The Effect of Elimination of Intersubunit Disulfide Bonds on the Activity, Assembly, and Secretion of Recombinant Human Acetylcholinesterase

EXPRESSON OF ACETYLCHOLINESTERASE CYS-580 → ALA MUTANT

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Site-directed mutagenesis was used to study the cysteine residue involved in the assembly of human acetylcholinesterase (HuAChE) catalytic subunits. Substitution of the cysteine at position 580 by alanine resulted in impairment of interchain disulfide bridge formation; the mutagenized enzyme (C580A) was secreted from recombinant cells in the monomeric form and failed to assemble into dimers. The mutant monomeric HuAChE did not differ from the native oligomeric enzyme neither in rate of catalysis nor in affinity to acetylthiocholine. Mutant monomers were also shown to retain the acetylcholinesterase characteristic sensitivity to high substrate concentrations. The mutation did not seem to affect the efficiencies of either synthesis or secretion of recombinant HuAChE polypeptides, as was demonstrated in cell lines derived from human embryonic kidney (293 cells) as well as from a human neuroblastoma (SK-N-SH). Furthermore, the mutation did not lead to an increase in accumulation of intracellular HuAChE polypeptides, suggesting that export of acetylcholinesterase from cells may not be coupled to subunit assembly.

Acetylcholinesterase (EC 3.1.1.7; abbreviated herein AChE) occurs in multiple molecular forms in different tissues of vertebrates and invertebrates. Some of these forms are soluble while others are associated with the extracellular surface of cells by different mechanisms of attachment (reviewed in Massouille and Bon, 1982; Silman and Futerman, 1987; Chatonnet and Lockridge, 1989). Members of one subset of attached forms consist of two or three tetratramers of catalytic subunits linked to a collagen-like structure that allows attachment to the basal lamina. A second class of cell-bound forms consists of tetratramers of catalytic subunits attached to a 20-kDa non-catalytic subunit embedded in the plasma membrane. The AChE forms that are not associated with structural subunits can be divided into two classes: those composed of hydrophilic subunits modified with a glycosphospholipid that attaches the enzyme to the cell membrane, and those composed of water-soluble secreted oligomers of hydrophilic catalytic subunits not associated with any cellular component. Butyrylcholinesterase (BuChE), a closely related cholinesterase (ChE) differing from AChE in acyl group selectivity, shares many of the structural features of AChE (Chatonnet and Lockridge, 1989).

Precursor-product relationship experiments indicate that the complex ChE forms are assembled in the cell from monomeric catalytic subunits (Brockman et al., 1986; Lazar et al., 1984). Active monomers were shown to constitute a major fraction of the intracellular AChE pool within various cells and tissues whereas the ectoenzyme, the enzyme exposed on the cell surface or secreted into the extracellular milieu, appears generally in multimeric forms (Taylor et al., 1981; Younkin et al., 1982; Lucas and Kreutzberg, 1985; Ralston et al., 1985; Atack et al., 1987; Koelle et al., 1987; Steiger et al., 1989). Free monomers of AChE and BuChE were identified in the circulation and in the supernatant of certain cultured cells (Atack et al., 1987; Lazar and Vigny, 1980), yet one cannot rule out the possibility that these were generated by extracellular disassembly of the complex oligomeric forms (Rotundo and Fambrough, 1979; Vigny et al., 1979; Allmand et al., 1981; Lockridge and La Du, 1982).

Correct tertiary and quaternary structure is required to make most membrane glycoproteins and many soluble secretory proteins competent for transport out of the endoplasmic reticulum (reviewed by Hurtley and Helenius, 1989). The pathway for assembly and export of AChE has not been fully defined as yet; globular dimers and tetratramers were shown to assemble in the rough endoplasmic reticulum, while the collagen-tailed forms are probably assembled in the Golgi apparatus (Rotundo, 1984). It is not clear, however, whether such an assembly is required for efficient export from the cell. Studies on the generation of multimeric ChE forms have been hindered by the complexity of the components involved. Cells can generate, through alternative splicing of one gene transcript, three species of catalytic subunits (Schumacher et al., 1988; Sikarov et al., 1988). These subunits can then possibly form, via different pathways, an array of complex, intracellular, surface-bound and secreted molecular forms. To allow dissection of these processes, we have recently established a recombinant expression system in which only one single species of HuAChE catalytic subunit is expressed. Human 293 kidney cells, transfected with a very efficient expression vector, carrying the cDNA sequences of the hydrophilic AChE polypeptide (Soreq et al., 1990), were found to secrete the...
soluble, oligomeric form of the human enzyme (Velan et al., 1991). This system may be very effective in studying HuAChE structure/function relationship by analyzing the effect of specific mutations on synthesis, activity, and secretion of AChE.

