Structure of 18S and 14S Acetylcholinesterase. Identification of Collagen-Like Subunits That Are Linked by Disulfide Bonds to Catalytic Subunits†

Terrone L. Rosenberry* and Jeanne M. Richardson‡

ABSTRACT: The 14S and 18S acetylcholinesterase (EC 3.1.1.7) forms present in 1.0 M ionic strength extracts of fresh electric organ of the eel *Electrophorus electricus* were purified by affinity chromatography. The purification procedure involved a highly efficient acridinium resin whose synthesis and use are introduced. Previous electron microscopy studies have determined that the 14S and 18S acetylcholinesterase species as well as an 8S species not observed in this study contain a 40-nm tail structure associated with discrete numbers of presumably catalytic subunits, and other reports have established that either trypsin or collagenase will convert the 8S, 14S, and 18S forms to 11S catalytic subunit tetramers which appear devoid of the tail structure. A comparative analysis of polypeptides present in 18S plus 14S preparations and 11S preparations by polyacrylamide gel electrophoresis in sodium dodecyl sulfate is presented in this report. Prior to disulfide reduction the predominant 11S component in sodium dodecyl sulfate was an apparent catalytic subunit dimer possessing an intersubunit disulfide bond; in contrast, only half the 18S plus 14S components corresponded to this dimer while the remainder were found primarily as two oligomers, A and B, of high molecular weight (>300 000). Exposure of either 11S or 18S plus 14S preparations to disulfide reduction in the absence of a denaturant selectively reduced interpolypeptide bonds and generated primarily 75 000 molecular weight catalytic subunit monomers which appeared identical in the two preparations. In addition, this selective reduction of the 18S plus 14S enzyme produced a faint indication of polypeptides E with *apparent* molecular weights, relative to noncollagen standards, of 40 000 and 44 000. These polypeptides, which could not be obtained from the 11S preparation, were more clearly seen after complete disulfide reduction in a denaturant. Polypeptides E are thus prime candidates for the tail subunits. Fractionation of the nonreduced 18S plus 14S preparation by gel exclusion chromatography in sodium dodecyl sulfate revealed that all fractions contained catalytic subunits while polypeptides E derived only from oligomers A and B and not from catalytic subunit dimers. Difference amino acid compositions indicated that polypeptides E are largely collagen-like, containing about 30% glycine, 15% proline, 7% hydroxyproline, and 7% hydroxylysine. A proposed model of 18S acetylcholinesterase considers this molecule to consist of one oligomer A unit, composed of three pairs of catalytic subunits disulfide bonded to the tail structure, and three catalytic subunit dimers. It follows that the catalytic subunits in both 18S and 11S acetylcholinesterase are arranged asymmetrically. Both the indicated amino acid composition of polypeptides E and the presence of intersubunit disulfide bonds linking noncollagenous glycoprotein catalytic subunits to the apparently collagen-like filamentous tail structure are characteristic of basement membrane components.

Acetylcholinesterase (EC 3.1.1.7) from the electric organs of electric fish can be extracted in several molecular species. These species are characterized by various sedimentation coefficients. From the eel *Electrophorus electricus*, 8S, 14S, and 18S forms have been purified and examined by electron microscopy (Rieger et al., 1973; Dudai et al., 1973). All three forms are highly asymmetric oligomers and appear as clusters of, respectively, 4, 6–8, and 10 or more subunits attached to an elongated tail. Species of 8, 14, and 16 S have been purified from extracts of *Torpedo californica* (Lwebuga-Mukasa et al., 1976). These enzyme forms from either fish can be converted to 11S species by treatment with trypsin or by an apparent autolysis on storage of crude enzyme solutions (Massoulié and Rieger, 1969). Electron micrographs of purified 11S enzyme from eel show globular structures of four subunits without the tail. Other properties of these enzyme glycoprotein species have been described in a recent review (Rosenberry, 1975).

Commercial collagenases will release acetylcholinesterase as a 10S species from the sarcolemmal membranes of rat muscle (Hall and Kelly, 1971; also see Betz and Sakmann, 1973). In retrospect, this observation first suggested the possible collagen-like nature of the tail structure in the asymmetric acetylcholinesterase species. Commercial collagenase was also shown to convert purified 18S and 14S eel species to an 11S form (Dudai and Silman, 1974a). A clear interpretation of collagenolytic action in these cases was prevented by the possibility that the conversion could arise from a protease contaminant in the commercial collagenases used. This question was resolved by Lwebuga-Mukasa et al. (1976), who showed that highly purified collagenase, free of caseinolytic or amidase activity, converted 16S torpedo enzyme to an 11S form with a molar efficiency greater than that of trypsin. Furthermore, purified preparations of both the 16S torpedo enzyme and the 18S eel enzyme were reported to contain about 1% hydroxyproline while the 11S species contained, at most, barely detectable amounts (Lwebuga-Mukasa et al., 1976; Anglister et al., 1976). These observations further indicated that the tail structure contained a collagen-like amino acid sequence, although they did not exclude the possibility that a collagen

† From the Departments of Biochemistry and Neurology, College of Physicians and Surgeons, Columbia University, New York, New York 10032. *Received February 9, 1977.* This investigation was supported, in part, by U.S. Public Health Service Grants NS-03304 and NS-11766, by National Science Foundation Grant No. PCM73-00744, by the Muscular Dystrophy Association, and by the New York Heart Association.

‡ Present address: Science Library, Department of Libraries, University of Kansas, Lawrence, Kans. 66045.

contaminant was carried through the purification procedure.

In this report we describe some modifications of an affinity resin introduced by Dudai and Silman (1974b) which have permitted us to purify and further characterize 18S plus 14S acetylcholinesterase from the electric eel. This characterization focuses on the subunit composition of these enzyme forms and identifies a tail subunit candidate which appears to be rich in amino acids that denote collagen.

Materials and Methods

Acetylcholinesterase. 14S and 18S enzyme species were purified from extracts of fresh electric organ tissue by affinity chromatography as described below. 11S acetylcholinesterase was purified from a crude lyophilizate (Rosenberry et al., 1972) by a previously described affinity chromatography procedure (Chen et al., 1974).

Affinity Resin. N^1-Benzyloxycarbonyl-1,3-propanediamine hydrochloride (I) was prepared from bis(benzyloxycarbonyl)-1,3-propanediamine essentially as described by Lawson et al. (1968). The time of heating under reflux was reduced to 20 min.

9-[N^γ-(Benzyloxycarbonyl)-γ-aminopropylamino]acridine hydrochloride (II) was prepared from I (11.0 g; 0.045 mol) and 9-phenoxyacridine (Albert, 1966b; 12.2 g; 0.045 mol) in phenol (40 g) according to a general procedure by Albert (1966c). The mixture was maintained at 110–130 °C for 90 min and briefly triturated with anhydrous ethyl ether (200 mL) to form a light brown gum. After discarding the solvent, anhydrous ethyl ether (800 mL) was added to the gum. This mixture was stirred until the gum was completely converted to a yellow precipitate (II) which was washed with additional ether (16.3 g; 86%; mp 116–118 °C; hygroscopic). Recrystallization was from ethanol–anhydrous ethyl ether [mp 155–157 °C; ultraviolet (UV) (0.1 M sodium phosphate, pH 7) λ_{max} 411 (ε 9800), 433 (ε 8400), 394 nm (ε 6200)]. Anal. (Galbraith Laboratories, Knoxville, Tenn.) Calcd for $C_{24}H_{24}ClN_3O_2$: C, 68.32; H, 5.73; N, 9.96; Cl, 8.40. Found: C, 68.48; H, 6.36; N, 9.60; Cl, 8.28. However, recrystallization was generally omitted.

