedmer et al. (1979), although these workers observed complete disaggregation at 0.02% Triton X-100. These data suggested that RBC AChE was interacting with Triton X-100 only when it formed micelles.

Quantitation of Bound Detergent—To test the suggestion that only Triton X-100 micelles interact with RBC AChE, binding studies with 

\[ ^{3}H \text{Triton X-100} \]

were conducted. After detergent binding measurements was the possibility that the eluting ligand could alter the amount of bound detergent, perhaps by partitioning into the micelle phase and reducing the amount of detergent/micelle. To examine this possibility, we made multiple binding measurements with two eluting ligands at various concentrations, as shown in Fig. 5. A

FIG. 3. Sucrose gradient centrifugation of RBC AChE before and after reduction of intersubunit disulfide bonds. One-half of the 11-μg purified AChE sample (Gz) was reduced with 10 mM dithiothreitol and alkylated with N-ethylmaleimide in the absence of erodophonium chloride (see “Materials and Methods”). Samples Gz (open area) and Gz (shaded area) were divided into equal 200-μl portions, applied to four sucrose gradients, and centrifuged as described under “Materials and Methods.” A, sedimentation in gradients containing 1% Triton X-100; B, sedimentation in gradients containing no Triton X-100. Arrows mark the position of catalase migration (11.4 S). Enzyme activity is units/fraction. Activity recoveries in A were 93% for Gz, and 47% for Gz. Activity recoveries for both the Gz and Gz aggregates in B were about 80% of the average value.

Summary of the hydrodynamic and detergent binding properties of RBC AChE

Purified RBC AChE was obtained as a native Gz form, and samples were converted to Gz by reduction under nondenaturing conditions as outlined under “Materials and Methods.” Hydrodynamic properties were measured in 1.0% Triton X-100 and detergent binding, in 0.1% 

\[ ^{3}H \text{Triton X-100} \]

Tabulated means for sedimentation coefficients (Sedvol) and Stokes radii were averages of three determinations and are listed with the standard error. Apparent sedimentation coefficients were corrected for detergent binding as outlined under “Materials and Methods.” Stokes radii (Sedvol) were estimated from the elution position on Sepharose CL-4B relative to two marker proteins (catalase, R= 5.2 nm; β-galactosidase, R= 8.2 nm; Bon et al., 1978) as in Fig. 6.

<table>
<thead>
<tr>
<th>Form</th>
<th>SEDvol</th>
<th>Sedvol</th>
<th>Detergent binding</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nm</td>
<td>g/a protein</td>
<td></td>
<td>M_M</td>
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<tr>
<td>Gz</td>
<td>6.5 ± 0.2</td>
<td>7.4 ± 0.2</td>
<td>0.63 ± 0.04</td>
<td>160,000 ± 8,000</td>
</tr>
<tr>
<td>Gz</td>
<td>4.3 ± 0.1</td>
<td>6.2 ± 0.1</td>
<td>0.61 ± 0.14</td>
<td>85,000 ± 6,000</td>
</tr>
</tbody>
</table>

* Detergent binding values were determined by affinity chromatography as outlined in Fig. 4. Tetrapropylammonium iodide (2 mM) was the eluting ligand.

b Molecular weights were estimated from the Svedberg equation assuming a Svedberg equation (see Bon et al., 1976, and “Materials and Methods”). Bound detergent was taken as 0.63 ± 0.03 for Gz, from a weighted average of the three tetrapropylammonium iodide eluates in Fig. 4, and 0.75 ± 0.07 for Gz, from the previous value for Gz plus a weighted average ratio of 1.18 ± 0.10 for (detergent bound to Gz)/(detergent bound to Gz) for all data in the table. Bound detergent was deducted from the tabulated values.

Same as Footnote a except decamethonium bromide (10 mM) was the eluting ligand. Weighted average of five estimates for Gz and three estimates for Gz. One estimate for Gz (0.41 ± 0.05) was made with enzyme reduced while adsorbed to the affinity resin and did not differ from the average value.