Disulfide bonds play a major role in the assembly of the various ChE forms, either by linking identical catalytic subunits or by binding the catalytic subunits to the structural subunits. Catalytic subunits contain 6 cysteine residues which are conserved in all cholinesterase characterized to date (Schumacher et al., 1986; Lockridge et al., 1987a; Prud'homme et al., 1987; Sikarov et al., 1987; Doctor et al., 1990; Rachinsky et al., 1990). These cysteines are involved in intrasubunit disulfide bonds as demonstrated for Torpedo AChE (MacPhee-Quigley et al., 1986) and for serum human BuChE (Lockridge et al., 1987b). A seventh cysteine, at the carboxyl terminus of the polypeptide, is conserved in hydrophilic cholinesterase catalytic subunits, but is absent in the Drosophila enzyme (Holl and Spierer, 1986). This amino acid was implicated in formation of interunit bonds by partial proteolysis and modification studies (Lockridge et al., 1979; Lockridge and La Du, 1982) and more recently by chemical sequencing (Roberts et al., 1991).

In this study we analyze the role of interunits disulfide bond formation in assembly of HuAChE oligomers. We employ site-directed mutagenesis to replace the COOH terminus proximal cysteine of the HuAChE catalytic subunit (amino acid position 580 in the human enzyme) by alanine. The mutagenized Cys580 → Ala subunit was expressed in two different human cell lines, and the effect of the modification on the activity, the molecular configuration, and the subcellular distribution of the enzyme was examined.

MATERIALS AND METHODS

Recombinant DNA Techniques—Production of plasmids, isolation of DNA fragments, cloning, and bacterial transformation were performed essentially as described in Current Protocols in Molecular Biology (Ausubel et al., 1987). Synthetic DNA oligodeoxynucleotides (~60 nucleotides long) were prepared using the automated Applied Biosystems DNA synthesizer, and then assembled by ligation to generate the full-length DNA fragment as described (Shafferman et al., 1987; Rachinsky et al., 1990). Sequence of the synthetic DNA was verified, after cloning, by the dideoxy sequencing method (US Sequenase kit).

Transfection and Selection of AChE Producing Cells—CaCl2 purified plasmids pL5CAN and pEmA580n were used to transfect 293 cells (transformed human embryonal kidney cells, ATCC CRL1573) by the calcium phosphate method (Wigler et al., 1977). Selection of stably transfected cells was achieved by exposing the cells, 48 h post transfection, to 0.8 mg/ml G418 in Dulbecco's modified Eagle's medium + 10% heat-inactivated fetal calf serum for 20 days. Transfection with 20 μg of plasmid DNA/100-mm culture plate, resulted in formation of 50–100 G418-resistant colonies. Pools of colonies were formed by trypsinizing the cells and allowing them to resettle in the original tissue culture dish. Upon reaching confluence, cell growth medium was substituted to Iscove's modified Dulbecco's medium + 2% Ultroser-G (GIBCO). Homogeneous cell lines expressing high levels of Aβ80 mutagenized AChE, were cloned from the colony pool by limiting dilution to 96-well microtiter plates. One such cell line, designated EmA580-D9, was used in some of the comparative experiments. Transfection of the human neuroblastoma cell line SK-N-SH (ATCC HTB11) was carried out using the Lipofectin Reagent (Becton Dickinson Research Laboratories) as recommended by the manufacturer. Selection for G418-resistant colonies was performed as described for 293 cells, except that only 0.5 mg/ml G418 was used.

