Effect of human acetylcholinesterase subunit assembly on its circulatory residence

Theodor CHITLARU, Chanoch KRONMAN, Baruch VELAN and Avigdor SHAFFERMAN[1]

Department of Biochemistry and Molecular Genetics, Israel Institute for Biological Research, Ness-Ziona 74100, Israel

Sialylated recombinant human acetylcholinesterase (rHuAChE), produced by stably transfected cells, is composed of a mixed population of monomers, dimers and tetramers and manifests a time-dependent circulatory enrichment of the higher-order oligomeric forms. To investigate this phenomenon further, homogeneous preparations of rHuAChE differing in their oligomerization statuses were generated: (1) monomers, represented by the oligomerization-impaired C580A-rHuAChE mutant, (2) wild-type (WT) dimers and (3) tetramers of WT-rHuAChE generated *in vitro* by complexation with a synthetic ColQ-derived proline-rich attachment domain ('PRAD') peptide. Three different series of each of these three oligoform preparations were produced: (1) partly sialylated, derived from HEK-293 cells; (2) fully sialylated, derived from engineered HEK-293 cells expressing high levels of sialyltransferase; and (3) desialylated, after treatment with sialidase to remove sialic acid termini quantitatively. The oligosaccharides associated with each of the various preparations were extensively analysed by matrix-assisted laser desorption ionization–time-of-flight MS. With the enzyme preparations comprising the fully sialylated series, a clear linear relationship between oligomerization and circulatory mean residence time (MRT) was observed. Thus monomers, dimers and tetramers exhibited MRTs of 110, 195 and 740 min respectively. As the level of sialylation decreased, this differential behaviour became less pronounced; eventually, after desialylation all oligoforms had the same MRT (5 min). These observations suggest that multiple removal systems contribute to the elimination of AChE from the circulation. Here we also demonstrate that by the combined modulation of sialylation and tetramerization it is possible to generate a rHuAChE displaying a circulatory residence exceeding that of all other known forms of native or recombinant human AChE.

Key words: MALDI–TOF-MS, N-glycosylation, oligomerization, pharmacokinetics.

INTRODUCTION

The propensity of some proteins to be retained for extended periods in the circulation, whereas others are rapidly directed towards clearance pathways, has major consequences for biological functions such as immunological responses and endocrine regulation, as well as for the generation of therapeutically efficient biomolecules. Among the latter, special attention has been focused in recent years on the enzymes acetylcholinesterase (AChE) and butyrylcholinesterase, whose therapeutic potential requires their long-term retention within the circulation [1–6].

The elimination of circulating proteins is an intricate process involving a variety of removal mechanisms. Specific characteristics of the circulating protein, including protein size, surface charge, hydrophobicity and the presence of specific amino acid and carbohydrate epitopes, might be decisive in determining its circulatory longevity. In addition to clearance systems, which are based on protein elimination via kidney glomerular filtration or protease degradation, different receptor-based systems that recognize and bind to protein-related epitopes are responsible for the active removal of the protein from the bloodstream [7,8].

In the case of AChE, the contribution of appended carbohydrates to circulatory residence was demonstrated by the finding that bacterially generated recombinant AChE, as well as N-glycanase-treated cholinesterases (ChEs) of animal cell origin, both being devoid of N-glycans, are cleared rapidly from the circulation of experimental animals [4,9]. However, the pharmacokinetic profiles of an array of mutated recombinant human acetylcholinesterase (rHuAChE) forms differing in their numbers of N-glycosylation sites [4] suggested that, although N-glycosylation in itself does have a role in determining circulatory residence, the structural features of the N-glycans have a decisive role in the circulatory retention of ChEs. In particular, we have shown that the capping of N-glycan β-galactose residues with sialic acid is required for the long-term retention of the protein. We also demonstrated that an inverse linear relationship exists between the number of exposed β-galactose residues in association with a given form of rHuAChE and its circulatory lifetime. In accordance with these findings, we demonstrated [6] that circulatory residence can be extended by generating rHuAChE or recombinant bovine acetylcholinesterase (recombinant BoAChE) in a modified cell line that expresses high levels of recombinant 2,6-sialyltransferase and thus allows improved sialylation of the co-expressed recombinant AChE products. However, we noted that although the enzyme produced in the sialyltransferase-modified cells displays increased retention, it is still removed from the circulation more rapidly than native serum-derived ChEs. The inability of the modified enzyme to be retained in the circulation over long periods in a manner comparable with that of native serum-derived ChEs might be an outcome of specific sugar-related epitopes or other post-translation factors. Specifically, the more rapid clearance of recombinant ChEs might result from the fact that recombinant ChEs produced in HEK-293 cells as well as in other cell systems

Abbreviations used: 2-AB, 2-aminobenzamide; AChE, acetylcholinesterase; BoAChE, bovine acetylcholinesterase; ChE, cholinesterase; HuAChE, human acetylcholinesterase; MALDI–TOF, matrix-assisted laser desorption ionization–time-of-flight; MRT, mean residence time; PRAD, proline-rich attachment domain; rHuAChE, recombinant human acetylcholinesterase; WT, wild-type.

[1] To whom correspondence should be addressed (e-mail avigdor@iibr.gov.il).

characteristically display a state of subunit assembly differing from that of circulatory long-lived native serum-derived enzyme. Whereas the recombinant enzyme is mostly in dimeric and monomeric forms, the native serum-derived enzyme is almost exclusively assembled into tetramers. The failure of recombinant ChEs to assemble efficiently into tetramers might accelerate their removal from the circulation. This was indeed recently shown to be true for BoAChE [10]. However, unlike the bovine enzyme, natural human tetrameric circulating AChEs are not known to exist; it is not yet clear whether the assembly of the human enzyme subunits into tetrameric forms confers any pharmacokinetic advantage on this enzyme.

In the present study we first employed refined matrix-assisted laser desorption ionization–time-of-flight MS (MALDI–TOF-MS) analyses to allow an accurate determination of the glycan structures of rHuAChE produced by HEK-293 and sialyltransferase overexpressor 293ST-2D6 cell lines, and quantitatively assessed the contribution of N-glycan terminal sialylation to the elimination of rHuAChE from the circulation. In addition, by using rHuAChE mutants that fail to form intersubunit disulphide bridges, or by inducing *in vitro* tetrameric assembly by the ColQ-derived proline-rich attachment domain (PRAD) [10–13], as well as by employing highly enriched dimeric enzyme preparations, we conducted a comparative pharmacokinetic analysis of an array of uniformly assembled rHuAChEs. These various oligomeric preparations, which differed one from another in their enzyme subunit organization, were prepared in different cellular backgrounds to probe in addition the combined effect of sialylation and subunit assembly on clearance. Thus we here determine a rank order for monomeric, dimeric and tetrameric rHuAChEs, which are highly sialylated, partially sialylated or totally non-sialylated, with regard to their ability to reside in the circulation. Finally, we demonstrate that by combining efficient sialylation with the induction of high-order oligomerization, it is possible to confer unprecedented circulatory residence abilities on the rHuAChE.

EXPERIMENTAL

Cell culture

The generation of HEK-293 cell lines stably expressing high levels of rHuAChE [14] was described previously [15]. The generation of highly sialylated rHuAChE [wild-type (WT) and C580A] *in vivo* was achieved by expressing the WT or mutated human acetylcholinesterase (HuAChE) genes in genetically modified 293ST-2D6 cells that stably expressed a recombinant rat Golgi version of 2,6-sialyltransferase [6,10]. Both HEK-293 and 293ST-2D6 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % (w/v) FCS (fetal calf serum).

Enzyme production, purification and quantification

Recombinant AChE was produced on a large scale by HEK-293 or 293ST-2D6 cells (10^8–10^9 cells/litre) cultured in tissue culture multitrays [16]. After the establishment of the anchor-dependent cultures, the cells were fed with production medium (containing 10 % FBS depleted of AChE). Conditioned medium harvested from confluent stationary cell cultures consistently contained higher proportions of tetrameric AChE (tetramer between 10 % and 15 %; dimer between 70 % and 80 %; monomer between 5 % and 20 %; for example preparation 4 in Table 2) than that of young subconfluent cultures of producer cells (for example preparation 8 in Table 2), which are characterized by very high levels of dimers (more than 95 %) ([16,17], and A. Lazar, C. Kronman, B. Velan and A. Shafferman, unpublished work).