9-γ-Aminopropylaminoacridine dihydrobromide (III) was obtained after dissolving II (15.3 g; 0.039 mol) in glacial acetic acid (100 mL) and adding 30–32% HBr in glacial acetic acid (Fisher; 50 mL) with swirling. The yellow crystals (III) obtained were washed repeatedly with anhydrous ethyl ether (15.9 g; 98%; mp 242–246 °C dec). For analysis, III was obtained by an identical procedure from VI [mp 268–270 °C; UV (0.1 M sodium phosphate, pH 7) λ_{max} 411 (ε 9100), 433 (ε 7600), 394 nm (ε 5900)]. Anal. (dried crystals appeared to be hydrated with one molecule of water) Calcd for $C_{16}H_{21}Br_2N_3O$: C, 44.57; H, 4.91; N, 9.75; Br, 37.06. Found: C, 45.04; H, 4.95; N, 9.64; Br, 37.25.

Sepharose 4B was activated with cyanogen bromide and coupled with 6-aminocaproic acid (2 μmol/mL packed gel) to give *N*-(Sepharose)-6-aminocaproic acid (IV) essentially as outlined by March et al. (1974). Coupling of III (10 μmol/mL packed gel) and IV by the *N*-hydroxysuccinimide ester activation procedure of Cuatrecasas and Parikh (1972) gave the affinity resin 9-[N^γ-(ε-Sepharose 4B-aminocaproyl)-γ-aminopropylamino]acridinium (V) with attached ligand concentrations of about 1 μmol/mL packed gel according to estimates of the unreacted ligand in the gel washings.

Enzyme Inhibitors. 9-[N^γ-(Benzyloxycarbonyl)-γ-aminopropylamino]acridine (VI) was obtained from aqueous solutions of recrystallized II by extraction into chloroform, evaporation of the chloroform, extensive drying of the resulting oil in vacuo, and crystallization from benzene–hexane [mp 104–106 °C; UV (0.1 M sodium phosphate, pH 7) λ_{max} 411 (ε 9800), 433 (ε 8300), 394 nm (ε 6200)]. Anal. Calcd for $C_{24}H_{23}N_3O_2$: C, 74.78; H, 6.01; N, 10.90. Found: C, 75.28; H, 6.21; N, 10.91.

10-Methyl-9-[N^γ-(benzyloxycarbonyl)-γ-aminopropylamino]acridinium *p*-toluenesulfonate (VII) was prepared by the addition of methyl *p*-toluenesulfonate (250 mg, 1.35 mmol) to VI (500 mg, 1.30 mmol) in dimethylformamide (5 mL) at 160 °C. After 10 min the reaction was cooled to 25 °C, and ethyl acetate (50 mL) was slowly added to initiate crystallization of VII [300 mg; 40%; mp 182–184 °C; UV λ_{max} 411 (ε 10 400), 433 (ε 8700), 394 nm (ε 6600)]. Anal. Calcd for $C_{32}H_{33}N_3O_5S$: C, 67.23; H, 5.82; N, 7.35; S, 5.61. Found: C, 66.85; H, 5.76; N, 7.65; S, 5.77.

Enzyme Assays. Competitive inhibition constants were obtained from pH-stat measurements with 11S acetylcholinesterase and acetylcholine as substrate in 0.1 M NaCl at pH 8.0 as outlined previously (Rosenberry and Bernhard, 1972; Rosenberry at al., 1972).

The activity of acetylcholinesterase during purification procedures was routinely monitored by the spectrophotometric method of Ellman et al. (1961) at 25 °C (0.50 mM acetylthiocholine iodide, 0.33 mM Nbs_2,[1] 0.1 M sodium phosphate, 3.00 mL) except that the assay was buffered at pH 7.0. The use of this pH significantly reduces the blank hydrolysis rate and allows daily premixing of the assay solution. Activities of purified enzyme samples were occasionally measured under standard pH-stat assay conditions (Rosenberry et al., 1972; 2.7 mM acetylcholine bromide, 0.1 M sodium chloride, 0.02 M magnesium chloride, pH 7.4, 25 °C). Conversion factors were calculated based on simultaneous measurements in the two assays. For 18S plus 14S acetylcholinesterase, 1 activity unit ≡ 1 μmol of acetylcholine hydrolyzed per minute in the pH-stat assay is equivalent to 3.94 ± $0.16\Delta A_{412nm}$ (1.0 cm)/min in the spectrophotometric assay. For 11S acetylcholinesterase, 1 unit in the pH-stat assay is equivalent to 3.42 ± $0.14\Delta A_{412nm}$/min in the spectrophotometric assay. The different conversion factors for the two enzyme forms may reflect the fact that 18S plus 14S enzyme can aggregate under the assay conditions and give rise to microenvironmental pH effects on the activity (see Silman and Karlin, 1967; Rosenberry, 1975).

Extraction of Enzyme. An electric eel was sacrificed and the main electric organ removed by dissection. The fresh tissue was immediately mixed with 1–2 vol of sodium phosphate (pH 7.0, 10 mM) and homogenized at 4 °C in a Waring Blendor. The homogenate was then centrifuged at 10 000*g* for 60 min. The supernatant (S_1), containing 5–10% of the total enzyme activity, was discarded, and the residue was rehomogenized with 0.8 vol (relative to the initial tissue volume) of 1.4 M NaCl, 23 mM sodium phosphate, pH 7.0. After centrifugation of this homogenate at 48 000*g* for 30 min, the supernatant (S_2) was collected, and the residue was rehomogenized with 0.4 vol of 1.0 M NaCl, 20 mM sodium phosphate, pH 7.0. This homogenate was also centrifuged at 48 000*g* for 30 min and the residue was discarded; the supernatant (S_3) was combined with S_2 as the crude onput to the affinity column.

Affinity Chromatography. All solvents used in affinity chromatography were Millipore filtered. A single chromatography column (2.5 × 45 cm) at 4 °C was packed with 200 mL of affinity resin in 1.0 M NaCl, 20 mM sodium phosphate, pH 7.0 (buffer I). The packed column was washed with 3 to 5 vol of buffer I, and the crude enzyme extract was introduced

[1] Abbreviations used are: iPr_2PF, diisopropylphosphoryl fluoride; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid).

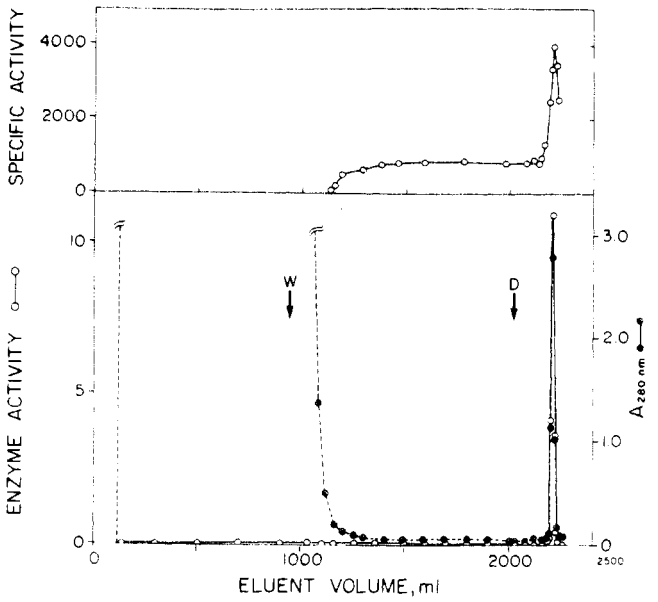

FIGURE 1: Affinity chromatography of 18S plus 14S acetylcholinesterase on an acridinium resin (V). Crude extracts S_2 and S_3 containing a total enzyme activity of 227 000 units in 1100 mL were introduced to the affinity column as outlined under Materials and Methods. Onset of the wash with buffer I is indicated by W, and introduction of 10 mM decamethonium bromide is indicated by D. Individual column fractions were 8.5 mL during enzyme elution. The lower profile gives the eluted enzyme activity (units/microliter) and the A_{280nm} for selected fractions. The upper profile gives the corresponding specific activity (units milliliter^{-1} A_{280nm}^{-1}).

to the column at a flow rate of 0.1–0.2 column vol per h. After entry of the crude extract, the column was washed with 4–6 vol of buffer I at a flow rate of 0.1–0.2 column vol per h. Elution of the retained enzyme was accomplished with decamethonium bromide (5 or 10 mM) at a flow rate of 0.05 column vol per h.[2]

The column could be regenerated with 6 M guanidine hydrochloride as outlined by Chen et al. (1974). Cycles of regeneration and affinity chromatography could be repeated at least four times over a 3-month period without noticeable change in the chromatographic properties.