Determination of AChE Activity—Activity of secreted AChE was determined 1–3 days post medium change to Iscove's modified Dulbecco's medium + 2% Ultroser-G, a medium containing a serum substitute, low in endogenous ChE activity. Cell-associated activity was determined after disruption of cells by freeze thawing (Velan et al., 1991) in 20 mM potassium phosphate buffer, pH 7.4, containing the protease inhibitors bacitracin (1 mg/ml), benzamidine (1 mM), and aprotinin (5% (v/v) of Sigma preparation). The soluble fraction was obtained by removal of cell debris by Eppendorf centrifugation. Bound enzyme was extracted with 0.5% Triton X-100 and 1 M NaCl. AChE activity was assayed according to Ellman et al. (1961). The reaction mixture contained 0.5 mM acetylthiocholine, 50 mM Na-phosphate buffer, pH 6.0, 0.1 mg/ml bovine serum albumin, and 0.3 mM 5,5'-dithiobis(2-nitrobenzoic acid). The assay was performed at 25 °C monitored by the absorbance monitored by the thermomax microplate reader (Spectronic Instruments). One unit is defined as the amount of enzyme hydrolyzing 1 μmol of acetylthiocholine/min. For activity titration, the mutagenized enzyme (0.6 unit/ml) was preincubated at 25 °C for 2.5 h, with varying concentrations of one of the cholinesterase inhibitors meperidine hydrochloride, and for varying durations of one of the cholinesterase inhibitors meperidine hydrochloride.

Sucrose Density Gradient Centrifugation—Analytical sucrose gradient centrifugation was performed on linear 5–25% sucrose gradients containing 50 mM Na-phosphate buffer, pH 8.0, or in presence of 1% Triton X-100, using SW41-Ti rotor (Beckman) for 22 h at 36,000 rpm, at 4 °C. 0.3-ml fractions were collected and assayed for AChE activity.

Preparation of Antibodies against HuAChE—Sequence-directed antibodies were prepared against a HuAChE segment fused to β-galactosidase. The fusion polypeptide was generated in Escherichia coli using a trp driven expression system, as described (Shafferman et al., 1990). A 291-base pair long DNA fragment, spanning codons 228–325 of HuAChE cDNA (Soreq et al., 1990; Rachinsky et al., 1990), was isolated following PstI digestion. This fragment was inserted into the PstI site of plasmid pTOZ (Leitner et al., 1991), in-frame with the coding sequence of β-galactosidase, to generate plasmid pAC2GAL. Expression of the recombinant hybrid polypeptide, designated AC2β-galactosidase, in E. coli strain MI1060 was induced by tryptophan starvation, and the polypeptide was extracted from cells and purified as described earlier (Grosfeld et al., 1989).

Three doses of purified AC2β-galactosidase were injected intramuscularly (100 μg/injection) to rabbits at biweekly intervals. First two injections contained complete Freund's adjuvant, while the last injection was with incomplete Freund's adjuvant. Sera were collected 4 weeks post last injection; anti-AChE titers, determined by enzyme-linked immunosorbent assay were 1:2,000–1:40,000.

Western Blots—Secreted, as well as cellular proteins from transfected cells were separated by 10% SDS-polyacrylamide gels. Samples were then electrophoresed onto nitrocellulose at 200 mA for 16 h in a 20 mm Tris, 150 mM glycine, pH 8.3, buffer containing 20% methanol. Prestained protein standards (Bio-Rad) were used as size markers. The nitrocellulose paper containing the samples was incubated 1 h at ambient temperature with either made of 0.1 M Tris-HCl, pH 7.4, 0.15 M NaCl, 10% bovine serum albumin (Fraction V, Sigma) or 0.2% Nonidet P-40. The paper was then washed three times with Tris-saline-Niobetid P-40-SDS. Second antibody binding was performed as described above using a 1:400 dilution of goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma). Following washing, paper was developed with Fast Blue RR salt staining (Sigma). In blots containing the cellular extracts, 2% Caudbury's low fat dried milk was used to replace bovine serum albumin and Ultroser-G in the blocking and immunoreaction buffers.