The purification of secreted rHuAChE was performed by affinity chromatography with Sepharose-bound procainamide, as described previously [4,15]. AChE activity was measured by the method of Ellman et al. [18]. Assays were performed in the presence of 0.5 mM acetylthiocholine/50 mM sodium phosphate buffer (pH 8.0)/0.1 mg/ml BSA/0.3 mM 5,5′-dithiobis-(2-nitrobenzoic acid). The assay was performed at 27 °C and monitored by a Thermomax microplate reader (Molecular Devices), as described previously [19].

Pharmacokinetics

Clearance experiments in mice (three to six ICR male mice per enzyme sample) and analyses of pharmacokinetic profiles were performed as described previously [4]. The study was approved by the local committee for animal experiments. Residual AChE activity in blood samples was measured and all values were corrected for background activity determined in blood samples withdrawn 1 h before performing the experiment. The clearance patterns of the various enzyme preparations were usually biphasic and fitted to a bi-exponential elimination pharmacokinetic model ($C_t = Ae^{-k\alpha t} + Be^{-k\beta t}$), as described previously [4]. This model permits the determination of the parameters A and B, which represent the fractions of the material removed from the circulation in the first (fast) and second (slow) elimination phases respectively, and $t_{1/2}\alpha$ and $t_{1/2}\beta$, which represent the circulatory half-life values of the enzyme in the fast and slow phases. In all cases, the correlation coefficient was greater than 0.997. The pharmacokinetic parameters mean residence time (MRT) (which reflects the average length of time for which the administered molecules are retained in the organism) and clearance (the proportionality factor relating the rate of substance elimination to its plasma concentration, equalling dose/area under the concentration–time curve) were obtained independently by analysing the clearance data in accordance with a non-compartmental pharmacokinetic model with the use of the WinNonlin program [20]. Elimination half-life values calculated by the non-compartmental analysis coincided with the $t_{1/2}\beta$ values obtained by the double-exponential elimination pharmacokinetic model.

Release, recovery, purification and labelling of N-glycans

N-glycans of purified enzyme preparations (approx. 100 μg of protein) were released by treatment with N-glycosidase F (Glyko, Novato, CA, U.S.A.) as described previously [15]. Deglycosylated protein was removed by precipitation with ethanol; glycans were recovered and purified from the supernatant as described by Kuster et al. [21]. To increase sensitivity [22,23], purified glycans were fluorescently labelled. Fluorescent labelling of purified glycans with 2-aminobenzamide (2-AB) was performed as described by Bigge et al. [24], with a commercial labelling kit (Glyko). During the 2 h labelling incubation, the temperature was kept at 55 °C to prevent heat-induced desialylation of the glycans.

Enzymic modification of N-glycans

Removal of sialic acid

Agarose-bound sialidase (0.04 unit; Sigma) was prewashed five times with water and incubated at room temperature for 16 h with 2-AB-labelled N-glycans released from 1.5–2.0 nmol of AChE. Sialidase was removed by Eppendorf centrifugation.

Desialylated N-glycans were dried under vacuum, resuspended in 30 μl of water and stored at −20 °C until use. Glycans prepared in this manner were subjected to MALDI–TOF-MS analysis or to further treatments with glycosidase followed by MALDI–TOF-MS analysis.

For the generation of non-sialylated rHuAChE preparations (C580A-HuAChE monomeric, WT enzyme and enzyme tetramerized *in vitro*) for pharmacokinetic study, a similar procedure was employed except that 100 nmol of AChE was incubated for 16 h with 1.2 unit of agarose-bound sialidase. The preparations of AChEs devoid of terminal sialic acid residues were extensively dialysed against PBS before administration to animals for the efficient removal of free sialic acid.

Removal of neutral monosaccharides

Desialylated 2-AB-labelled N-glycans obtained from 0.05–0.07 nmol of AChE (in 1 μl of water) were incubated for 24 h with 1 μl of bovine kidney fucosidase (1.3 units/ml; Glyko), β-galactosidase (5 units/ml; Glyko) or green coffee bean α-galactosidase (0.5 unit/ml; Sigma). Water was added to a final volume of 10 μl; samples were stored at −20 °C until analysed by MALDI–TOF-MS.

Esterification of sialic acids

To permit the concomitant measurement by MALDI–TOF-MS analysis of both neutral and acidic glycans, the carboxylate groups of sialylated 2-AB-labelled glycans were converted into their neutral methylated forms by esterification with methyl iodide, essentially as described by Kuster et al. [21]. We note that in this procedure the 2-AB moiety itself undergoes methylation and therefore both neutral and acidic glycans invariably display an increment in molecular mass of 14.015 Da in addition to the increase in mass size resulting from sialic acid methylation of acidic glycans. Esterified glycans were purified as described [21] and stored at −20 °C until MALDI–TOF-MS analysis.

MS

Mass spectra were acquired on a Micromass TofSpec 2E reflectron time-of-flight (TOF) mass spectrometer. 2-AB-labelled desialylated or 2-AB-labelled esterified glycan samples were mixed with an equal volume of freshly prepared 2,5-dihydroxybenzoic acid [10 mg/ml in 70 % (v/v) acetonitrile] and loaded on the mass spectrometer target. Routinely, 1 μl of glycan samples diluted 1:10 in water were subjected to analysis. Dried spots were recrystallized by adding 0.5 μl of ethanol and allowed to dry again. Neutral glycans were observed as $[M+Na]^+$ ions. Peptide mixture (1 μl) [renin substrate, corticotropin fragment 18–39 and angiotensin (10 pmol/μl), all from Sigma], which served as a three-point external calibrant for mass assignment of the ions, was mixed with freshly prepared α-cyano-4-hydroxycinnamic acid (10 mg/ml in 49.5 % acetonitrile/49.5 % ethanol/0.001 % trifluoroacetic acid), loaded on the mass spectrometer target and allowed to dry. All oligosaccharides were analysed at 20 kV with a single-stage reflectron in the positive-ion mode. Between 100 and 200 scans were averaged for each of the spectra shown.

PRAD peptide synthesis

The PRAD peptide CLLTPPPPPLFPPPFFRG was synthesized manually by fluoren-9-ylmethoxycarbonyl chemistry, as described previously [25]. The peptide was dialysed against 0.05 % trifluoroacetic acid for 48 h. Quality control of the peptide was performed by MALDI–TOF-MS; the concentration of the peptide was evaluated by its A_{215} after reverse-phase HPLC.

Tetramerization of rHuAChE *in vitro*

Previous studies by Giles et al. [13] demonstrated that rHuAChE can be tetramerized by a PRAD-derived peptide. The conditions used in the present study for the tetramerization of sialylated or non-sialylated rHuAChE were based on incubation of the affinity-chromatographically purified rHuAChE with the synthetic PRAD peptide for 12–16 h at room temperature in the presence of 5 mM phosphate buffer, pH 8.0. In analytical tetramerization experiments designed for sucrose-gradient analysis of tetramer formation, 0.06 nmol of rHuAChE (equivalent to 25 units) was mixed at different ratios with the PRAD peptide (AChE to PRAD mole ratios of 10:1, 4:1 and 1:4) in a final volume of 70 μl. Preparative tetramerization for the generation of milligram amounts of tetrameric rHuAChE for pharmacokinetic studies included 14.4 nmol (equivalent to 6000 units) of rHuAChE (mostly dimeric), which were incubated with 28.8 nmol of PRAD peptide in a final volume of 2 ml. Before administration to mice, rHuAChE tetramerized *in vitro* was dialysed extensively against PBS.

Sucrose-density-gradient centrifugation

Analytical sucrose-density-gradient centrifugation was performed on 5–25 % (w/v) sucrose gradients containing 0.1 M NaCl/50 mM sodium phosphate buffer (pH 8.0). Centrifugation was performed in an SW41 Ti rotor (Beckman) for 26 h at 160000 ***g***. Fractions of 0.2 ml were collected and assayed for AChE activity. Alkaline phosphatase, catalase and β-galactosidase were used as sedimentation markers. For determination of the rHuAChE assembly status in the circulation of mice at various time points after administration, 10 μl samples removed from five mice, each injected with 1000 units intravenously at zero time, were pooled and applied to sucrose gradients.