Gel Exclusion Chromatography in 1% Sodium Dodecyl Sulfate. Chromatography in 1% sodium dodecyl sulfate containing 0.1 M NaCl, 20 mM sodium phosphate (pH 7.0) was carried out on Sepharose 4B at 30 °C in a jacketed 0.9 × 145 cm glass column fitted with Teflon inlet and outlet tubing. The flow rate of about 2.0 mL/h was determined by a 50–60-cm hydrostatic pressure differential.

Analytical Procedures. Density gradient ultracentrifugation was performed according to Martin and Ames (1961). Electrophoresis in 3.5% polyacrylamide gels containing 1% sodium dodecyl sulfate followed the procedure of Fairbanks et al. (1971). Protein concentrations were estimated by the absorbance (A) at 280 nm. While not measured precisely, the $\epsilon_{280nm}^{1\%}$ for 18S acetylcholinesterase appears close to the value of 18.0 previously reported for the 11S enzyme (Rosenberry, 1975). Acylation of 18S plus 14S enzyme (0.5–1.5 mg/mL) was carried out with [^{32}P]iPr$_2$PF[1] (Amersham-Searle; 0.25 mM final) in buffer I containing 2% 2-propanol until no detectable enzyme activity remained.

Results

Inhibition of Enzyme Activity. The affinity resin V introduced in these experiments differs from a similar resin reported by Dudai and Silman (1974b) in two significant respects. It is synthesized by a different series of intermediates that are readily obtainable, and it is not methylated at the 10 position on the acridine ring. To test the effect of methylation at this position on the affinity of ligands for the catalytic site of acetylcholinesterase, the competitive inhibition constants (K_{comp}) for 9-[N^{γ}-(benzyloxycarbonyl)-γ-aminopropylamino]acridinium hydrochloride (II) (which had been recrystallized as the free base VI) and the corresponding 10-methylated analogue (VII) were measured. Both compounds are essentially in the cationic acridinium forms at pH values below 8, since the pK_a for the protonated ring nitrogen in 9-aminoacridine hydrochloride is 10.0 (Albert, 1966a). K_{comp} for II was 0.17 ± 0.05 μM, and K_{comp} for VII was 0.18 ± 0.13 μM; thus, replacing the methyl group with a proton at the 10 position has little effect on the enzyme affinity.

Enzyme Purification. The total enzyme activity in extracts S_2 + S_3 averaged 350 000 units per kg of electric organ. A typical profile of the affinity chromatography is shown in Figure 1. About 7% of the crude enzyme applied to the affinity column was not retained, about 15% was recovered in the buffer wash, and about 60% was eluted sharply with decamethonium. The purified enzyme had a specific activity of 3800 units mL^{-1} A_{280nm}^{-1}, about 75 times that of the crude onput, and was free of detectable protein contaminants as judged by sodium dodecyl sulfate gel electrophoresis (see below). The primary contaminant in the purification was a UV absorbing material with a λ_{max} of 260 nm, probably nucleic acid, that was readily apparent in the buffer wash. The purified enzyme eluted with decamethonium appeared relatively free of this contaminant and was characterized by a λ_{max} of 280 nm and an A_{280nm}/A_{250nm} ratio of 2.4.

The peak specific activity of 4000 units mL^{-1} A_{280nm}^{-1} observed in Figure 1 has been quite reproducible among several preparations in our laboratory. When partially purified material eluted in the buffer wash was subjected to a second cycle of affinity chromatography, 72% of the onput activity was eluted with decamethonium and was characterized by the same specific activity and UV absorbance as the other enzyme pools eluted by decamethonium. These observations suggest that additional affinity chromatography of enzyme eluted with decamethonium in the first chromatography cycle of our procedure is unnecessary.

The maximum observed specific activity, while relatively reproducible, may be somewhat less than the true value. Preliminary experiments indicate that the total activity of 18S plus 14S acetylcholinesterase decreases during transfer and dilution in a second container, in contrast to that of 11S enzyme treated similarly. Such experiments suggest that the 18S and 14S enzyme forms are partially removed from solution by adsorption on container walls. Similar observations have been reported by Massoulié et al. (1975). Furthermore, the intrinsic activity of the 18S and 14S species may be decreased by aggregation in the assay solvent (see Materials and Methods). Such aggregation has been demonstrated at the 0.1–0.2 M ionic strength used in the assays here (Massoulié and Rieger, 1969; Dudai et al., 1973), and its effect on activity measurements is unknown.

Sucrose Gradient Centrifugation. A typical experiment with enzyme freshly purified is shown in Figure 2. Note the onput

[2] A disadvantage in this procedure is that the column sometimes became clogged when crude enzyme onputs contained several hundred milliliters. This problem has been virtually eliminated by our current use of Sepharose CL-4B (Pharmacia) for the synthesis of affinity resins and of Pharmacia flow adapters in the construction of affinity columns (P. Barnett, T. L. Rosenberry, and C. Mays, unpublished observations).

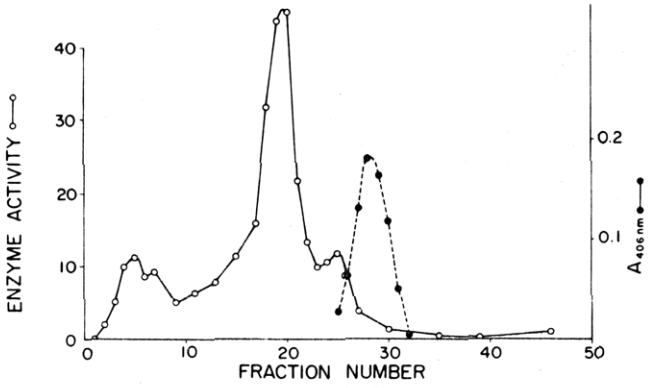

FIGURE 2: Sucrose gradient centrifugation of acetylcholinesterase freshly purified by affinity chromatography. Enzyme eluted in the peak fraction with decamethonium was diluted tenfold with buffer I (to 0.20 A_{280nm}), and 2.0 mL containing 50 μL stock catalase (25 mg/mL, Sigma) was applied to a 24.0-mL gradient of 5–20% sucrose in buffer I over 5.0 mL of 60% sucrose in buffer I. The gradient was centrifuged at 23 000 rpm in an SW 25.1 rotor for 28.5 h. Equal fractions of 0.7 mL were collected dropwise by puncturing the bottom of the tube. Catalase was determined by its A_{406nm}. Acety lcholinesterase activity (units/milliliter) was determined by Ellman assay. The top of the gradient is at the right of the plot.