RESULTS AND DISCUSSION

Expression of the Human Acetylcholinesterase Mutant Aβ80-AChE in Human Kidney and Neuroblastoma Cell Lines—Construction of the expression vector for the mutant Aβ80-AChE is depicted in Fig. 1. A 187-base pair long synthetic DNA duplex carrying codon GCC(Ala) instead of codon TGG(Cys) at position 580 was introduced into the plasmid vector pL5CA (Velan et al., 1991), to replace the native HuAChE cDNA sequence between the unique restriction sites BssHII and SalI. The mutagenized HuAChE cDNA was then used to replace the HuAChE sequence within the expression

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vector pLS5C generating the expression vector pEmA580n (Fig. 1). pLS5C and pEmA580n drive the synthesis of wild type and mutated catalytic subunits, respectively, via the cytomegalovirus immediate-early promoter-enhancer (Focek and Hofstetter, 1986).

Both vectors carry the neo selection marker under the control of the mouse major histocompatibility antigen H2Ld' promoter-enhancer. The murine H2Ld' promoter was shown to function weakly within rodent cells (Vogel et al., 1986) and to be repressed in 293 cells (Lewis and Manley, 1985). We reasoned that expression of the neo under the weak (and repressed) promoter will confer G418 resistance only upon cells which have integrated the plasmid into sites of active transcription within the chromosome. This will allow high levels of transcription of the neo gene as well as of the proximal ache cDNA. The success of this approach for expression of high levels of rHuAChE (5-25 pg/cell) with various HuAChE-linked promoters will be described elsewhere. The H2Ld'-neo cassette was generated by ligation of the XbaI-BamHI fragment from pLd (Evans et al., 1983) to a BglII-BamHI fragment encompassing the neo gene and the downstream SV40 polyA signal from a derivative of pSV2NEO (Southern and Berg, 1982).

Plasmids pLS5C and pEmA580n were used to transfect the human embryonal kidney cell line 293. Stably transfected cells were selected by exposure to G418 colony pools, stemming from about 100 colonies, were formed as described under "Materials and Methods." AChE activity secreted into the growth medium from pLS5C- and pEmA580n-transfected cell pools was determined. Similar amounts of enzymatic activity were secreted by the two pools, 1.5-2 units/10^6 cells/24 h from the pLS5C-transfected cells and 2-2.5 units/10^6 cells/24 h from the pEmA580n-transfected cells. The ability of recombinant 293 cells to express efficiently the mutated A580-AChE was further substantiated by studies with a cloned cell line selected from the pEmA580n-transfected cell pool. Such an isolated clone was found to produce AChE at levels of 20 units/10^6 cells/24 h. This value is within the range of AChE activities recovered from isolated clones expressing the wild type HuAChE catalytic subunit. It should be noted that in most experiments described below we have used pools of transfected colonies rather than isolated clones. This was done to avoid misinterpretations originating from variations between individual clones.

293 cells were specifically chosen for manipulation of recombinant products due to their potential for high expression and efficient secretion (Alwine, 1985). As these cells are neither cholinergic nor of neuronal origin, studies were extended to the human neuroblastoma cell line SK-N-SH (Biedler et al., 1973). These neuroblastoma cells, which are devoid of detectable endogenous AChE activity, were transfected by...
the plasmids pL5CAN and pEmA580n, stable colony pools were formed, and cell supernatants were analyzed for secreted AChE activities. Results summarized in Table I indicate that the expression efficiency of rHuAChE in SK-N-SH cells is 10-fold lower than in 293 cells, but like the 293 cells, SK-N-SH cells secrete the native and the mutagenized enzyme forms at comparable quantities. Experiments described below were performed with enzyme produced by 293 cells, yet all major observations were confirmed in SK-N-SH cells as well.

The observation that two different cell lines produce and secrete an active A580-AChE mutant provides the first indication that substitution of cysteine 580 by alanine does not hamper the catalytic activity nor the synthesis and secretion of the AChE subunit.

