

Purification and Crystallization of a Dimeric Form of Acetylcholinesterase from *Torpedo californica* Subsequent to Solubilization with Phosphatidylinositol-specific Phospholipase C

A dimeric form of acetylcholinesterase from *Torpedo californica* was purified to homogeneity by affinity chromatography subsequent to solubilization with a phosphatidylinositol-specific phospholipase C of bacterial origin. Bipyramidal crystals of the enzyme were obtained from solutions in polyethylene glycol 200. The crystals diffract to 2.0 Å (1 Å = 0.1 nm) resolution. They were found to be orthorhombic, space group $P222_1$, with $a = 163.4(\pm 0.2)$ Å, $b = 112.1(\pm 0.2)$ Å, $c = 81.3(\pm 0.1)$ Å.

The principal biological role of acetylcholinesterase (AChE†, acetylcholine hydrolase, EC 3.1.1.7) is the termination of impulse transmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine (Barnard, 1974). In keeping with this requirement, AChE is characterized by a remarkably high specific activity, especially for a serine hydrolase (Rosenberry, 1975), functioning at a rate approaching that of a diffusion-controlled reaction (Hasinoff, 1982). The powerful acute toxicity of organophosphate poisons (and of carbamates and sulfonyl halides which function analogously) is attributed primarily to their serving as potent inhibitors of AChE (Koelle, 1963). This effect is achieved by their covalent attachment to a serine residue within the active site. Elucidation of the three-dimensional structure of AChE is thus of fundamental interest for understanding its remarkable catalytic efficacy, and of importance in devising therapeutic approaches to organophosphorus poisoning.

The electric organs of the electric fish, *Torpedo* and *Electrophorus*, provide rich sources of AChE. The forms of AChE in the electric organ are structurally homologous to those in vertebrate nerve and muscle, and highly purified preparations of AChE from these sources have provided much of our knowledge concerning the structure and function of the enzyme (Froede & Wilson, 1980; Silman & Futerman, 1987). Furthermore, AChE from *Torpedo* is the only vertebrate AChE which has been cloned (Schumacher *et al.*, 1985) and the only AChE for which complete amino acid sequence data have been reported (MacPhee-Quigley *et al.*, 1985). In *Torpedo* electric organ tissue one of the principal forms of AChE is a disulphide-linked

catalytic subunit dimer belonging to a recently described class of membrane proteins which are linked to the plasma membrane *via* covalently attached phosphatidylinositol (PI) (Low *et al.*, 1986). In this dimer, the PI is attached to the COOH-terminus of each catalytic subunit through an intervening oligosaccharide sequence, which is apparently added post-translationally, and the diglyceride moiety of the PI serves as the hydrophobic anchor (Silman & Futerman, 1987). PI-anchored proteins can often be solubilized selectively by a PI-specific phospholipase C (PIPLC) of bacterial origin (Low *et al.*, 1986), and this is also the case for the *Torpedo* AChE dimer (Futerman *et al.*, 1983).

The only preparation of AChE crystallized so far is the 11 S tetramer from *Electrophorus electricus* (Leuzinger & Baker, 1967), which crystallized in two forms. One crystal form is hexagonal. It diffracts to 3.9 Å (1 Å = 0.1 nm) and belongs to space group $P6_222$, with cell dimensions of $a = b = 187$ Å, $c = 292$ Å (Chothia & Leuzinger, 1975). The other form is orthorhombic with diffraction that does not extend equally in the three dimensions and belongs to space group $F222$ with cell dimensions of $a = 141.7$ Å, $b = 202.6$ Å, $c = 235.3$ Å (Schrag *et al.*, 1987). The 11 S form of AChE from *Electrophorus* is derived, by proteolytic degradation, from native, "asymmetric" forms present in the fresh tissue in which one, two or three catalytic subunit tetramers are attached to a collagenous tail (Massoulié *et al.*, 1970; Dudai *et al.*, 1972). The catalytic subunit tetramer so obtained itself possesses an asymmetric character, and also contains "nick" sites due to its method of preparation (for a discussion see Anglister & Silman, 1978; Anglister *et al.*, 1980). Furthermore, no sequence data are available for *Electrophorus* AChE, whether from direct chemical sequencing or from cloning studies. It thus seemed reasonable to select, as a candidate for crystallization, the AChE dimer from *Torpedo californica*. The