RESULTS AND DISCUSSION

Comparison of the N-glycans associated with rHuAChE produced by HEK-293 cells and sialyltransferase-modified HEK-293 cells

We found previously [4] that the terminal sialylation of the N-glycans appended to the ChE has a decisive role in determining the circulatory behaviour of ChEs. This result prompted us to generate the novel 293ST-2D6 cell line [6,10] engineered to overexpress a heterologous Golgi-associated α-2,6-sialyltransferase gene [26] that acts upon co-transfected glycoproteins and generates highly sialylated glycoforms [10]. An examination of rHuAChE produced by HEK-293 cells and sialyltransferase-modified HEK-293 cells by high-pH anion-exchange chromatography pulsed amperometric detection revealed that in the latter case the glycans were sialylated to a greater extent [6]. However, this method permitted neither an accurate quantitative evaluation of the sialylation extent nor an elucidation of the precise glycan structures associated with the rHuAChE product. In the present study we deciphered the entire spectrum of N-glycan forms associated with rHuAChE produced from the different cell lines by employing refined MALDI–TOF-MS techniques. We recently used this technique successfully to analyse the N-glycan structures of BoAChE [10].

To permit the visualization by MALDI–TOF-MS of the entire range of N-glycans associated with rHuAChE, 2-AB-labelled glycans were converted into neutral forms by two different

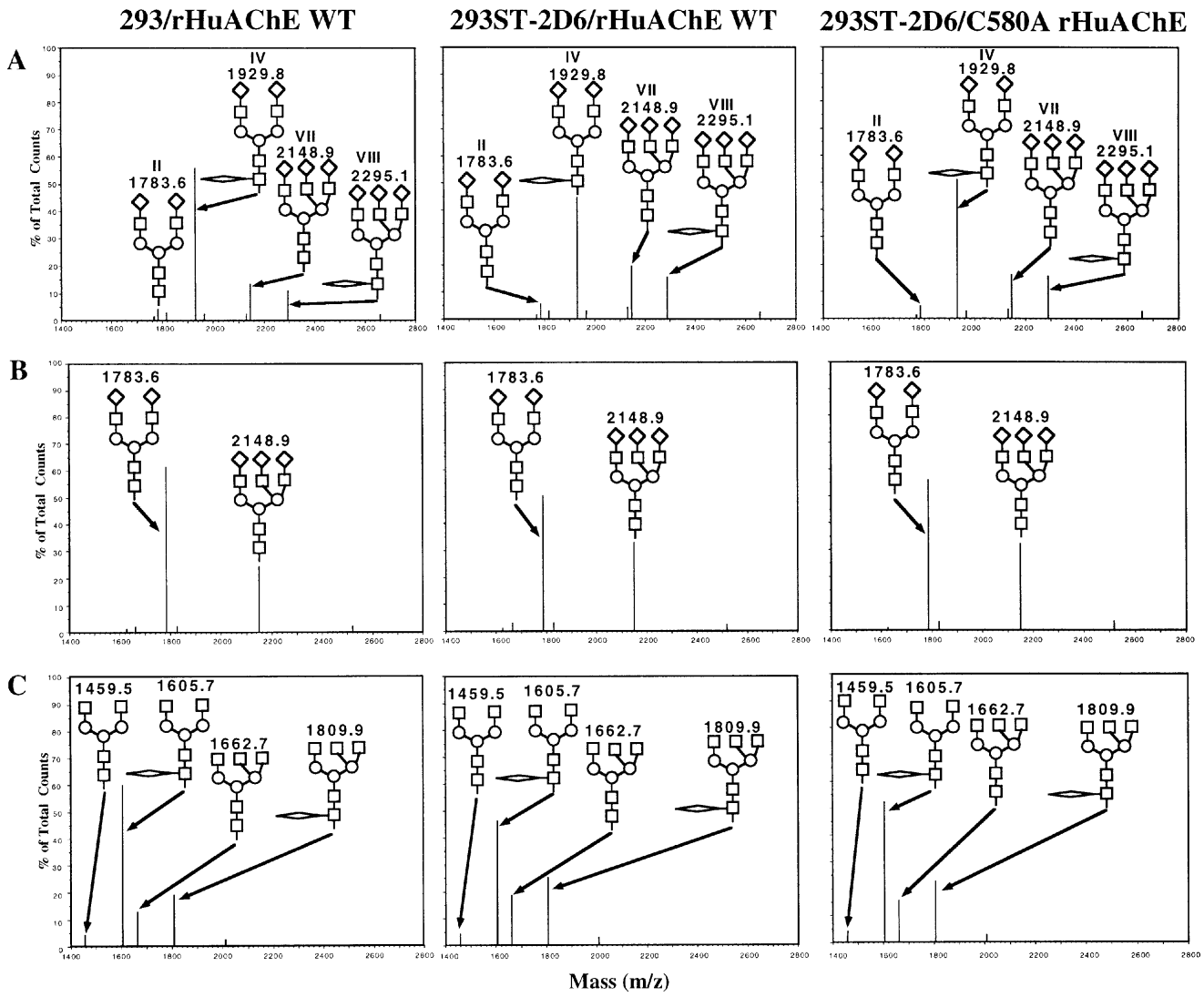

Figure 1 MALDI–TOF mass spectra of N-glycans associated with recombinant forms of human AChE after desialylation

N-glycans purified from WT rHuAChE produced in HEK-293 cells or 293ST-2D6 cells or from the mutated C580A rHuAChE generated in 293ST-2D6 cells were subjected to treatment with sialidase and labelling with 2-AB before MALDI–TOF-MS analysis. Molecular masses and structural details are shown for the major glycan forms. Molecular masses represent monoisotopic masses of the respective $[M+Na]^+$ ions of the glycan species. (**A**) N-glycans without additional exoglycosidase digestion. Roman numerals refer to the peak designations assigned to the various major forms in Table 1. (**B**) N-glycans after digestion with bovine kidney fucosidase. (**C**) N-glycans after digestion with bovine testes β-galactosidase. Symbols: □, GlcNAc; ○, Man; ◇, β-Gal; ⬭, Fuc.

methods. In the first mode, sialic acid residues were removed from the purified N-glycans to form a non-charged glycan pool that could be readily analysed by MALDI–TOF-MS in very low quantities and in a highly reproducible manner (S.D. $\leqslant 2\%$) (although by this method we obviously lost all information on the state of sialylation of the various glycan forms). In an alternative mode of operation, the glycans were subjected to an esterification procedure in which sialic acid residues were converted into their non-charged methyl ester forms. With this method, the relative quantities and structures of both sialylated and non-sialylated glycan forms were determined within a single MALDI–TOF-MS spectrum. However, because this procedure entailed some loss of sensitivity as well as a lower level of reproducibility (S.D. $\leqslant 10\%$), the data gathered with this protocol were validated by comparing them to the data acquired with a MALDI–TOF-MS analysis of desialylated glycans. Accordingly, the relative quantities of the different sialylated and non-sialylated esterified glycan forms were considered to be accurate when the sum of sialoforms corresponding to each of the basic glycan structures coincided with the relative quantities of the corresponding glycan structures determined for desialylated glycans.

Inspection of the MALDI–TOF-MS profiles of desialylated glycans of rHuAChE produced by HEK-293 cells and sialyltransferase-modified 293ST-2D6 cells revealed that in both cases the corresponding glycan pools comprised an array of varied glycan structures (Figure 1A). Subsequent treatment of the glycans with exoglycosidase (Figures 1B and 1C) allowed us to determine the exact structures of the different glycans (Table 1). In both cases the major glycan species (49–56% of total glycans) corresponded to the complex-type biantennary fucosylated form (Table 1, peak IV). Triantennary glycans (25–33% of the total glycans) consisted of similar amounts of fucosylated and non-fucosylated species (Table 1, peaks VII and

Table 1 Comparison of the basic glycan structures of desialylated WT rHuAChE and C580A-rHuAChE

	Abbreviation	Structure	MW $[M + Na]^+ + 2AB$	N-Glycan abundance (% of total)		
				HEK-293 produced rHuA ChE	293ST-2D6 produced rHuA ChE	293ST-2D6 produced C580A
I	NG1A2F		1767.8	1.68 ± 0.2	1.55 ± 0.4	1.0 ± 0.2
II	NA2		1783.6	4.89 ± 0.8	5.2 ± 0.6	4.6 ± 0.5
III	NG2(GalNac)A2F		1809.9	3.2 ± 1.0	3.6 ± 0.5	‹ 0.8
IV	NA2F		1929.8	56.0 ± 4.1	49.0 ± 4.8	51.0 ± 4.6
V	NG1(GalNAc)A2F		1971.8	3.3 ± 0.76	3.90 ± 0.7	2.0 ± 0.8
VI	NA2BF		2132.6	3.0 ± 0.45	4.6 ± 1	4.1 ± 1
VII	NA3		2148.9	13.6 ± 1.0	18.8 ± 0.9	16.3 ± 1.1
VIII	NA3F		2295.1	11.2 ± 1.6	14.5 ± 1.9	15.9 ± 2.1
IX	NA4F		2660.6	2.23 ± 0.29	2.8 ± 0.8	3.3 ± 1.0

VIII). Higher-branched glycans (Table 1, peak IX), bisecting glycans (Table 1, peak VI) and GalNAc-containing glycans (Table 1, peaks III and V) were present in both preparations at low levels only.