FIG. 4. Binding of 

\[ ^{3}H \text{Triton X-100} \]

to RBC AChE. Purified Gz enzyme (1.8 mg) was totally adsorbed by passage through 8 ml of affinity resin and eluted with 2 mM tetrapropylammonium iodide as described under “Materials and Methods.” Fractions (0.9 ml) are numbered from the introduction of the tetrapropylammonium iodide to the column. The enzyme activity (units/ml, C), absorbance at 290 nm (A_290, ), and scintillation counts/min in 100-μl aliquots (CPM, ) are shown for each fraction. The estimate of bound detergent was calculated from fractions 11–24 according to Equations 1 and 2 and is indicated in Fig. 5 and Table IV. The line through the counts/min is the calculated least squares fit from Equation 1.
constant amount of bound detergent was measured at all three concentrations of tetratpropylammonium iodide (0.63 ± 0.03 g/g of protein), but progressively smaller amounts of detergent were bound with increasing concentrations of decamethonium bromide. Measurements of bound detergent also were made with G2 RBC AChE produced by reduction either of the purified G2 enzyme prior to adsorption to the affinity resin or of the G2 enzyme adsorbed to the resin. Calculated values of bound detergent determined for the G2 and the G1 enzymes iodide are listed in Table IV. Binding experiments in sucrose gradients containing [3H]Triton X-100 demonstrated that decreasing the [3H]Triton X-100 concentration to 0.01% (and thus below the critical micelle concentration) resulted in no significant binding (<10% of the binding at 0.05%, data not shown).

Stokes Radius Determinations—Gel exclusion chromatography on Sepharose CL-4B was employed to further characterize the hydrodynamic properties of RBC AChE. The chromatographic profiles in Fig. 6 parallel the gradient runs in Fig. 3, except that the G1 sample generated by disulfide reduction was the [14C]N-ethylmaleimide-labeled G1 (50 µg; same stock as in Fig. 2) were applied consecutively to a 135-ml Sepharose CL-4B column (1.5 x 80 cm) equilibrated at 4 °C in 20 mM sodium phosphate, pH 7, plus or minus Triton X-100. A, the G1 sample was mixed with nonreduced G2 enzyme (300 µg) and standards and applied to the column equilibrated with 1.0% Triton X-100. Aliquots (1.0 ml) were scintillation counted (CPM, shaded area) and assayed for enzyme activity (units/ml, open area). The calculated small contribution of G1 to the activity was deducted, and the indicated activity corresponds to the G2 enzyme only (C). B, same sample and analysis as in A except G2 enzyme was omitted and the column was equilibrated (24 h) without Triton X-100. Both enzyme activity and counts/min correspond to the G1 enzyme (B). The elution positions of standards are indicated by arrows (from left, β-galactosidase, catalase, and K2Cr2O7). Recovery of ouput enzyme activity was 100% in A and 34% in B.

DISCUSSION

Evaluation of the Affinity Chromatography Procedure—Specific activities for purified RBC AChE reported in Table I are comparable to those reported previously with resins utilizing m-(acylaminophenyl)trimethylammonium affinity ligands (Ott et al., 1975; Grossmann and Lieflander, 1975; Ott et al., 1982). In contrast to these reports, however, the procedure outlined here results in essentially pure RBC AChE with a higher activity recovery after only one cycle of affinity chromatography. The "second cycle" of affinity chromatography conducted in the course of the [3H]Triton X-100 binding experiments in Table IV resulted in only an average 15% increase in specific activity over that listed for the hydroxyapatite column fractions in Table I. Combined with high volume molecular filtration for the production of large quantities of erythrocyte membranes (Rosenberry et al., 1981) and see "Materials and Methods"), this procedure permits the isolation of about 5 mg of RBC AChE from 10 liters of outdated erythrocytes in about 4 days.