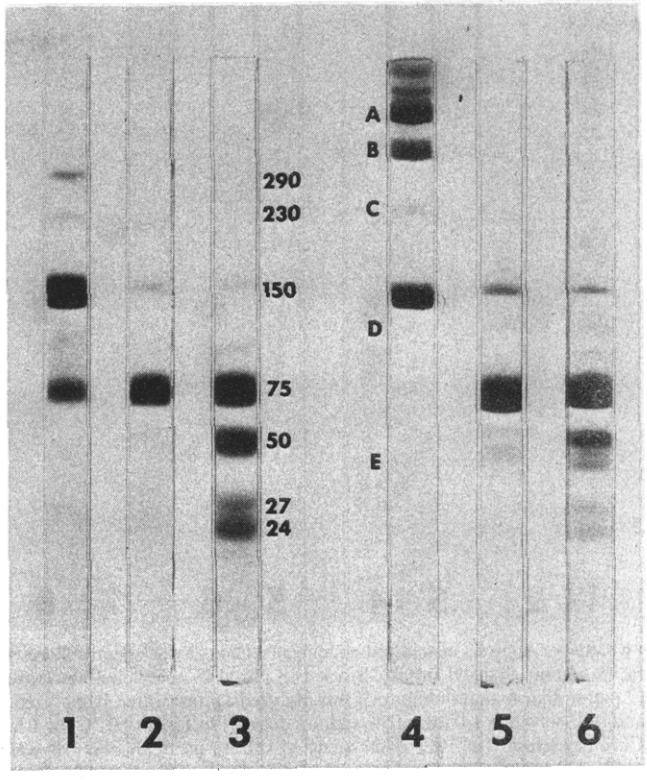

FIGURE 3: Gel electrophoresis of purified 11S acetylcholinesterase (gels 1–3) and 18S plus 14S acetylcholinesterase (gels 4–6) in three states of disulfide reduction. After the degree of disulfide reduction had been defined, sample preparation and electrophoresis in 1% sodium dodecyl sulfate on 3.5% acrylamide gels and gel staining with Coomassie Brilliant Blue R were carried out as described in Rosenberry et al. (1974). Previously identified bands are labeled with their apparent molecular weight in thousands. Each onput sample contained 25 μg of protein in 12.5 μL. Gels 1 and 4, nonreduced: stock 11S and dialyzed and lyophilized 18S plus 14S samples were incubated directly in 1% sodium dodecyl sulfate sample buffer without reducing agent at 50 °C for 1 h. Gels 2 and 5, selective partially reduced: stock 11S and 18S plus 14S solutions were diluted 5% by the addition of Tris-chloride (100 mM final) and dithiothreitol (100 mM final), pH 8.0; incubated at 25 °C for 30 min; mixed with crystalline *N*-ethylmaleimide (500 mM final) for 15 min; dialyzed against water and lyophilized; and redissolved in 1% sodium dodecyl sulfate sample buffer without reducing agent. Gels 3 and 6, completely reduced: stock 11S and dialyzed and lyophilized 18S plus 14S samples were incubated directly in 1% sodium dodecyl sulfate sample buffer containing dithiothreitol (100 mM) at 50 °C for 1 h. Electrophoresis was conducted at 3 mA/tube. Bottoms of displayed gels correspond to the observed dye front. Stock 11S enzyme (Chen et al., 1974) had been stored as a frozen concentrate for 2 years, during which the slight degradation corresponding to stained protein between the 150 000- and 75 000-dalton bands occurred. Stock 18S plus 14S enzyme is the peak fraction eluted with decamethonium in Figure 1 and used without further treatment. Bands are labeled as outlined in the text.

enzyme was at a concentration which corresponded to 0.2 A_{280nm}. Enzyme onputs more concentrated than this show increased amounts of aggregate at the bottom of the gradient (McCann and Rosenberry, 1977). In Figure 2 the major peak sediments at 17.8 S. A second peak corresponding to 13.8 S also can be seen. These values are in ag6eement with the 18S and 14S values determined previously in other laboratories (Massoulié and Rieger, 1969; Dudai et al., 1972; Morrod et al., 1975). Ratios of the 18S to the 14S species are typically close to 3 in our preparations (McCann and Rosenberry, 1977).

Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate. 11S acetylcholinesterase is characterized by intersubunit disulfide bonding (Froede and Wilson, 1970; Rosenberry et al., 1974; Rosenberry, 1975) and this feature is even more pervasive in the 18S and 14S enzymes. To illustrate this point, three states of disulfide reduction can be defined for either the 11S species or the 18S plus 14S mixture obtained by the present affinity chromatography procedure, and the six sodium dodecyl sulfate gel electrophoresis profiles corresponding to these states are shown in Figure 3. Gels 1–3 refer to 11S enzyme and gels 4–6 refer to the 18S plus 14S forms. The enzyme samples on gels 1 and 4 were electrophoresed without exposure to disulfide reducing agents. The samples on gels 2 and 5 were selective partially reduced (Rosenberry, 1975; T. L. Rosenberry, manuscript in preparation), a process involving exposure to the reducing agent dithiothreitol followed by complete alkylation with *N*-ethylmaleimide in the *absence* of a denaturant. The samples on gels 3 and 6 were completely reduced by exposure to dithiothreitol in the presence of sodium dodecyl sulfate. The apparent molecular weights of the polypeptide bands from the 11S enzyme generated by these procedures have been estimated from protein standards run in parallel on sodium dodecyl sulfate gels (Rosenberry et al., 1974; Rosenberry, 1975), and the values obtained[3] are entered in Figure 3 beside the corresponding bands. Additional polypeptide bands are generated from the 18S plus 14S enzyme samples; these bands are labeled A–E in Figure 3.

The primary species in the 11S enzyme prior to reduction (gel 1) is a 150 000-dalton dimer of catalytic subunits; faint bands corresponding to catalytic subunit trimers (approximately 230 000 daltons) and tetramers (approximately 290 000 daltons) and a moderate band close to catalytic subunit monomers (75 000 daltons) are also seen (Rosenberry et al., 1974). In contrast, 18S plus 14S enzyme shows two major oligomeric species A and B in addition to the 150 000 dimer prior to reduction (gel 4). The apparent molecular weights of A and B are both in excess of that of the 290 000-dalton tetramer in gel 1. Faint traces of catalytic subunit monomers, of apparent aggregates larger than band A, and of a species C with an apparent molecular weight, relative to the 11S oligomers, of 250 000 are also seen on gel 4.

[3] It should be noted that the apparent molecular weights of the species designated here as 24 000 through 75 000 average 5–15% lower when estimated by gel exclusion chromatography in 6 M guanidine hydrochloride (Rosenberry et al., 1974).

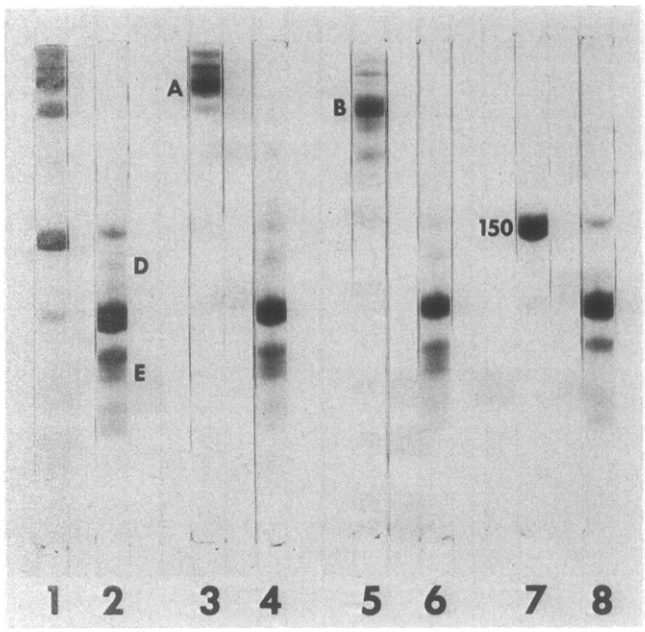

FIGURE 4: Analysis of selected oligomer fractions by gel electrophoresis in 1% sodium dodecyl sulfate. Stock 18S plus 14S acetylcholinesterase (7 mg; similar to that in Figure 3) was dialyzed against water, lyophilized, and redissolved in 1.0 mL of 5% sodium dodecyl sulfate at 50 °C for 1 h. Chromatography in 1% sodium dodecyl sulfate on Sepharose 4B was conducted as outlined under Materials and Methods, and selected fractions which showed the greatest homogeneity of oligomers A, B, and catalytic subunit dimers, respectively, were dialyzed against water and lyophilized. The lyophilized fractions were dissolved (1.6 mg/mL) in 1% sodium dodecyl sulfate sample buffer without reducing agent, and to part of each dissolved fraction was added dithiothreitol (40 mM final). Gels 1, 3, 5, and 7: 15 μg of nonreduced sample onput in 10 μL. Gels 2, 4, 6, and 8: 24 μg of completely reduced sample onput in 20 μL. Gels 1 and 2: unfractionated sample introduced to Sepharose 4B chromatography. Gels 3 and 4: oligomer A fraction. Gels 5 and 6: oligomer B fraction. Gels 7 and 8: catalytic subunit dimer fraction. Electrophoresis conditions and observed dye front as in Figure 3.