Examination of the MALDI–TOF-MS spectra of esterified glycans of rHuAChE produced by HEK-293 cells (Figure 2A, upper panel) and sialyltransferase-modified 293ST-2D6 cells (Figure 2A, lower panel) revealed that the two glycan pools differed significantly only in their state of sialylation. Whereas only 63 % of the glycans associated with the enzyme product of unmodified HEK-293 cells were fully sialylated (Figure 3A, upper panels), nearly 95 % of the glycans associated with the rHuAChE of 293ST-2D6 cells displayed full sialylation (Figure 3A, lower panels). Only 5.5–5.7 % of the glycans (Figure 3A, peaks I and III) consisted of immature forms lacking a galactose moiety and was therefore refractive to further sialylation.

Taken together, these results clearly establish that the sialyltransferase-modified 293ST-2D6 cell line promotes the highly efficient sialylation of rHuAChE co-expressed at high levels. Furthermore, sialylation-refractive glycans are present at low levels only, suggesting that incomplete sialylation is probably not the main reason for the fact that highly sialylated rHuAChE are cleared more rapidly than native serum-derived ChEs (Table 1; see also [6]).

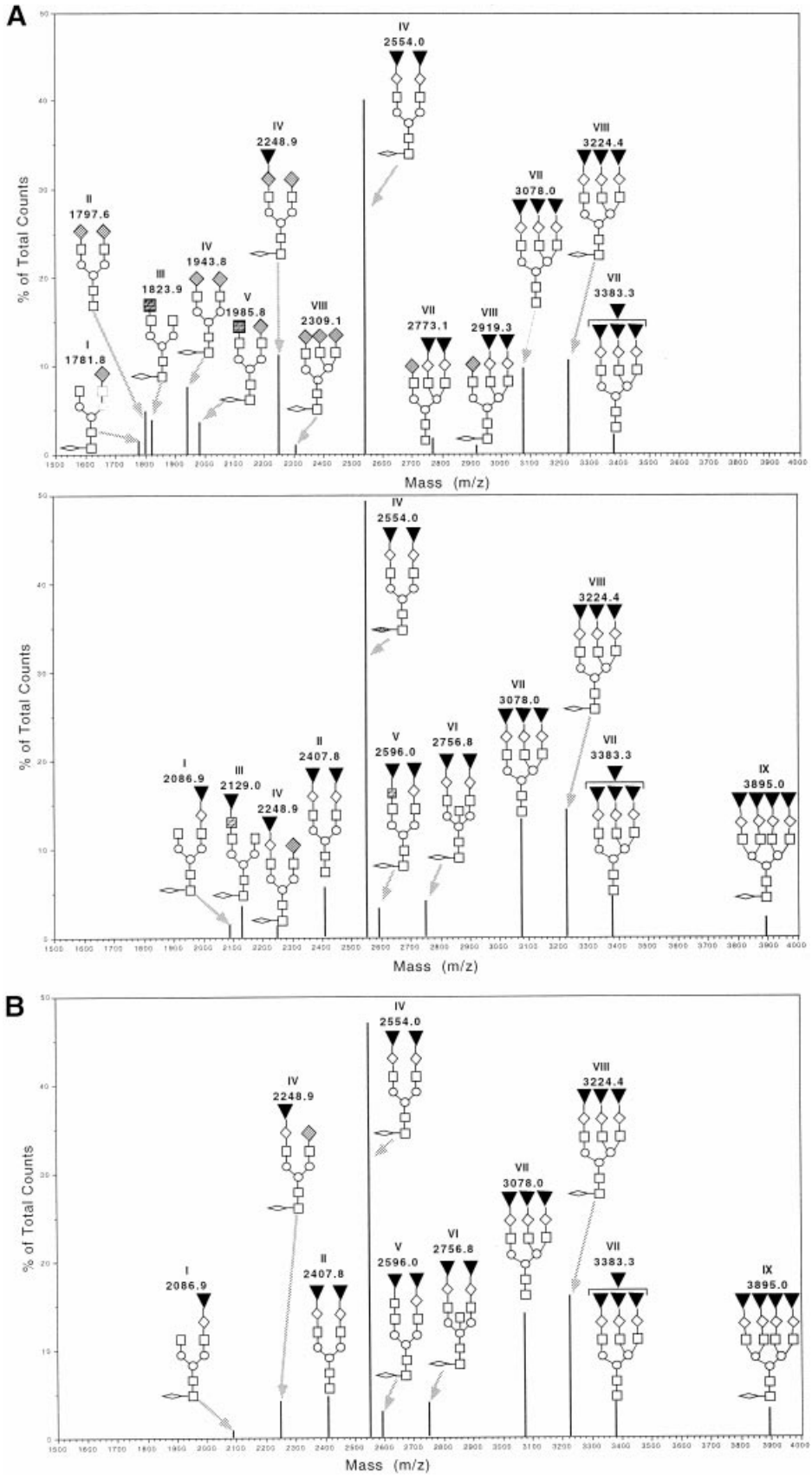

Figure 2 For legend see facing page

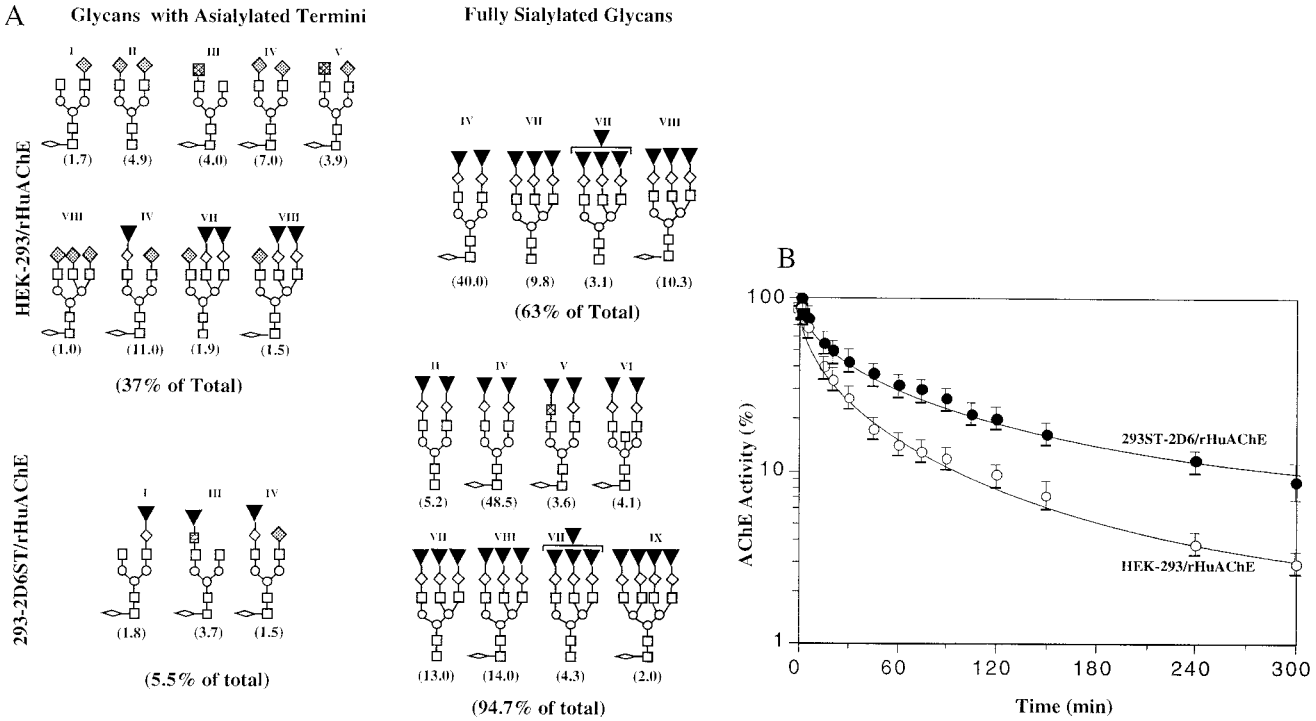

Figure 3 Glycan structures and pharmacokinetic profiles of rHuAChE produced by HEK-293 or 293ST-2D6 cells