The acridinium affinity resin described here involves the same ligand used for the purification of eel electric organ acetylcholinesterase (Rosenberry et al., 1982). An important development was the synthesis of the intact affinity ligand 9-(N'-c-aminocaproyl-γ-aminopropylamino)acridine dihydrobromide. This ligand could be attached to the resin in a single coupling step at a controlled concentration (0.8-1.0 amol/ml of packed resin) that was about twice the maximal that could be obtained by the old two-step coupling procedure. Furthermore, the amount of CNBr required to couple the ligand was
only about 40 mg/ml of packed resin, about one-fifth that used in the two-step procedure. CNBr has been shown to introduce nonspecific positively charged groups on agarose (Murphy et al., 1977), and these charges appear to reduce the efficiency of RBC AChE purification at the low ionic strengths employed here. Recoveries of purified RBC AChE increased from about 20 to 71% with the new resin, and specific activities of the purified enzyme were increased about 50%.

**Subunit Structure of RBC AChE and Number of Intersubunit Disulfides**—The conversion of G2 RBC AChE to a G1 form by reduction under nondenaturating conditions produced 1.7 mol of sulfhydryl group that could be alkylated with [14C]N-ethylmaleimide/70,000 g of protein (Table II). Similar experiments with purified eel electric organ AChEs generated 1.1 reduced sulfhydryl group per 11 S (G4) catalytic subunit and 1.0 per 18 S (A2) catalytic subunit and indicated that only a single sulfhydryl group on each catalytic subunit was involved in intersubunit disulfide bonding (Rosenberry, 1975). Related experiments have also been reported following nonreducing reduction of G4 human serum cholinesterase with incorporation values corresponding to 1.0 mol of sulfhydryl group alkylated per mol of catalytic subunits (Lockridge et al., 1979). Considerable efforts were required to obtain selective reduction of only the intersubunit disulfides in the eel AChEs, and incorporation values between 1 and 2 were quite common following reduction at high concentrations of dithiothreitol or in the absence of edrophonium chloride. Selective reduction of the intersubunit disulfides (Rosenberry, 1975; Bon and Massoulié, 1976; Vigny et al., 1979; Lee et al., 1982; Grassi et al., 1982). In view of these difficulties, our data appear consistent with conclusions from the previous studies that a single sulfhydryl group on each catalytic subunit is involved in intersubunit disulfide bonding.

There are two precedents shown in Fig. 7 for the way in which AChE catalytic subunits with one sulfhydryl group could be assembled in a disulfide-linked dimer. Assembly may involve either a single disulfide bond between the catalytic subunits (Fig. 7A) or two disulfide bonds, each linking a catalytic subunit to an intervening small noncatalytic subunit (Fig. 7B). Eel 11 S AChE, which is generated by an endogenous protease from asymmetric AChEs, is composed of one dimer A and one dimer B. The intervening structure in dimer B is an 8-kDa fragment cleaved from the collagen-like noncatalytic subunits of the asymmetric AChEs by the endogenous protease (Rosenberry, 1975; Barnett and Rosenberry, 1978; Rosenberry et al., 1980). Of concern to us was the possibility that the hydrophobic domain responsible for the membrane association of RBC AChE was located on such an intervening peptide in dimer B rather than within the catalytic subunit primary sequence. According to Fig. 7B, such a peptide should be labeled by [14C]N-ethylmaleimide following selective reduction with a stoichiometry equal to the catalytic subunits, but it need not be released from the catalytic subunits in the absence of denaturation. The absence of label outside the 75-kDa catalytic subunit band following PAGE in SDS in Fig. 2 argued against such a peptide, and the identity of the labeling stoichiometry in the denatured 75-kDa band with the labeled G1 aggregate from gel exclusion chromatography in Table II provided quantitative confirmation that the label was confined to the catalytic subunits and consistent with Fig. 7A. We cannot completely rule out a noncovalently linked noncatalytic hydrophobic subunit that remains associated in Triton and following reduction, but we see no evidence for it from RBC AChE samples run on PAGE gels in SDS by protein staining or by amine group radiolabeling or Edman degradation (Hass and Rosenberry, 1984).

**RBC AChE Meets Several Criteria for an Amphipathic Membrane Protein**—Integral membrane proteins that have been termed amphipathic exhibit several common characteristics that can serve as criteria for this class of proteins. 1) The intact protein requires detergent for extraction from membranes, binds to detergent micelles only, aggregates but does not generally precipitate when detergent is removed, and can be reconstituted into liposomes. 2) The larger hydrophilic domain is oriented on one side of the membrane only and is solubilized by protease cleavage, generally with full retention of associated enzymatic activities but complete loss of aggregation or detergent or liposome binding properties. 3) The hydrophobic domain is quite small (generally <10 kDa) and is located at the NH2 or COOH terminus of the primary structure. In this paper, we focus on the first of these criteria while the remaining two are addressed in the accompanying paper (Dutta-Choudhury and Rosenberry, 1984).