After complete disulfide reduction, many of the species obtained from the 11S enzyme (gel 3) and the 14S plus 18S enzyme mixture (gel 6) are similar. In addition to the 75 000-dalton intact catalytic subunit, both gels show the 50 000-, 27 000-, and 23 500-dalton catalytic subunit fragments previously identified for the 11S enzyme (Rosenberry et al., 1974). The amounts of these fragments relative to the intact catalytic subunit band are much lower in gel 6 than in gel 3, indicating that the presumably proteolytic degradation which generates the fragments has proceeded to a smaller extent in the rapidly isolated "native" 18S plus 14S enzyme preparation than in the 11S preparation, but the relative amounts of these fragments appear quite constant among several 18S plus 14S preparations. Two new and rather faint banding regions, D and E, are apparent on gel 6 but absent on gel 3. The polypeptides corresponding to these bands are prime candidates for the structures which distinguish 18S plus 14S enzyme from the 11S form. Polypeptide(s) D has an apparent molecular weight of 115 000 to 120 000 and polypeptide(s) E, of 38 000 to 45 000, based on their gel migration distances relative to the 11S polypeptide standards (see Figure 5 below). Moreover, the radiolabeling profiles of [^{32}P]iPr_2PF-labeled 18S plus 14S enzyme after complete reduction and electrophoresis as in gel 6 reveal that polypeptide(s) D, but not E, is labeled with ^{32}P (P. Barnett and T. L. Rosenberry, unpublished observations); thus, polypeptide(s) D is probably a residual aggregate of a catalytic subunit monomer and polypeptide(s) E.

Selective partial reduction in Figure 3 utilized a high dithiothreitol concentration (100 mM), but virtually identical banding patterns are observed for dithiothreitol concentrations between 2 and 200 mM. After selective partial reduction, virtually all of the higher oligomeric 11S enzyme bands in gel 1 are converted to catalytic subunit monomers (gel 2). This reduction generates a single free sulfhydryl group in the catalytic subunits which can be radioalkylated (Rosenberry, 1975; T. L. Rosenberry, manuscript in preparation). Little or no disulfide reduction of internal disulfide bonds within the catalytic subunit occurs under these conditions. This conclusion can be drawn from the failure to observe catalytic subunit fragments on gel 2 and from the observation that almost none of the five internal half-cystine residues in the 50 000-dalton catalytic subunit fragment are labeled following complete reduction of *N*-[^{14}C]ethylmaleimide-labeled selectively reduced 11S enzyme (Rosenberry, 1975; T. L. Rosenberry, manuscript in preparation). Selective partial reduction of 18S plus 14S enzyme (gel 5) leads to the disappearance of bands A–C and to the emergence of bands D and E, although E appears less intense than on gel 6. By analogy with the 11S enzyme, this result suggests that intersubunit disulfide bonds not only in the catalytic subunit dimer but also in oligomers A–C can be selectively reduced to yield catalytic subunit monomers. In addition, selective reduction of oligomers A–C also yields polypeptides D and E.

The banding patterns in gels 4–6 are highly reproducible among several 18S plus 14S preparations, although slight differences do occur in the relative staining intensity of band E.

Chromatography in Agarose Gels in 1% Sodium Dodecyl Sulfate. The nonreduced oligomeric species in 18S plus 14S enzyme can be fractionated by Sepharose 4B gel exclusion chromatography in 1% sodium dodecyl sulfate. Gels 3, 5, and 7 in Figure 4 correspond to samples of individual column fractions enriched, respectively, in oligomers A, B, and catalytic subunit dimers. The gel corresponding to the nonreduced column output of 18S plus 14S enzyme is also given as gel 1. Adjacent to each gel of a nonreduced sample is a corresponding gel of an identical sample after complete disulfide reduction. Completely reduced oligomer A (gel 4) and completely reduced oligomer B (gel 6) appear to generate similar patterns of catalytic subunit monomers, catalytic subunit fragments, and polypeptides D and E. Completely reduced catalytic subunit dimers (gel 8) generate only catalytic subunit monomers and catalytic subunit fragments. These observations confirm the suggestion in the previous section that catalytic subunits and polypeptides D and E are covalently linked by disulfide bonds in oligomers A and B.

The banding pattern corresponding to gels 2, 4, and 6 in Figure 4 shows increased resolution when the electrophoresis is carried out on 5.8% polyacrylamide gels (Figure 5). In particular, band E is revealed to be a doublet of two closely spaced polypeptides of apparent molecular weights 44 000 and 40 000 relative to catalytic subunits and catalytic subunit fragments.

Amino Acid Compositions. The composition of 18S plus 14S acetylcholinesterase is compared to an average of previously published 11S enzyme compositions in Table I. In agreement with the amino acid content of 16S *Torpedo californica* acetylcholinesterase reported previously (Lwebuga-Mukasa et al., 1976), the 18S plus 14S eel enzyme contains small amounts of hydroxyproline and hydroxylysine. Anglister et al. (1976) have also reported 0.83% (w/w) hydroxyproline (0.73 mol %) in the 18S plus 14S eel enzyme by a colorimetric procedure.

TABLE I: Amino Acid Compositions of Acetylcholinesterase Preparations.

Residue	18S plus 14S[a] (mol %)	11S[b] (mol %)	Catalytic subunit dimers[c] (mol %)
Hyp	0.6		0
Asp	11.6	11.8	12.0
Thr	3.6	4.2	4.3
Ser	6.4	6.7	7.7
Glu	10.1	10.1	9.7
Pro	6.7	6.8	6.7
Gly	10.6	8.3	8.1
Ala	5.2	6.3	5.5
Val	6.7	6.8	7.2
Met	2.4	2.8	2.2
Ile	3.7	3.8	3.7
Leu	8.5	8.4	8.8
Tyr	3.3	3.4	3.7
Phe	4.7	5.1	5.3
Hyl	0.7		0
Lys	4.0	4.5	3.8
His	2.3	2.2	2.2
Arg	5.3	5.1	5.4
Half-Cys		1.2	
Trp		2.4	

[a] Determined by Mr. Walter Schrepel, Department of Biochemistry, Columbia University; uncorrected 16-h hydrolysis at 116 °C in 6 N HCl containing 0.08 M mercaptoethanol under argon. Average of two analyses. Listed numbers based on a total of 96.37 because Cys and Trp were not measured. [b] Average of three analyses in Table VI of Rosenberry (1975). [c] Determined by Dr. Richard Berg, Department of Biochemistry, Rutgers Medical School, Piscataway, N.J.; uncorrected 16-h hydrolysis at 116 °C in 6 N HCl containing 0.08 M mercaptoethanol under nitrogen. Average of four analyses. Listed numbers based on a total of 96.37 because Cys and Trp were not measured.

The relative amounts of hydroxyproline and proline in 18S plus 14S acetylcholinesterase were determined with high precision by means of a fluorescamine-monitored amino acid analysis of oxidized samples.[4] A molar ratio of 4-hydroxyproline to proline of 0.099 ± 0.004 was observed. No 3-hydroxyproline could be detected. Application of this analysis procedure to 11S eel acetylcholinesterase[4] confirmed the absence of hydroxyproline; the molar ratio of hydroxyproline to proline was <0.001 (see Lwebuga-Mukasa et al., 1976; Anglister et al., 1976).

An isolation of the polypeptides corresponding to band E in Figures 3–5 has not yet been achieved. However, a reasonable preliminary estimate of their amino acid composition can be made from the difference in amino acid content between oligomers A or B and the 150 000-dalton catalytic subunit dimers. As expected from the reduced polypeptide compositions in Figures 3–5, the amino acid composition of these dimers is virtually identical with that of the 11S enzyme (Table I).[5] To ensure that the difference was calculated for amounts of oligomers A or B and dimers which were equal in catalytic subunit content, [^{32}P]iPr_2PF-labeled 18S plus 14S enzyme was

[4] These analyses were carried out by Dr. Stanley Stein at the Roche Institute, Nutley, N.J.