Substitution of Cys-580 Abolishes Dimerization of HuAChE Subunits — The molecular configurations of secreted native and A580-mutant recombinant enzymes were compared by sucrose density gradient and SDS-polyacrylamide gel electrophoresis analyses. The sucrose gradient profile of A580-AChE is distinctly different from that of the native enzyme carrying cysteine at position 580 (Fig. 2). The activity of the wild type enzyme segregates, upon centrifugation, into two major peaks sedimenting at 6 S and 11 S (Fig. 2A) corresponding to the soluble globular dimers and tetramers of mammalian ChEs. These were accompanied by a smaller 4 S peak corresponding to the monomeric form of rHuAChE (Velan et al., 1991). In contrast, the A580-HuAChE activity resides, almost exclusively, in the 4 S peak fraction (>95% of activity). The 6 S peak, corresponding to the subunit dimer, is practically absent in medium of cells expressing A580-AChE. We could detect, however, a minor peak at the 11 S region (Fig. 2B). These results suggest that substitution of cysteine at position 580 indeed affects the ability of two AChE catalytic subunits to associate into the dimeric form. The existence of the minor 11 S peak, which cosediments with the tetrameric form of the native enzyme is intriguing and will be discussed later.

Involvement of Cys-580 in oligomerization of AChE subunits was also demonstrated by SDS-polyacrylamide gel electrophoresis analysis at non-reducing conditions. When AChE is visualized by blotting and immunostaining (see "Materials and Methods"), the migration profiles of the native and mutated enzymes are different (Fig. 3). The secreted native AChE migrates, at non-reducing conditions, as a band of 130 kDa, lacks the 130-kDa band, and is characterized by two bands with apparent molecular mass of 67 and 70 kDa

| Table I | Secreted and cellular AChE activity in recombinant cells producing native or A580-mutated HuAChE catalytic subunits |
|---------|----------------------------------------------------------------------------------------------------------|
| AChE activity | SK-N-SH cells |
| 293 cells | pEmA580n transfected | pEmA580n SK-N-SH cells | pL5CAN transfected | pL5CAN SK-N-SH cells |
| Secreted | 5500 ± 610 | 4800 ± 550 | 470 ± 55 | 500 ± 45 |
| Cellular LS soluble | 320 ± 30 | 230 ± 25 | 20 ± 10 | 50 ± 25 |
| Cellular HSD soluble | 220 ± 30 | 230 ± 15 | 30 ± 5 | 40 ± 15 |

Fig. 2. Comparative sucrose gradient centrifugation analysis of native AChE (A) and A580-AChE (B) secreted by recombinant 293 cells. Cell growth supernatants of 293 cells transfected by pL5CAN (A) or pEmA580n (B) were loaded (0.2 ml) on 5–25% sucrose gradients in 50 mM Na-phosphate buffer, pH 8.0. Supernatants were collected 24-h post medium change; enzymatic activity was 1 unit/ml in both supernatants. Arrows mark the following sedimentation markers from left to right: β-galactosidase (16 S), bovine intestinal alkaline phosphatase (6 S), and bovine serum albumin (4 S). Activity in individual fractions is expressed as percent of total recovered activity.

Fig. 3. Western blot of AChE secreted into cell growth medium by recombinant 293 producing native and A-580 AChE. Aliquots of cell supernatant (30 μl) in Ultrorser-G-based medium were boiled in sample loading buffer (Laemmli, 1970) containing 1% SDS but no β-mercaptoethanol. After electrophoresis, gels were blotted and immunostained with anti HuAChE antibodies as described under "Materials and Methods." Migration of the prestained molecular weight markers (Bio-Rad) phosphorylase B (110 kDa), BSA (84 kDa), ovalbumin (47 kDa), and carbonic anhydrase (35 kDa) is marked by arrows. Lanes contain the following samples: 1, supernatant of pooled colonies producing A580-AChE (~1.5 unit/ml); 2, supernatant of non-transfected 293 cells; 3, supernatant of pooled colonies producing native AChE (~1.5 unit/ml); 4, supernatant of clone 10–3C2-D8, a 293 transfected producing high levels (~30 unit/ml) of native AChE. by two bands with apparent molecular mass of 67 and 70 kDa (a third faint 62-kDa band represents cross-reacting material present in the 293 growth medium as it appears also in the
control lane containing nontransfected cell supernatant, lane 2). These two bands correspond to glycosylation variants of the rHuAChE subunit polypeptide, and comigrate with the bands of the monomeric fraction in the native HuAChE (compare lanes f and d in Fig. 3). It should be noted that the monomeric form is present in low abundance in native preparations (Fig. 2A) and can be identified by SDS-polyacrylamide gel electrophoresis (Fig. 3, lane d) only in supernatants of high producer clones selected from the recombinant 293 cell pool.