(**A**) Glycan structures were deduced from the mass spectral data shown in Figure 2, and classified according to their sialic acid occupancy. The various glycans are annotated by a roman numeral that refers to the basic structures described in Figure 1. The monoisotopic masses of the respective $[M+Na]^+$ ions of the 2-AB-labelled glycan species after iodomethane-mediated esterification are as follows: I (non-sialylated), 1781.8; I (sialylated), 2086.9; II (non-sialylated), 1797.6; II (sialylated), 2407.8; III (non-sialylated), 1823.9; III (sialylated), 2129.0; IV (non-sialylated), 1943.8; IV (partly sialylated), 2248.9; IV (sialylated), 2554.0; V (non-sialylated), 1985.8; V (sialylated), 2596.0; VI (sialylated), 2756.8; VII (partly sialylated), 2773.1; VII (fully sialylated), 3078.0; VII (hyper-sialylated), 3383.3; VIII (non-sialylated), 2309.1; VIII (partly sialylated), 2919.3; VIII (fully sialylated), 3224.4; IX (sialylated), 3895.0. Note that methylation of the common 2-AB fluorescent moiety results in an increase of 14.015 Da in all glycan species. Abundances refer to the relative amount (percentage of total glycans) of each particular glycan species. Symbols: □, GlcNAc; ○, Man; ◇, β-Gal; ▦, GalNAc; <, Fuc; ▼, sialic acid. Terminally exposed (non-sialylated) β-Gal and GalNAc residues are tinted. (**B**) Pharmacokinetic profiles of purified AChE from HEK-293 cells (○) or from 293ST-2D6 cells (●). Results are presented as means ± S.D.

In a previous study [4] we assumed that the predominant forms of the glycans appended to various rHuAChE enzymes produced in HEK-293 cells were of the biantennary type. On the basis of this assumption, and on the chemical quantification of sialic acid of an array of rHuAChE mutants that differed in their state of sialylation, a value representing the number of glycan termini not occupied by sialic acid residues was calculated. This allowed us to find an inverse linear relationship between the number of non-sialylated glycan termini per enzyme subunit and the residence time of the different rHuAChE forms examined. Using the refined MALDI–TOF-MS technique for glycan analysis, we can now determine accurately the actual number of glycan termini per enzyme subunit, and their extent of sialic acid capping. First, we find that the actual average number of N-glycan termini per AChE subunit (containing three used N-glycosylation sites) is 6.4. Furthermore, we have established (Figure 3A) that 2.2 glycan termini per enzyme subunit of rHuAChE produced by the unmodified HEK-293 cells are devoid of sialic acid, whereas only 0.21 glycan termini per enzyme subunit of rHuAChE produced by the sialyltransferase-modified 293ST-2D6 cell line do not contain terminal sialic acid moieties (of these, only 0.045 glycan termini per enzyme subunit of the highly sialylated rHuAChE contain exposed galactose that can serve for sialic acid capping). When we now introduce the accurately determined values of non-sialylated glycan termini (Figure 3A) of the two forms of WT rHuAChE (from 293ST-2D6 and unmodified HEK-293 cells) on a graph (Figure 4) redrawn on the basis of the relationship described previously (Figure 5 in [4]) between $t_{1/2}\beta$ and the number of non-sialylated glycan termini, we find that: (1) the experimental values determined here are in good agreement with the values derived from the previously determined slope of the linear relationship and (2) the $t_{1/2}\beta$ of approx. 130 min observed for the rHuAChE produced by the sialyltransferase-modified cells is indeed very close to the predicted value extrapolated for fully sialylated molecules.

Figure 2 MALDI–TOF mass spectra of total N-glycan pools associated with WT rHuAChE produced by HEK-293 or 293ST-2D6 cells and C580A-AChE produced by 293ST-2D6 cells

(**A**) Purified N-glycans of WT AChE produced in HEK-293 cells (upper panel) or in the genetically modified 293ST-2D6 cells (lower panel), and (**B**) C580A-AChE generated in 293ST-2D6 cells, were subjected to labelling with 2-AB and iodomethane-mediated esterification before MALDI–TOF-MS analysis. Molecular masses and structural details are shown for the glycan forms. Molecular masses represent monoisotopic masses of the respective $[M+Na]^+$ ions of the glycan species. Roman numerals refer to the peak designations of the various major forms in Table 1. Note that an increase of 14.015 Da in all glycan species occurs owing to the methylation of the common 2-AB moiety. Symbols: □, GlcNAc; ○, Man; ◇, β-Gal; ▦, GalNAc; <, Fuc; ▼, sialic acid. Terminally exposed (non-sialylated) β-Gal and GalNAc residues are tinted.

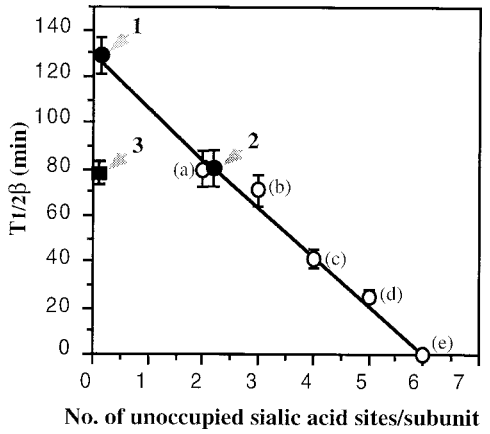

Figure 4 Relationship between the circulatory residence time of WT rHuAChE produced by HEK-293 or 293ST-2D6 cells and that of C580A AChE produced by 293ST-2D6 cells, and their degree of sialic acid occupancy

The $t_{1/2}\beta$ values of WT rHuAChE produced by 293ST-2D6 cells (1) or HEK-293 cells (2) and monomeric C580A AChE produced by 293ST-2D6 cells (3) were plotted against the number of unoccupied glycan termini as calculated from the MALDI–TOF-MS analysis data (Figure 3A). Points a–e: rHuAChE preparations consisting mostly of dimeric forms of WT rHuAChE (a), D61N rHuAChE (b), S541N rHuAChE (c), D61N/S541N rHuAChE (d) and sialidase-treated WT rHuAChE (e) (Figure 5 in [4]). The estimates of the numbers of unoccupied glycan termini in points a–e were based on sialic acid measurement and the assumption that the N-glycans associated with HEK-293 AChE were mainly of a biantennary type. Results are presented as means $\pm$ S.D.

The fact that the $t_{1/2}\beta$ of rHuAChE produced by the sialyltransferase-modified 293ST-2D6 cell line is in accordance with the value predicted for an enzyme devoid of unoccupied sialylation sites substantiates our conclusion that extension of circulatory residence by improved sialylation has reached an upper limit and that post-translation-related factors other than glycan sialylation impose a limitation on its ability to reside in the circulation for longer periods, in a manner similar to that of native ChEs.

Circulatory residence of rHuAChE is affected by its state of assembly

Naturally occurring circulatory and membrane-bound ChEs are assembled as multimeric complexes [27–33]. Homodimers of enzyme subunits, formed by covalent disulphide bonding of cysteine residues located at the C-terminus of the enzyme, are further assembled into quaternary forms. An examination of fetal BoAChE by sucrose-gradient analysis reveals that this circulatory long-lived enzyme is arranged entirely in the tetrameric configuration. In contrast, recombinant ChEs produced in various cell culture systems contain variable amounts of tetramers, dimers and monomers [4,17,32,34,35]. For example, rHuAChE produced in HEK-293 or 293ST-2D6 cells exhibits at most 5–15 % tetramers and more than 60 % dimers of AChE subunits, suggesting that the inability of recombinant human ChE to assemble efficiently into stable tetramers might contribute to its relatively rapid removal from the circulation. We have recently shown [10] that, indeed, for BoAChE, the aptitude of the native fetal serum-derived enzyme to reside for prolonged durations in the circulation depends not only on its sialylation level but also on its tetrameric quaternary configuration. It should be noted that, in contrast with the bovine enzyme, human native circulatory AChE counterparts are not available and therefore the impact of tetramerization cannot be probed directly by comparison with a serum-resident enzyme of human origin.