† Abbreviations used: AChE, acetylcholinesterase; PI, phosphatidylinositol; PIPLC, PI-specific phospholipase C; PEG, polyethylene glycol; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

two subunits in this dimer are very similar or identical, and the primary sequence and suggested arrangement of intrachain disulfides have been reported (MacPhee-Quigley *et al.*, 1985, 1986). Furthermore, selective solubilization by PIPLC provides a novel approach, which yields significant purification prior to affinity chromatography and precludes any nicking or other proteolytic damage to the protein, since the only lytic step involved in solubilization is the cleavage, from the covalently attached PI molecule, of the diglyceride which serves as hydrophobic anchor (Futerman *et al.*, 1985). Use of such a preparation would also supply a bonus, inasmuch as elucidation of its structure would yield information about the location and conformation of the oligoglycan part of the anchoring domain of a PI-anchored protein.

Purification of AChE, subsequent to solubilization by PIPLC from *Staphylococcus aureus*, was carried out essentially as described (Futerman *et al.*, 1985). Thus, the particulate fraction from a homogenate of frozen electric organ tissue from *Torpedo californica* (Pacific Biomarine, Venice, CA) in 0.1 M-NaCl, 0.01 M-Tris (pH 8.0) was exposed to PIPLC (1 µg/ml) for 18 hours at room temperature. Almost quantitative solubilization of the AChE dimer was thus obtained. After centrifugation at 78,000 g for one hour, at 4°C, the supernatant was passed over an affinity-chromatography column consisting of the affinity ligand (*m*-amino-phenyl)trimethylammonium coupled to Sepharose 2B via a dicaproyl spacer. After extensive washing, the bound AChE was eluted from the column with 2 mM-decamethonium bromide in the application buffer. The purified enzyme was then dialyzed exhaustively against 0.1 M-NaCl, 0.01% (w/v) sodium azide, 1 mM-2-(*N*-morpholino)ethanesulfonic acid (Mes) (pH 6.5) and concentrated up to 10 to 15 mg/ml in a Centricon 30 microconcentrator. The purified AChE displayed a single polypeptide band of apparent molecular weight 65,000 on SDS/polyacrylamide gel electrophoresis, and had a specific activity of >2500 units per mg with 3 mM-[³H]acetylcholine as substrate, at pH 7.4 and 25°C. Approximately 7 mg of AChE so purified could be obtained routinely from 450 g of electric organ tissue.

We have crystallized the above preparation of *Torpedo* AChE by the vapor diffusion method using hanging drops (5 µl) at room temperature. The precipitating reservoir contained 35% polyethylene glycol (PEG) 200 and the protein drop contained 8 to 10 mg AChE/ml, 18% PEG 200 and was brought to a final concentration of ~0.1 M-Mes by addition of 0.5 M-Mes (pH 6.0). Bipyramidal crystals grow spontaneously, as well as by seeding, up to 0.5 mm in their longest dimension, over a period of two to three weeks after nucleation. They diffract to 2.0 Å resolution and lose ~25% of their diffracting power within 24 hours in the X-ray beam at room temperature. Unit cell dimensions were determined via X-ray studies using a Rigaku AFC5-R rotating-anode diffractometer operated at 10 kW. The

crystals were found to be orthorhombic, space group $P222_1$, with $a = 163.4(\pm 0.2)$ Å, $b = 112.1(\pm 0.2)$ Å, $c = 81.3(\pm 0.1)$ Å. Assuming the unit cell to contain four AChE dimers we calculate the ratio of the volume of the asymmetric unit to the molecular weight of the dimer (130,000) to be $V_m = 2.95$ Å³/dalton, well within the range outlined by Matthews (1968).

Several other different crystal forms of AChE were obtained when the enzyme was crystallized from higher molecular weight PEGs by vapor diffusion at room-temperature and their diffracting properties are currently being investigated. Hexagonal crystals grew from 30% PEG 400, 0.014 M-spermine, 0.1 M-Mes buffer at pH 5.6; plate-shaped crystals grew from 40% PEG 400, 0.1 M-Mes buffer at pH 5.6; rod-shaped crystals grew from 20% PEG 1500, 0.1 M-Mes buffer at pH 6.5. X-ray data collection of the orthorhombic crystal form, which has a considerably smaller unit cell and diffracts to higher resolution than the crystals of AChE reported previously, is planned for the near future.

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J. L. Sussman¹
M. Harel¹
F. Frolow¹
L. Varon²
L. Toker²
A. H. Futerman²
I. Silman²

Departments of Structural Chemistry¹ and
Neurobiology²
Weizmann Institute of Science
Rehovot 76100, Israel

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