[5] A small 8000-dalton polypeptide has been identified in the 11S enzyme which is not present in these dimers. This polypeptide appears to be a residual tail subunit fragment (Rosenberry, 1975; T. L. Rosenberry, manuscript in preparation). Its presence in the 11S enzyme appears to have little effect on the overall amino acid composition.

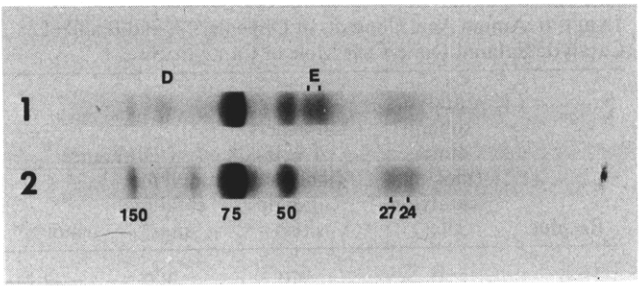

FIGURE 5: Identification of subunits which appear to correspond to the tail structure of 18S plus 14S acetylcholinesterase by gel electrophoresis in 1% sodium dodecyl sulfate. Selected fractions from 1% sodium dodecyl sulfate gel chromatography as in Figure 4 which contained either a mixture of oligomers A and B comparable to stock 18S plus 14S enzyme (gel 1) or catalytic subunit dimers (gel 2) were prepared for electrophoresis in 1% sodium dodecyl sulfate on 5.8% acrylamide gels in the completely reduced state as described in Figure 4. Each onput sample contained 25 μg of protein. Electrophoresis conditions and observed dye front as in Figure 3.

prepared and fractionated by sodium dodecyl sulfate column chromatography. The additional amino acid residues in oligomers A or B can then be attributed to the band E polypeptides. The results (Table II) should be considered tentative, because the difference between two large numbers is subject to considerable error (see footnote *b* in Table II). These results indicate that nearly a third of these additional residues are glycine, that proline is the next most prevalent amino acid, and that a high percentage of the lysine residues are hydroxylated. Such a composition implies that the band E polypeptides are largely collagen-like.

Discussion

An affinity chromatography purification of asymmetric acetylcholinesterase forms was first reported by Dudai et al. (1972), who showed that a resin linked to *N*-methylacridinium selectively purified 18S plus 14S eel enzyme species from 1 M NaCl extracts. Affinity chromatography of these forms at low to moderate ionic strength (<0.4 M) has also been reported (Morrod et al., 1975; Chen et al., 1973; Massoulié and Bon, 1976; Lwebuga-Mukasa et al., 1976). We selected the *N*-methylacridinium resin as a candidate for our use but were unable to reproduce its synthesis (Dudai and Silman, 1974b). Consequently, we developed the acridinium resin introduced here and found its synthesis to be highly reliable. With this resin maximal purification of eel 18S plus 14S forms is achieved after one affinity chromatography cycle, and the specific activity achieved is the same as that reported by Dudai and Silman (1974b) after two cycles of affinity chromatography. This specific activity corresponds to about 65% of the maximal value for eel 11S acetylcholinesterase, and, considering the problems of adsorption and aggregation noted in the Results section, this difference is likely to arise from assay difficulties rather than from impurities in the 18S plus 14S preparation. In support of this conclusion, preliminary experiments with the titrating agent 1-methyl-7-dimethylcarbamoyloxyquinolinium iodide (Rosenberry and Bernhard, 1971) indicate that purified 18S plus 14S enzyme has about the same equivalent weight as the purified 11S enzyme. Our purified 18S plus 14S eel preparations have a somewhat higher proportion of the 18S species than those previously reported and show no tendency toward either conversion to the 11S form or fragmentation of the catalytic subunits on storage, in contrast to those of Morrod et al. (1975) and Bon and Massoulié (1976).

The sodium dodecyl sulfate gel electrophoresis experiments

TABLE II: Amino Acid Contents of Oligomers A and B and of Catalytic Subunit Dimers per Mole of Catalytic Sites.[a]

Residue	Catalytic subunit dimers (mol/mol catalytic sites)	Oligomers A + B (mol/mol catalytic sites)	Difference mol/mol catalytic sites[b]	Difference mol %[c]
Hyp	0	8.0	8.0	(7.2)
Asp	72.9	76.3	3.4	(3.0)
Thr	26.2	27.0	0.8	(0.7)
Ser	46.6	44.1	−2.5	0
Glu	59.1	69.8	10.7	(9.6)
Pro	40.6	56.9	16.3	(14.6)
Gly	49.4	83.5	34.1	(30.6)
Ala	33.5	38.8	5.3	(4.8)
Val	43.9	48.6	4.7	(4.2)
Met	13.3	15.2	1.9	(1.7)
Ile	22.7	23.5	0.8	(0.7)
Leu	53.2	58.3	5.1	(4.6)
Tyr	22.5	22.1	−0.4	0
Phe	32.0	30.8	−1.2	0
Hyl	0	7.6	7.6	(6.8)
Lys	23.0	28.4	5.4	(4.8)
His	13.6	14.7	1.1	(1.0)
Arg	32.7	39.0	6.3	(5.7)

[a] [^{32}P]iPr$_2$PF-labeled 18S plus 14S acetylcholinesterase was fractionated by gel exclusion chromatography in 1.0% sodium dodecyl sulfate. Column fractions were pooled such that oligomers A and B were combined in the same ratio indicated in the original 18S plus 14S preparation. Samples of this combined pool and of the catalytic subunit dimer fraction were dialyzed against water, lyophilized, and prepared for amino acid analyses as indicated in footnote *c* of Table I. Aliquots of each hydrolyzed sample were removed for ^{32}P determination to ensure that the amino acid contents would be based on equivalent amounts of catalytic sites. The contents shown are calculated on the basis of a total of 585 of the indicated residues per catalytic site in the catalytic subunit dimer, a value previously found to correspond to an assumed 70 000-dalton catalytic subunit polypeptide (excluding carbohydrate residues) in the 11S enzyme (Rosenberry, 1975). Average of four analyses for each sample, determined by Dr. Richard Berg (see Table I). [b] The standard error of these values did not exceed the larger of the following estimates: 2 residues or 3% of the sum of the corresponding values in columns 1 and 2. [c] The calculation of the mole percent values for the additional amino acid residues in oligomers A and B ignores negative difference values.

in Figures 3-5 identify two polypeptide species E which are prime candidates for tail subunits previously indicated only by electron micrographs. These putative tail subunits appear to be linked to catalytic subunits in the native enzyme by disulfide bonds. The amino acid composition of these subunits has been estimated indirectly (Table II) and indicates that much of their structure is collagen-like. Because the molecular weights of collagen-like polypeptides are usually overestimated relative to noncollagen protein standards in sodium dodecyl sulfate gels or on sodium dodecyl sulfate columns by as much as a factor of two (Furthmayer and Timpl, 1971; Ohno et al., 1975), the apparent molecular weights of 44 000 and 40 000 for these polypeptides should be considered quite tentative. The difference amino acid composition in Table II indicates that the oligomer A and B mixture contains additional amino acids which presumably correspond to the band E polypeptides. The observed amino acid difference corresponds to a little over mol wt 20 000 per pair of catalytic subunits, and inclusion of carbohydrate residues would increase this rough estimate. Thus, it appears that one or perhaps two band E polypeptides are associated with each pair of catalytic subunits in oligomers A and B. It is noteworthy that tail subunits of 40 000 daltons were calculated from the length of the tail structure in electron micrographs of eel 8S acetylcholinesterase. This calculation assumed that the tail subunits displayed an α helix configuration (Cartaud et al., 1975). Furthermore, the reported difference in glycine content between 16S and 11S torpedo enzymes corresponds to a collagen amino acid sequence of 40 000 daltons for every 360 000 daltons of 16S enzyme (Lwebuga-Mukasa et al., 1976).