The small fraction of monomeric forms found in the native AChE preparations (Fig. 2A) could originate from direct secretion of monomers into cell supernatant. Alternatively, monomers could represent protease-mediated disassembly products of the oligomeric molecules. Generation of monomers by COOH-terminal proteolysis of complex forms was reported for various ChEs (Vigny et al., 1979; Allmand et al., 1981) and may occur in 293 cell supernatants as well. When AChE-containing supernatants, collected from transfected 293 cells, were incubated over monolayers of non-transfected cells, we observed a gradual decrease in the dimeric AChE peak and a concomitant increase in the monomer peak (data not shown). Within 72 h all the dimers were converted to monomers suggesting that naive 293 cells secrete into the medium a protease responsible for this transition.

Gradient centrifugation analyses of native and mutated AChE in SK-N-SH supernatants yielded similar results to those described for 293 cells, although the 4 S activity peak was not observed in the supernatants of cells secreting native enzyme. Dimers were never observed in A580-AChE preparations, corroborating the involvement of the cysteine moiety in this position in formation of interchain disulfide bridges. A cysteine residue located at an analogous position was implicated in interchain disulfide bond formation in HuBuChE and bovine AChE by alkylation analysis and chemical sequencing (Lockridge and La Du, 1982; Roberts et al., 1991). Yet, the site-directed mutagenesis experiment of HuAChE described here allows the direct identification of this ChE conserved cysteine as responsible for subunit dimerization. It is believed that this same cysteine is involved not only in catalytic subunit dimerization but also in binding of the catalytic subunits to structural components of cell surface-associated forms (MacPhee-Quigley et al., 1986). To prove this unequivocally, a cell system where the A580-AChE is coexpressed with a structural subunit should be devised.

The nature of the fast sedimenting activity in supernatant of A580-AChE-producing cells is unclear. This peak could represent tetramers generated directly from mutant monomers without prior dimerization. Alternatively, this peak may represent complexes between catalytic monomers and unrelated proteins. Hybrid molecules of this type, generated by attachment of BuChE monomers and serum-derived components were implicated in the molecular heterogeneity of HuBuChE (Scott and Powers, 1974; Masson, 1989). We have noted that the absolute amount of the A580-AChE fast sedimenting material was constant in all preparations examined, regardless of the total amount of AChE secreted by the cell. This may suggest the involvement of an additional factor, present in the medium at limiting concentration, in the formation of this molecular form. Additional evidence to the peculiar character of this form resides in its time dependent inactivation curve described below.

The A580-AChE Monomer Resembles the Native Enzyme in Catalytic Properties—Catalytic activity of the rHuAChE was not impaired by preventing oligomerization of subunits (Table 1). This result was expected from a large body of data gathered on activity of ChEs: a, catalytic differences between diverse structural forms originating from the same species have not been recorded (Rotundo and Fambrough, 1979), b, the interchain bond by itself is not required for activity, and c, limited proteolysis of various ChE yielded active monomers (Vigny et al., 1979; Allmand et al., 1981; Lockridge and La Du, 1982).