**Aggregation**—When purified RBC AChE was depleted of detergent and concentrated by hydroxypatite chromatography, it retained only about 3% of the Triton X-100 that was bound to each G2 dimer when Triton X-100 was above its critical micelle concentration. The preparations were aggregated as demonstrated in Fig. 3, and aggregates in such preparations consisted of discrete oligomers of three to seven dimeric protomers that interconverted quite slowly on storage (Ott et al., 1975; Ott and Brodbeck, 1978). In contrast to Ott et al. (1975), we found that the aggregated RBC AChE could be chromatographed on Sepharose CL-4B in the absence of detergent. The aggregate appeared as a somewhat broadened peak corresponding to an average Stokes radius of about 10 nm that was well separated from the column void volume. Recoveries of activity from this gel exclusion chromatography were typically 50–70%, significantly less than when the column was equilibrated with 1% Triton X-100, and recoveries were even lower and peak trailing worse for the first exposure of a fresh column to aggregated RBC AChE (compare Fig. 5B to data in Dutta-Choudhury and Rosenberry, 1984). In con-

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Fig. 7. Alternative schemes for the intersubunit disulfide linkage in RBC AChE.

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A homogeneous micelle of Triton X-100 bound per g of G₂ RBC AChE in Table IV corresponded to 140 Triton X-100 molecules/140 kDa of RBC AChE protein, a reasonable estimate for the G₂ dimer mass of 160,000 reported by Ott et al. (1975), and 128 (7-glutamyl transpeptidase, Hughey and Curto, 1981). Other proteins from Footnote b in Table IV) and corresponded to about 80 Triton X-100 molecules/70 kDa of GI protein. Other values reported for the moles of bound Triton X-100/mol of protein for amphipathic protein-micelle complexes include 100 (cytochrome b₅, Robinson and Tanford, 1975), 49 (major sialoglycoprotein of the human red cell membrane, Clarke, 1975), and 128 (γ-glutamyl transpeptidase, Hughey and Curthoys, 1976). Our observed detergent binding value for G₂ falls within this range, indicating that each molecule of this enzyme form interacts with a single micelle. The fact that the G₂ form binds nearly twice as much detergent per molecule as G₁ strongly argues that this form interacts with two micelles, presumably with one on each subunit as diagrammed in Fig. 8. This point provides further evidence that each subunit in the dimer contains a hydrophobic domain, and it is noteworthy because it indicates that these subunit domains appear sufficiently separated to permit each individually to bind a 45-kDa micelle.

Comparison of RBC AChE to Globular AChEs in Other Tissues—G₂ AChEs are the predominant globular membrane-bound AChE forms in human erythrocytes and torpedo electric organ tissue, and these two AChE forms exhibit several common properties consistent with amphipathic structures that have been noted above (also see Bon and Massoulié, 1980; Massoulié and Bon, 1982). G₁ forms are also prevalent in human skeletal muscle (Carson et al., 1979) but are quite minor compared to G₁ and G₄ forms in other mammalian tissues, particularly in the central nervous system. Bovine brain G₁ and G₄ forms are primarily membrane-bound and bind nonionic detergents (Grassi et al., 1982), but after partial purification both the bovine brain G₁ and G₄ and the rat brain G₄ enzymes fail to aggregate in detergent-free sucrose gradients. The bovine brain G₁ and G₄ forms nonetheless appear to be amphipathic, as Pronase digestion abolishes their detergent interactions (Grassi et al., 1982). The G₂ AChE form which predominates in chicken muscle also does not aggregate but does appear to bind detergent (Allemand et al., 1981). Thus, aggregation in the absence of detergent may not be a feature common to all amphipathic globular AChEs, and structural distinctions among this class of AChEs remain to be pursued.

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