To establish whether tetrameric forms of rHuAChE can exhibit a significant pharmacokinetic advantage, inspection of the subunit assembly state of administered rHuAChE was performed. Recombinant AChE was injected into mice and blood samples, withdrawn at various time points, were applied to sucrose gradients to determine the state of assembly of the residual enzyme (Figure 5A). The clearance study was performed with mice that were administered a high dose (1000 units per mouse) of AChE instead of the standard dose of 100 units [4,6] of highly sialylated rHuAChE preparation produced by the 293ST-2D6 cell line. The rHuAchE used was purified from the cell-conditioned medium of confluent stationary cells and contained a higher than usual proportion (10–15 %) of tetramers (see the Experimental section). This high input dose was necessary because approx. 60 % of the injected activity is cleared from the circulation in the fast phase of the removal process [4], yielding a residual activity that would probably be too low for accurate analysis of blood samples by sucrose-gradient sedimentation. It should be noted that the use of a fully sialylated preparation is required for this type of experiment because, as shown previously, the effect of *N*-glycan undersialylation on clearance of AChE from the circulation overrides any other potential factor contributing to circulatory longevity [6].

The pharmacokinetics of the individual oligoforms was followed by their evolution from the sedimentation profiles obtained at the various time points (Figure 5A). An enrichment in the relative amount of rHuAChE tetramers at each successive time point was clearly observed (Figure 5B), indicating that the tetrameric form of the enzyme was being removed much more slowly than the dimers and the monomers. Thus, whereas the tetrameric form constituted only approx. 12 % of the input enzyme, they constituted 65 % and almost 100 % of the circulatory enzyme at 9 and 24 h after injection respectively (Figure 5B).

Plotting the residual AChE activity of the tetrameric form against the time elapsed since enzyme administration allowed us to estimate the pharmacokinetic profile of the tetrameric form of rHuAChE (Figure 5C). Thus the slow-phase half-life ($t_{1/2}\beta$) was found to be 600 ± 100 min. Although this type of evaluation of the circulatory behaviour of the multimeric forms of rHuAChE clearly indicated that the tetrameric form of the enzyme is retained in the bloodstream much more efficiently than monomers and dimers, the poor resolution of the peaks representing the dimeric and monomeric forms of the enzyme precluded the determination of individual pharmacokinetic profiles for these two forms. Yet we can establish from this experiment that the combined pharmacokinetic profile of rHuAChE dimers and monomers exhibited a $t_{1/2}\beta$ value of 100 ± 20 min. Furthermore, although difficult to quantify, a clear-cut enrichment of the dimeric fraction over the monomeric fraction was observed. For example, at 9 h after administration the monomeric portion was virtually undetectable, whereas dimeric AChE could still be observed (Figure 5A).

To confirm the results obtained in these experiments, and to determine accurately the pharmacokinetic behaviour of AChE with respect to its subunit assembly, we set up a system for the inspection of homogeneous preparations of the various rHuAChE oligomeric forms.

Pharmacokinetic profile of monomeric and dimeric rHuAChE

To determine the pharmacokinetic behaviour of non-assembled rHuAChE, the C580A mutated enzyme [17], which is impaired in

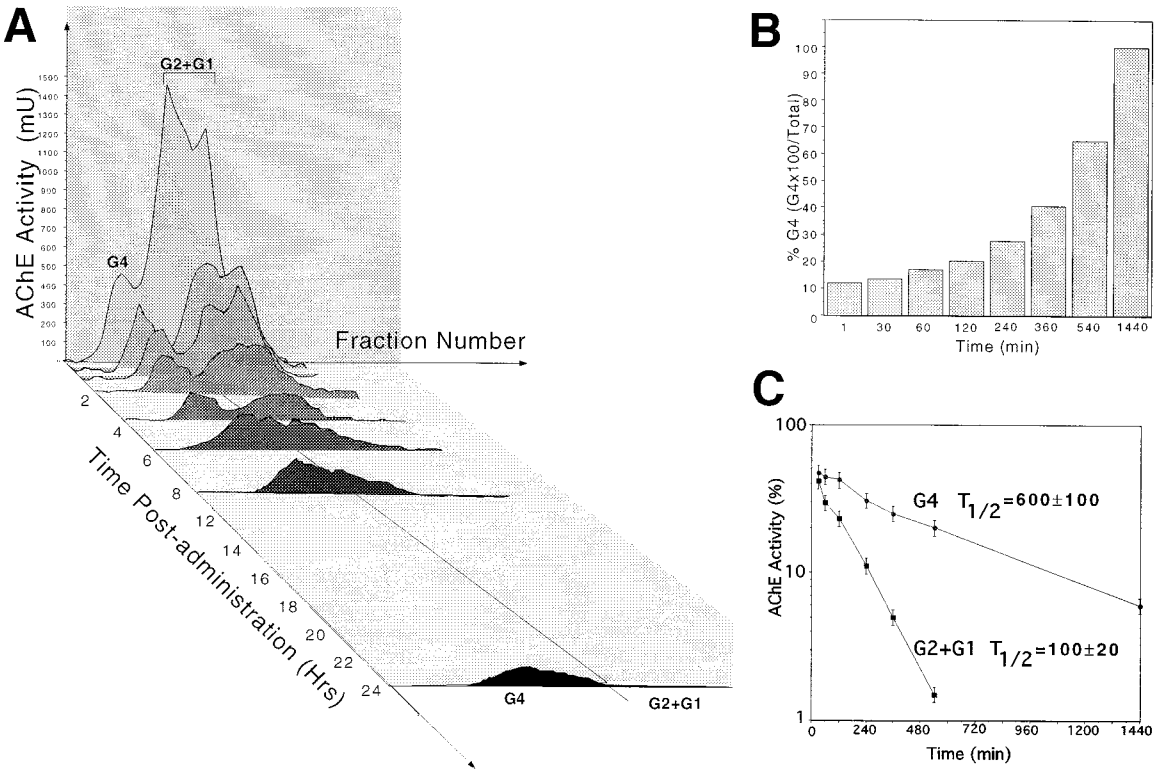

Figure 5 Enrichment of the tetrameric AChE fraction in the circulation after its administration to mice

A purified heterogeneous (in its subunit assembly status) rHuAChE preparation produced in 293ST-2D6 cells, containing 12% tetramers and 88% dimers/monomers, was administered to five mice (1000 units per mouse; Table 2, preparation 6). At the indicated time points after administration, 10 μl samples were withdrawn from each mouse, pooled and subjected to oligomeric status determination by sucrose gradient centrifugation. (**A**) AChE sedimentation profiles in the blood samples. (**B**) Percentages of the tetrameric AChE fraction (G4) in the blood samples. Note that the input material (at 1 min after administration) contained only 12% tetramers, whereas the sample 24 h after administration consisted entirely of tetrameric (G4) forms. (**C**) Exponential activity decay curves calculated individually for the tetrameric fraction (G4) and the combined monomeric/dimeric fractions (G1 + G2). Results are presented as means ± S.D.

its ability to form the interchain disulphide bridge necessary for enzyme dimerization, was expressed at high levels in the sialyltransferase-modified 293ST-2D6 cell line and in HEK-293 cells. In view of the demonstrated impact of glycosylation, and in particular sialylation, on the pharmacokinetics of AChE, identical glycan repertoires are a prerequisite for conducting a pharmacokinetic comparison between the dimeric WT and monomeric C580A AChEs. Analysis of the N-glycans appended to the enzyme product revealed that the basic structures (Figure 1), relative abundances (Table 1) and, most importantly, the degree of sialylation (Figure 2) of C580A-HuAChE were indeed indistinguishable from those of the WT rHuAChE.