The recombinant expression system described here provides a source for a homogenous intact monomeric HuAChE preparation that can be directly analyzed for its catalytic properties. Moreover, this system enables comparison of AChE monomers and oligomers of identical and defined genetic origin differing only by a single amino acid. Various enzymatic analyses of the Human A580-AChE mutant monomer are depicted in Fig. 4. The $K_m$ for acetylthiocholine was deduced from a Lineweaver-Burk plot (Fig. 4A), and the apparent first order rate constant for catalysis ($k_{cat}$) was calculated by active site titrations (Fig. 4B) with the irreversible inhibitors MEFP and Soman. The values obtained, $K_m$ of 125 $\mu M$ and $k_{cat}$ of $4.2 \times 10^7$ $1/min$ are essentially identical to those of native recombinant HuAChE (117 $\mu M$ and $3.9 \times 10^7$ $1/min$). The results were confirmed with sucrose gradient purified molecular forms as well; the monomeric mutant did not differ from native dimers and tetramers in affinity to substrate (acetylthiocholine) and in rate of catalysis. Other features of the enzymatic reaction such as pH dependence and temperature dependence (activity ratio at 27/39°C) were compared and found not to be different in the mutant monomer (data not shown). The inhibitory effect of high substrate concentra-

![Fig. 4. Kinetic parameters of the A-580 mutant monomer of rHuAChE. A, double reciprocal Lineweaver-Burk plot; B, active site titration with MEFP and Soman; C, substrate inhibition profile $K_m$ for acetylthiocholine was calculated from the Lineweaver-Burk plot. Inhibition by increasing amounts of acetylthiocholine was evaluated as described previously (Velan et al., 1991). $k_{cat}$ was calculated from the active site titration curve: A580-AChE at a concentration of 0.59 unit/ml (hydrolyzing 0.59 $\mu M$ of acetylthiocholine/min) was titrated with various concentrations of MEFP (○) or Soman (○). The intercepts for zero activity were determined by extrapolation. Active site concentration was found to be $1.4 \times 10^{-12}$ $mol/ml$ for MEFP titration and $2.8 \times 10^{-12}$ $mol/ml$ for Soman titration (note that the latter value should be divided in two since only one of the autonomic forms of Soman is an active inhibitor).](image-url)
tions, characteristic to AChEs, was retained by the A580 mutation (Fig. 4C and Velan et al., 1991), confirming previous studies on monomers of bovine AChE (Vigny et al., 1979).

As noted for ChEs from other sources (Edwards and Brimijoin, 1983; Caut et al., 1987), oligomerization of HuAChE subunits was found to have a significant effect on the thermostability of the enzyme (Fig. 5). The time-dependent loss of activity of the monomeric mutant at 55 °C can be described by a monophasic exponential function from which a half-life of 2 min is deduced. In contrast, the time-dependent inactivation of the sucrose gradient-purified native tetramer is biphasic, with calculated half-life components of 10 and 35 mins (the native sucrose gradient purified dimers behave very much like the purified tetramers, data not shown). It is worthwhile noting that the inactivation of the fast sedimenting (11 S) A580-AChE peak (Fig. 2B) did not follow the pattern of the native 11 S tetramer inactivation; the curve was found to be monophasic with a calculated half-life of about 15 min.

Blocking of Subunit Assembly Does Not Lead to Accumulation of AChE in the Cell—The possible effect of an assembly block on efficiency of AChE synthesis and export was assessed by comparing cellular enzyme levels in recombinant cells producing mutant A580-AChE and native C580-AChE. AChE contents in cell extracts was monitored by enzymatic assays as well as by immunological assays which are not dependent on activity.

Homologous cultures of 293 cells secreting comparable amounts of C580-AChE and A580-AChE were found to contain similar levels of cell-associated enzymatic activity (Table I). The distribution of activity in the soluble fraction and the membrane-bound fraction were also similar in both cultures. As expected from previous studies (Velan et al., 1991), total cellular activity in the two cultures was very low in comparison to the activity secreted into the medium. Essentially the same observations could be made by comparing recombinant cultures of SK-N-SH cells producing the native and the mutant AChE (Table I). Total cellular antigenic mass of HuAChE within the recombinant cells was assessed by Western blotting of SDS/β-mercaptoethanol-treated recombinant 293 cell extracts. Immunostaining was performed with anti-HuAChE antibodies directed to nonconformational linear epitopes on HuAChE. The two characteristic glycosylation forms of the AChE subunit polypeptide were identified in the extracts of cells producing native and mutant AChE (Fig. 6, compare lanes 2 and 3 to the background in lane 1). Although accurate quantitation is difficult, the relative intensity of the AChE bands in both extracts was found to be very close (Fig. 6), indicating again that the A580 mutation does not affect synthesis levels.