A striking feature of 18S plus 14S acetylcholinesterase is the asymmetry of catalytic subunits prior to disulfide reduction. Catalytic subunits are present in oligomers A and B as well as in 150 000-dalton catalytic subunit dimers. Separation of the 18S and 14S components in the purified preparations by the extensive use of sucrose gradient centrifugation reveals that oligomer A arises primarily from the 18S form and oligomer B, from the 14S form (McCann and Rosenberry, 1977). These studies also demonstrate that the oligomer B band is a closely spaced doublet of two bands. Gel slicing analyses of [^{32}P]iPr$_2$PF-labeled 18S plus 14S enzyme run on sodium dodecyl sulfate gels without disulfide reduction indicate that about 50% of this catalytic site label occurs in the catalytic subunit dimer band while the remaining 50% occurs in the higher molecular weight bands (P. Barnett and T. L. Rosenberry, unpublished observations). Our observations are similar to those recently reported for nonreduced [^{32}P]iPr$_2$PF-labeled 18S plus 14S enzyme (Silman and Dudai, 1975) except that we see virtually no subunit monomer band. Thus, one-half of the catalytic subunits appears linked as subunit dimers while the remaining half is covalently associated with putative tail subunits.

We observe that selective partial reduction of 18S plus 14S enzyme converts virtually all catalytic subunits to 75 000-dalton monomers. A similar result was obtained with 11S enzyme, where this conversion was accompanied by the exposure of one half-cystine per catalytic subunit (Figure 3; Rosenberry, 1975; T. L. Rosenberry, manuscript in preparation). As described in the Results section, selective partial reduction of either the 18S plus 14S or the 11S enzyme results in little observable reduction of intrasubunit disulfide bonds. These selective partial reduction data appear much less ambiguous than those recently reported for 16S and 11S torpedo acetylcholinesterase (Lwebuga-Mukasa et al., 1976). In particular, both their 16S and 11S samples showed only partial conversions to catalytic subunit monomers after selective partial reduction. The discrepancy between the eel and torpedo enzymes on the effects of selective partial reduction may arise from the somewhat lower concentrations of reducing and alkylating agents or the pH used in their study, but differences between the two species may also exist in the accessibility of intersubunit disulfide bonds to exogenous reducing agents prior to denaturation.

We propose the schematic model of 18S eel acetylcholinesterase shown in Figure 6 as a summary both of our data and of previous literature reports, particularly the electron micrographs of Cartaud et al. (1975). Twelve catalytic subunits are arranged as three tetrameric groups. Within each tetramer, two catalytic subunits are directly linked while the remaining two are covalently attached to the collagen-like tail structure. In 1% sodium dodecyl sulfate the subunit dimers dissociate from the remaining six catalytic subunits which maintain their attachment to the tail structure in an aggregate that corresponds to oligomer A. Selective partial reduction breaks all the disulfide bonds shown and also appears to at least partially dissociate the tail structure to polypeptides E. The tail structure may exist in a collagen-like three-stranded helical conforma-

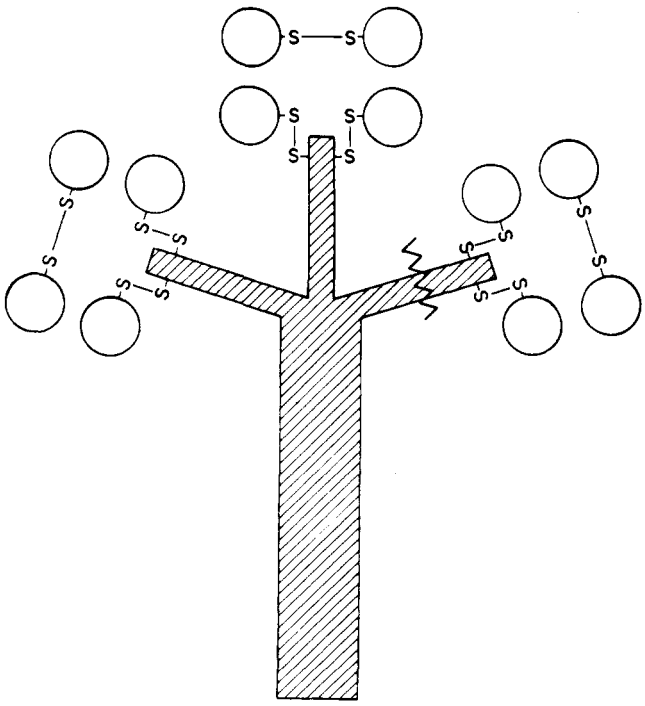

FIGURE 6: Schematic model of 18S eel acetylcholinesterase. The circles refer to catalytic subunits, and the cross-hatched region, to an as yet undefined combination of polypeptides E which comprise the tail structure. Partial cleavage of catalytic subunits (Rosenberry et al., 1974) is ignored. Catalytic subunits may be considered in dimeric pairs. In half of these pairs, subunits are disulfide linked directly to each other, while in the other half, a disulfide linkage is mediated via the tail subunits. Both a 14S and an 11S species are generated from an 18S molecule by cleavage at the indicated tail structure site.

tion, but no specific information has been obtained on this point.

The high proportion of 18S enzyme in our preparations supports the suggestion of Massoulié et al. (1971) that the 14S and 8S enzyme forms occur as intermediates in the degradation of the 18S enzyme to the 11S form. As proposed earlier by Rieger et al. (1973), this degradation appears to involve the sequential removal of one 11S tetramer from the 18S form to give the 14S species (Figure 6) and of a second 11S tetramer to give the 8S species. The 11S tetramers so formed appear to retain a residual tail fragment linked asymmetrically by disulfide bonds to only two of the catalytic subunits.[5] Estimated molecular weight differences between the 18S, 14S, and 8S eel forms relative to the 11S species suggest a total tail molecular weight of about 100 000 (Bon et al., 1976). According to the model in Figure 6, the total tail structure is included in oligomer A, and our data from Table II are consistent with a similar estimate for the total tail molecular weight.

The indication that tail subunits of 16S torpedo acetylcholinesterase have at least a partial collagen-like structure led Lwebuga-Mukasa et al. (1976) to suggest that tail subunits are responsible for an in vivo localization of acetylcholinesterase in the extracellular basement membrane matrix. A similar suggestion based on more tentative evidence had been made earlier (Dudai and Silman, 1974a). The plausibility of this suggestion is further enhanced by the parallels in intersubunit disulfide bonding between 18S acetylcholinesterase and a citrate-soluble fraction of lens capsule basement membrane (Olsen et al., 1973). This fraction was composed of two components, collagenous filament structures and noncollagenous globular structures, which apparently could be dissociated by reduction of disulfide bonds. Pervasive intersubunit disulfide bonding is also a characteristic of procollagen (Byers et al., 1975).

The identification of putative tail subunits must now be followed by the isolation and characterization of these structures. Further studies are also required to quantitate the stoichiometry between catalytic and tail subunits, to describe the conversions to 14S and 8S enzyme forms, and to investigate the relationship between tail subunits and basement membrane components.

Acknowledgments

We are grateful to Dr. Philip Barnett and to Ms. Carol Mays for their technical assistance in the latter parts of this investigation. We also thank Dr. Richard Berg, Dr. Stanley Stein, and Mr. Walter Schrepel for their determinations of amino acid compositions.