When administered to mice, the highly sialylated C580A enzyme was cleared from the circulation more rapidly than highly sialylated WT enzyme (Table 2 and Figure 6). Specifically, the slow-phase half-life ($t_{1/2}\beta$) and the MRT for C580A-HuAChE were 80 ± 6 and 110 min respectively (Table 2, preparation 3), in comparison with 133 ± 9 and 195 min respectively for the WT dimeric enzyme (Table 2, preparation 6). Because both the fully sialylated C580A-HuAChE and the dimeric WT enzyme are virtually indistinguishable in their N-glycosylation (Figures 1 and 2 and Table 1), the apparent differences in their circulatory residence are most probably an outcome of the difference in their subunit assembly. It therefore seems that the non-assembled monomeric form of the enzyme is removed more readily from the circulation than the assembled forms of the enzyme. Indeed, plotting the $t_{1/2}\beta$ of highly sialylated C580A against the number of unoccupied sialic acid sites per enzyme subunit (Figure 4) reveals that in this case $t_{1/2}\beta$ deviates from the linear relationship observed in the past between the number of unoccupied glycan termini and the clearance rates of the other differently sialylated forms of the enzyme examined. It should be noted that all the AChE sialoforms used for the pharmacokinetic studies that generated the inverse linear relationship (Figure 4) were composed mainly of dimers. It is also worth noting that this difference between the monomers and dimers becomes less pronounced with partly sialylated products derived from HEK-293 cells (Table 2, preparations 2 and 5), and is completely abolished when the enzyme glycans are totally devoid of sialic acid residues (Table 2, preparations 1 and 4).

Pharmacokinetic profile of tetrameric rHuAChE generated *in vitro*

Tetrameric forms of AChE are encountered as postsynaptic complexes localized in neuromuscular junctions. In these complexes, subunit multimerization and membrane anchorage of AChE is mediated by the ColQ protein (the non-catalytic AChE subunit [34–37]). The recognition and tetramerization of AChE depends on the PRAD of ColQ, which recruits four AChE subunits by interacting with their C-terminal regions [11,12,38]. The ability of a synthetic peptide comprising the PRAD region of ColQ to promote the assembly of rAChE subunits into tetramers *in vitro* was shown recently [10,13].

Table 2 Pharmacokinetic parameters of recombinant HuAChEs differing in their level of sialylation and state of assembly

Abbreviations: G1, G2 and G4 represent the monomeric, dimeric and tetrameric forms respectively. Results are presented as means ± S.D.

Enzyme type	Preparation no.	State of assembly	Sialylation status	*A* (% of total)	$t_{1/2}\alpha$ (min)	*B* (% of total)	$t_{1/2}\beta$ (min)	Clearance (ml/h per kg)	MRT (min)
Monomer (C580A)	1	G1 > 99%	Non-sialylated	100*	3.5 ± 0.2	–	–	1170	3.6
	2	G1 > 99%	Partly sialylated	65 ± 3	3.8 ± 0.7	35 ± 2	50 ± 8	199	73
	3	G1 > 99%	Fully sialylated	75 ± 6	5.9 ± 1.5	28 ± 4	80 ± 6	138	110
Dimer (WT)	4	G2 > 95%	Non-sialylated	100*	3.3 ± 0.15	–	–	1210	3.5
	5	G4 = 15%; G2 + G1 = 85%	Partly sialylated	74 ± 5	7.3 ± 0.7	25 ± 1.5	80 ± 4	124	102
	6	G4 = 12%; G2 + G1 = 88%	Fully sialylated†	59 ± 4	14 ± 2	42 ± 3	133 ± 9	93	195
	7	G2 > 95%	Fully sialylated‡	62 ± 4	13 ± 2.5	40 ± 3	129 ± 8	99	186
Tetramer (WT + PRAD peptide)	8	G4 > 99%	Non-sialylated	100*	3.5 ± 0.18	–	–	1185	3.5
	9	G4 > 99%	Partly sialylated	55 ± 6	6.9 ± 2	46 ± 4	129 ± 13	83	194
	10	G4 > 99%	Fully sialylated	59 ± 4	30 ± 5	40 ± 4	595 ± 40	13	740

* Sialidase-treated AChE displays a single-phase clearance profile.
† Conditioned medium of a dense (stationary) culture.
‡ Conditioned medium of a young (exponential phase) subconfluent culture (see the Experimental section).

Incubation of rHuAChE in the presence of synthetic PRAD peptide resulted in the quantitative conversion of the mostly dimeric preparation into tetrameric form (Figure 7); no residual monomeric or dimeric forms could be detected. The reaction seemed (Figure 7B) to follow the 1:4 [PRAD]/[AChE] stoichiometry implied by the tetrameric nature of the AChE multimeric complex. As reported for the bovine AChE tetramers [10], the tetramers of rHuAChE generated *in vitro* with PRAD are highly stable, withstanding prolonged dialysis and several cycles of freezing and thawing. Furthermore, the tetramerization reaction *in vitro* can be scaled up for the generation of sufficient amounts of tetrameric AChE to permit pharmacokinetic studies. The fact that human AChE possesses the ability to tetramerize *in vitro* in the presence of the PRAD peptide is of particular interest because native circulating tetrameric forms of human AChE have not been reported. The only tetrameric AChE complexes known in humans are the neuromuscular membrane-bound forms of the enzyme.

Tetramerization of ChE subunits mediated by the ColQ protein has been shown to involve the C-terminal region of the enzyme [39–41] which contains the tryptophan amphiphilic tail ('WAT') domain, which directly recognizes and interacts with the PRAD of ColQ. In line with these reports, we find that incubating, in the presence of PRAD, a truncated AChE form (ΔC rHuAChE) [42] lacking the C-terminal region did not result in any detectable tetramer formation (Figure 7B).

Highly sialylated rHuAChE produced by 293ST-2D6 cells was subjected to PRAD-mediated tetramerization *in vitro* and its pharmacokinetic behaviour was monitored (Figure 6B). The enzyme was retained in the circulation for considerably longer periods than either dimeric or monomeric forms of rHuAChE (Figure 6B). The $t_{1/2}\beta$ and MRT values of tetrameric rHuAChE (Table 2, preparation 9) were 595 and 740 min respectively; thus the tetrameric form of the enzyme displays roughly 4-fold and 7-fold increases in circulatory retention over pure fully sialylated dimeric and monomeric forms of rHuAChE respectively. Notably, the $t_{1/2}\beta$ (595 min) of highly sialylated rHuAChE uniformly tetramerized by interaction with the synthetic PRAD peptide is very similar to that estimated for the tetrameric fraction of the heterogeneous preparation of highly sialylated rHuAChE (Figure 5C; $t_{1/2}\beta = 600$ min). The agreement in the values obtained by the two independent experimental approaches is remarkable in view of the fact that the mechanism of assembly of the tetramers of the heterogeneous population derived from the 293ST-D6 cells is unknown and such tetramers most probably do not include PRAD-like peptides. Similarly, for BoAChE, we have shown recently [10] that native serum-derived tetramers and *in vitro*-generated bovine rAChE tetramers obtained by incubation with PRAD exhibit identical circulatory residence times. This identical pharmacokinetic behaviour strengthens the notion that tetramerization itself is the factor that confers long-term circulatory residence.

Relationship between N-glycan sialylation and oligomerization in establishing the residence time of HuAChE in the circulation

Taken together, the pharmacokinetic experiments performed with differently assembled forms of rHuAChE clearly establish that the complexation of enzyme subunits into forms of higher order contributes significantly to their ability to reside in the circulation for long periods. We observe a clear and direct correlation between the pharmacokinetic characteristics of the different preparations (Figure 6; see also the clearance parameters in Table 2) and their multimerization state. These observations are consistent with the recently reported results obtained with