The ratio of enzymatic activity to steady state level of rHuAChE, as estimated by immunostaining, seems to be identical in cell-associated and secreted fractions. Pools of inactive intracellular AChE polypeptides were, however, revealed in avian myotubules and in Torpedo electric organ (Stieger et al., 1987; Rotundo, 1988; Rotundo et al., 1989), and such a pool could be present in the recombinant 293 cells as well. Yet, if inactive forms exist in the transfectants, no indication to an increase in their amount due to Cys-580 substitution was found.

In many multisubunit membrane proteins, intracellular oligomerization is a prerequisite for transport out of the endoplasmic reticulum (Kreis and Lodish, 1988; Copeland et al., 1988; Lippincott-Schwartz et al., 1988). The structural requirements for export of secretory proteins seem to be less stringent. Some secretory proteins such as β-2-microglobulin and immunoglobulin light chain can be secreted as monomers,
forms could never be detected in cell extracts of A580-AChE producers but were revealed as a very faint band of 130 kDa in extracts of native enzyme producers when these were overloaded on the gel (Fig. 6, lane 7).

Sucrose gradient analyses of the cell-associated AChE yielded similar results. Buffer-soluble as well as detergent-soluble extracts (both extractions were carried out in the presence of an anti protease mixture) from cells producing the A580 mutant enzyme, exhibited exclusively the monomeric form (Fig. 7, C and D). Buffer-soluble extracts from recombinant 293 cells producing native AChE are characterized by a major peak of the monomeric form, accompanied by much smaller peaks of the dimeric and tetrameric AChE forms (Fig. 7A). The detergent-soluble fraction from these same cells contain only the monomeric form (Fig. 7B).

It appears from these findings that, independent of the presence of cysteine at position 580, the predominant intracellular molecular form in transfected 293 cells is a monomer. This observation is intriguing, considering the fact that most of the native recombinant enzyme secreted from 293 cells is recovered in the medium as dimers and tetramers (Fig. 2A). Accurate kinetic and cytochemical studies will be required, therefore, to determine at what rate and in which cellular compartments are monomers converted into the oligomeric forms. It should be noted that in other cell systems (Lazar et al., 1984; Rotundo, 1988) most of the AChE monomers synthesized in the cell are not destined for assembly but are rapidly turned over presumably through intracellular degradation. One could speculate that also in 293 cells the large pool of monomers is rapidly degraded and that only the dimers and tetramers recovered in the soluble cellular extract (Fig. 7A) are destined for secretion. The effective secretion of A580 mutant AChE proves, nevertheless, that this oligomerization is not a prerequisite for export and that monomers synthesized in the cell can be secreted as such.

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whereas other polypeptides such as fibrinogen, luteinizing hormone β-chain, and platelet-derived growth factor A and B chains may need to acquire the appropriate quaternary structure before efficient release from the endoplasmic reticulum. (For review see Hurtley and Helenius, 1989.) The establishment of recombinant clones of 293 cells and SK-N-SH cells that secrete catalytic subunits blocked in dimerization capability, clearly indicates that HuAChE, or at least its hydrophilic form, does not need to acquire an oligomeric form in order to be released from the cell. This is further substantiated by experiments where cell-associated activity was monitored; interference with dimer formation does not lead to an increase in intracellular retention of enzyme molecules or to staggering of secretion. Moreover, the A580 mutation does not seem to elicit formation of any intracellular degradation products detectable by Western blotting.

Monomers Are the Major Component of the Cellular AChE Pool in Recombinant Cells Producing Either A580 Mutant or Native Enzyme—The molecular configuration of cell-associated AChE in recombinant 293 cells was analyzed by Western blotting of non-reduced cell extracts (Fig. 6, lanes 4–6). Both extracts were found to contain the monomeric bands. Dimeric