References

Albert, A. (1966a), The Acridines, Arnold, London, p 170.
Albert, A. (1966b), The Acridines, Arnold, London, p 282.
Albert, A. (1966c), The Acridines, Arnold, London, p 307.
Anglister, L., Rogozinski, S., and Silman, I. (1976), *FEBS Lett. 60,* 129-132.
Betz, W., and Sakmann, B. (1973), *J. Physiol. 230,* 673-688.
Bon, S., Huet, M., Lemonnier, M., Rieger, F., and Massoulié, J. (1976), *Eur. J. Biochem. 68,* 523-530.
Bon, S., and Massoulié, J. (1976), *FEBS Lett. 71,* 273-278.
Byers, P. H., Click, E. M., Harper, E., and Bornstein, P. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 3009-3013.
Cartaud, J., Rieger, F., Bon, S., and Massoulié, J. (1975), *Brain Res. 88,* 127-130.
Chen, Y. T., Rosenberry, T. L., and Chang, H. W. (1973), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 32,* 554.
Chen, Y. T., Rosenberry, T. L., and Chang, H. W. (1974), *Arch. Biochem. Biophys. 161,* 479-487.
Cuatrecasas, P., and Parikh, I. (1972), *Biochemistry 11,* 2291-2299.
Dudai, Y., Herzberg, M., and Silman, I. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2473-2476.
Dudai, Y., and Silman, I. (1974a), *J. Neurochem. 23,* 1177-1187.
Dudai, Y., and Silman, I. (1974b), *Methods Enzymol. 34,* 571-580.
Dudai, Y., Silman, I., Shinitzky, M., and Blumberg, S. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 2400-2403.
Ellman, G. L., Courtney, K. D., Andres, V., Jr., and Featherstone, R. M. (1961), *Biochem. Pharmacol. 7,* 88-95.
Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971), *Biochemistry 10,* 2606-2617.
Froede, H. C., and Wilson, I. B. (1970), *Isr. J. Med. Sci. 6,* 179-184.
Furthmayer, H., and Timpl, R. (1971), *Anal. Biochem. 41,* 510-516.
Hall, Z. W., and Kelly, R. (1971), *Nature (London), New Biol. 232,* 62-63.
Lawson, W. B., Leafer, M. D., Jr., Tewes, A., and Rao, G. J. S. (1968), *Hoppe-Seyler's Z. Physiol. Chem. 349,* 251-262.
Lwebuga-Mukasa, J. S., Lappi, S., and Taylor, P. (1976), *Biochemistry 15,* 1425-1434.
March, S. C., Parikh, I., and Cuatrecasas, P. (1974), *Anal. Biochem. 60,* 149-152.
Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236,* 1372-1379.
Massoulié, J., and Bon, S. (1976), *Eur. J. Biochem. 68,*

531-539.
Massoulié, J., Bon, S., Rieger, F., and Vigny, M. (1975), *Croat. Chem. Acta 47*, 163-179.
Massoulié, J., and Rieger, F. (1969), *Eur. J. Biochem. 11*, 441-455.
Massoulié, J., Rieger, F., and Bon, S. (1971), *Eur. J. Biochem. 21*, 542-551.
McCann, W. F. X., and Rosenberry, T. L. (1977), *Arch. Biochem. Biophys.* (in press).
Morrod, P. J., Marshall, A. G., and Clark, D. G. (1975), *Biochem. Biophys. Res. Commun. 63*, 335-342.
Ohno, M., Riquetti, P., and Hudson, B. G. (1975), *J. Biol. Chem. 250*, 778-787.
Olsen, B. R., Alper, R., and Kefalides, N. A. (1973), *Eur. J. Biochem. 38*, 220-228.
Rieger, F., Bon, S., Massoulié, J., and Cartaud, J. (1973), *Eur. J. Biochem. 24*, 539-547.
Rosenberry, T. L. (1975), *Adv. Enzymol. 43*, 103-218.
Rosenberry, T. L., and Bernhard, S. A. (1971), *Biochemistry 10*, 4114-4120.
Rosenberry, T. L., and Bernhard, S. A. (1972), *Biochemistry 11*, 4308-4321.
Rosenberry, T. L., Chang, H. W., and Chen, Y. T. (1972), *J. Biol. Chem. 247*, 1555-1565.
Rosenberry, T. L., Chen, Y. T., and Bock, E. (1974), *Biochemistry 13*, 3068-3079.
Silman, H. I., and Karlin, A. (1967), *Proc. Natl. Acad. Sci. U.S.A. 58*, 1664-1668.
Silman, I., and Dudai, Y. (1975), *Croat. Chem. Acta 47*, 181-200.

Rapid Kinetic Studies of Partial Reactions in the Heme Free Derivative of L-Lactate Cytochrome *c* Oxidoreductase (Flavocytochrome b_2); the Flavodehydrogenase Function†

Motohiro Iwatsubo,‡ Maryvonne Mével-Ninio, and Françoise Labeyrie*

ABSTRACT: The flavodehydrogenase, the "heme-free" derivative of flavocytochrome b_2 (L-lactate cytochrome *c* oxidoreductase, EC 1.1.2.3, present in aerobic yeast), has been successfully reconstituted from the apoprotein. Stopped-flow experiments show that the behavior of this flavodehydrogenase toward the substrate L-lactate is indistinguishable from that of the flavocytochrome b_2 containing its heme groups (both have the same lactate K_m and V_m values). In contrast, its behavior with a one-electron acceptor such as ferricyanide is very different: the apparent bimolecular rate constant for the reoxidation of the fully reduced enzyme by ferricyanide is 200 times lower for the flavodehydrogenase than for the flavocytochrome b_2 itself. Analysis of the reoxidation by ferricyanide of the reduced flavodehydrogenase in terms of two successive steps of one-electron transfer indicates that this acceptor reacts at least 20 times faster with the flavin semiquinone than with the hydroquinone. The reactions of ferricyanide with native flavocytochrome b_2 and its heme-free derivative are compared and discussed to shed light on the role of the heme within that multicomponent enzyme. The role of the cytochrome b_2 moiety when the enzyme reacts with an acceptor such as ferricyanide could be to act as the best acceptor of one electron from the substrate-reduced flavin, and allow the transformation of a poorly reactive electron donor (flavin hydroquinone) into two much more reactive one-electron donors (ferroheme and flavin semiquinone). The cytochrome b_2 moiety acts as the specific donor to cytochrome *c*. The assumption that heme does not modify the kinetic parameters of the flavodehydrogenase, but simply, by its presence, adds new possibilities, especially when cytochrome *c* is the acceptor, is discussed.

During the last 20 years, yeast mitochondrial flavocytochrome b_2 or L-lactate cytochrome *c* reductase has been the object of numerous investigations which have shed light on several facets of its structure and function. Catalysis leads to electron transfer from L-lactate to various acceptors, such as ferricyanide, dyes, and cytochrome *c*, the latter being its natural acceptor. The specific role of each prosthetic group—flavin mononucleotide and protoheme—in the catalytic function and the interactions between them are very intriguing problems. The protoheme and flavin groups, because of their much higher redox potentials, can both be completely reduced by L-lactate (Baudras, 1965c; Iwatsubo et al., 1968; Capeillère-Blandin et al., 1975); the enzyme then accepts a total of three electrons per protomer, i.e., 12 for the stable active tetramer. Both kinds of prosthetic groups are quantitatively reoxidized by external acceptors (Morton and Sturtevant, 1964). Thus a priori, both are able to take part directly in the transfer of the electrons from L-lactate to the external acceptors during the catalytic reaction.

To elucidate the role of each prosthetic group in the overall electron transfer, two experimental approaches were used. The first approach consisted of detailed kinetic studies of the holo(FMN[1]-heme)enzyme. Combined stopped-flow absorbance and rapid freezing EPR experiments were carried out on the

† From the Centre de Génétique Moléculaire du CNRS, 91190-Gif-sur-Yvette, France. *Received March 7, 1977*. This work has been supported in part by a grant from the Délégation Générale à la Recherche Scientifique et Technique, Comité d'Intéractions Moléculaires en Biologie.

‡ Deceased April 23, 1977.

[1] Abbreviations used are: F_{HQ}, flavohydroquinone; F_{SQ}, flavosemiquinone; F_{ox}, flavoquinone; H_{ox}, oxidized heme; H_{red}, reduced heme; EDTA, (ethylenedinitrilo)tetraacetic acid; FMN, flavin mononucleotide.