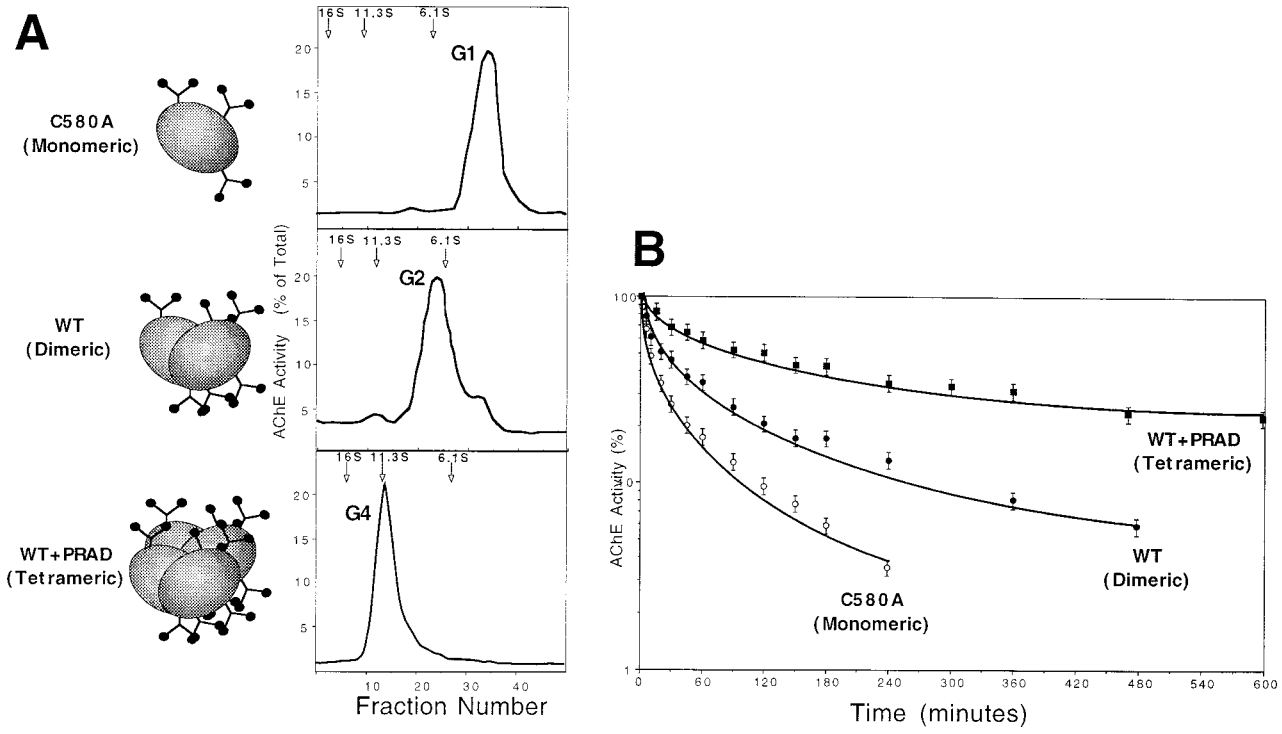

Figure 6 Pharmacokinetic behaviour of monomeric, dimeric and tetrameric homogeneous preparations of fully sialylated rHuAChE

(**A**) Schematic representation and sucrose-gradient sedimentation profiles of monomeric C580A AChE, dimeric WT AChE (more than 95% dimers) and tetrameric AChE. (**B**) Circulatory clearance profiles of the three preparations in mice (see also Table 2). Results are presented as means ± S.D.

BoAChE (both recombinant and native FBS), whose residence in the circulation was shown to be affected by subunit assembly and sialylation. These two elements were shown to exert their pharmacokinetic influence in a hierarchical manner, sialylation being the overriding factor [10].

Here we find that the circulatory longevity of human AChE follows a simple tetramer > dimer > monomer rule. However, as in with the bovine enzyme, this rule is manifested in a sialylation-dependent manner, as indicated by the following observations: (1) treatment of rHuAChEs differing in their oligomerization state with sialidase leads to their equally rapid clearance from the circulation within minutes (Table 2, preparations 1, 4 and 8); (2) partly sialylated forms such as those produced by HEK-293 cells exhibit only a modest increase in retention time on assembly of the monomers to dimers and of the dimers to tetramers (note the MRTs of 73, 102 and 194 min for preparations 2, 5 and 9 in Table 2); (3) dimeric forms of fully sialylated AChE (produced in 293ST-2D6 cells) are retained in the circulation significantly longer than fully sialylated monomeric forms or partly sialylated dimeric forms; and (4) a marked synergistic pharmacokinetic effect is observed on the tetramerization of fully sialylated rHuAChE (note the MRT of 740 min in Table 2, preparation 10; see also Figure 6).

Although sialylation prevents clearance by blocking the recognition of glycoproteins by the liver asialoglycoprotein receptor [43], the mechanism by which circulatory residence time is enhanced through multimerization is not entirely clear. On the one hand, large multimeric complexes are known to be retained owing to the size exclusion, which limits the glomerular filtration removal process in the kidney. It has been reported that proteins smaller than 70 kDa are effectively removed from the circulation by the kidneys [44]. This would imply that glomerular filtration affects dimers and tetramers equally and cannot explain the marked difference in the removal rates of the two forms because both dimeric and tetrameric AChE exceed 70 kDa (the molecular masses of dimeric and tetrameric rHuAChE are approx. 140 and 280 kDa respectively). On the other hand, the assembly of catalytic subunits into tetramers might mask clearance-mediating amino acid epitopes such as those involved in the removal of other glycoproteins [45–49]. The possible epitope-masking effect of tetramerization is compatible with the recently published crystal structure of the AChE tetramer [50]. The proposed structure implies a highly regulated and stable architecture of the subunits in which certain domains of all four AChE molecules participating in a tetramer are sequestered within the quaternary structure. The possible involvement of such epitopes as a third circulatory residence factor (in addition to sialylation and oligomerization) participating in the clearance of AChE is also implied from the difference in the pharmacokinetic behaviours of fully sialylated tetrameric forms of BoAChE [10] and HuAChE (whose MRTs are 1340 and 740 min respectively). It should be noted in this context that all of the divergent amino acids between the human and bovine AChEs are located at the surface of the molecules and are situated mostly within two distinct topological clusters [32]; they might therefore confer different patterns of interaction with other proteins. The possible involvement of amino acid epitopes in the clearance of AChE is currently under study in our laboratory.

The therapeutic use of recombinant enzymes of human genetic origin, generated in human heterologous cell culture systems (e.g. HEK-293 and 293ST-D6), entails a significant immunogenic advantage. However, the paucity of a long-lived form of human

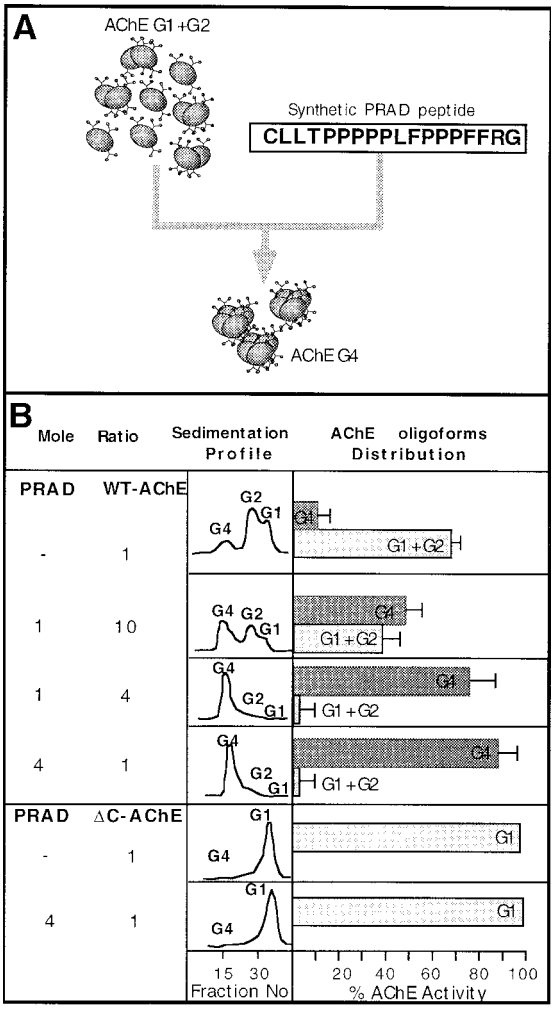

Figure 7 PRAD-mediated tetramerization of rHuAChE *in vitro*

(**A**) Scheme of the tetramerization process mediated by the ColQ-derived synthetic PRAD peptide. (**B**) Sucrose-gradient analysis after incubation of purified rHuAChE produced by HEK-293 cells in the presence of a highly uniform synthetic PRAD preparation at the molar ratios presented in the table at the left. The sedimentation profiles of the various reactions are shown in the middle panel. The percentages of the tetrameric (G4) and residual monomeric/dimeric (G1 + G2) fractions after each of the reactions are represented in the histograms at the right.

AChE represents a serious concern for the development of a recombinant bioscavenger of human origin. The significant enhancement of the circulatory residence time of rHuAChE by combined genetic oversialylation and biochemical tetramerization presented here therefore represents a significant step towards the development of a therapeutically relevant formulation that will permit the exploitation of the bioscavenging abilities of AChE. These studies might serve as a basis for the generation of genetically modified cultured cells with a high potential for promoting both sialylation and tetramerization. This will permit the high-level expression of a circulatory long-lived AChE product or the improvement of the pharmacokinetic performance of other recombinant glycoproteins of therapeutic value, the use of which might have been complicated by their short residence time.

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