Overloading and removal of N-glycosylation targets on human acetylcholinesterase: effects on glycan composition and circulatory residence time

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Optimization of post-translational modifications was shown to affect the ability of recombinant human acetylcholinesterase (rHuAChE) produced in HEK-293 cells to be retained in the circulation for prolonged periods of time [Kronman, Velan, Marcus, Ordentlich, Reuveny and Shafferman (1995) Biochem. J. **311**, 959–967; Chitlaru, Kronman, Zeevi, Kam, Harel, Ordentlich, Velan and Shafferman (1998) Biochem. J. **336**, 647–658; Chitlaru, Kronman, Velan and Shafferman (2001) Biochem. J. **354**, 613–625]. To evaluate the possible contribution of the number of appended N-glycans in determining the pharmacokinetic behaviour of AChE, a series of sixteen recombinant human AChE glycoforms, differing in their number of appended N-glycans (2, 3, 4 or 5 glycans), state of assembly (dimeric or tetrameric) and terminal glycan sialylation (partially or fully sialylated) were generated. Extensive structural analysis of N-glycans demonstrated that the various glycan types associated with all the different rHuAChE glycoforms are essentially similar both in structure and abundance, and that production of the various glycoforms in the sialyltransferase-overexpressing 293ST-2D6 cell line resulted in the generation of enzyme species that carry glycans sialylated to the same extent. Pharmacokinetic profiling of the rHuAChE glycoforms in their fully tetramerized and sialylated state clearly demonstrated that circulatory longevity correlated directly with the number of attached N-glycans (mean residence times for rHuAChE glycoforms harbouring 2, 3, and 4 glycans = 200, 740, and 1055 min respectively). This study provides evidence that glycan loading, together with N-glycan terminal processing and enzyme subunit oligomerization, operate in a hierarchical and concerted manner in determining the pharmacokinetic characteristics of AChE.

Key words: pharmacokinetics, protein oligomerization, MALDI-TOF mass spectrometry.

INTRODUCTION

The distinctive residence time courses for different proteins in the circulation are determined by a set of parameters that characterize the proteins and affect their susceptibility to diverse elimination systems. These parameters include primary traits (e.g. protein size, charge, hydrophobicity and the presence of specific amino-acid epitopes), as well as post-translational processing-related characteristics, such as side-chain carbohydrate appendage and enzyme subunit assembly. More often, protein removal is mediated by more than one mechanism [1–4]. The intricate pattern of co-existent elimination processes confer a typical pharmacokinetic profile characterizing the protein.

Acetylcholinesterase (AChE; EC 3.1.1.7), an enzyme that plays a crucial role in the termination of nerve impulse transmission, is mostly present in the form of cell-bound catalytic hetero-oligomers that are tethered to post-synaptic cells via structural non-catalytic subunits [5,6]. In the fetal stage, soluble homo-tetrameric AChE can be found at appreciable levels in the circulation as well [7], whereas in adults, circulating AChE is substituted by the homo-tetrameric form of the related enzyme, butyrylcholinesterase (BChE). The biological role of circulating cholinesterases and the stage-dependent occurrence of the AChE and BChE are, as yet, poorly understood.

Previous studies have demonstrated the therapeutic potential of plasma-derived cholinesterases as efficient bioscavengers of organophosphate compounds. In addition to their high reactivity towards this group of toxic agents, the native forms of cholinesterases were retained in the circulation of experimental animals for extended periods of time, exhibiting mean residence times (MRTs) of more than 1000 min [8–10]. In contrast, recombinant human or bovine AChE, produced in stably transfected cell lines of the human embryonal kidney (HEK)-293 cell line, were eliminated from the bloodstream of experimental animals within much shorter periods of time (MRTs of recombinant human [11,12] and of recombinant bovine [10,13] AChEs are approx. 100 and 60 min respectively). Glycan processing and enzyme assembly were both found to be decisive factors in determining the circulatory fate of AChEs [10–12,14]. Specifically, the inefficient terminal sialylation of the N-glycans appended to the recombinant AChEs contributed greatly to the rapid elimination of these forms of the enzyme. Indeed, genetic modulation of AChE producer cell lines by the introduction of heterologous α-2,6 sialyltransferase resulted in the generation of efficiently sialylated recombinant AChE, which resided in the circulation for extended periods of time [10,14]. Likewise, induction of enzyme subunit oligomerization by the *in vitro* complexation of recombinant bovine or human AChE (rHuAChE), together with a synthetic Col-Q-derived proline-rich attachment domain (PRAD) peptide, also resulted in the formation of recombinant AChEs with improved pharmacokinetic performances. N-glycan sialylation and subunit assembly of the recombinant enzymes

Abbreviations used: 2-AB, 2-aminobenzamide; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; FBS, fetal bovine serum; Gal, galactose; HEK, human embryonic kidney; MALDI-TOF, matrix-assisted laser desorption ionization-time-of-flight; MRT, mean residence time; N350Q, mutant bearing a substitution of $Asn^{350} \rightarrow Gln$, etc.; PRAD, proline-rich attachment domain; rHuAChE, recombinant human acetylcholinesterase; WT, wild type.

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acted in an hierarchical manner with respect to circulatory residence time.

The biological activity of many proteins is influenced by N-glycosylation [15,16]. In some cases, N-glycans are directly involved in catalytic activity [17–19], whereas in other cases they determine the ability of the respective proteins to be retained in the circulation. AChEs provide an attractive system for exploring the effect of N-glycan loading on glycoprotein pharmacokinetics, since their enzymic activity is not impaired by N-glycan modulation [20–22]. Human AChE carries three N-glycosylation consensus (Asn-Xaa-Ser/Thr) sequences [23], all of which are utilized in the recombinant version of the enzyme [22]. We have reported previously [11] the generation of a series of glycosylation-variants of recombinant human AChE, which are either underglycosylated or overglycosylated. The preliminary pharmacokinetic analyses of the various glycosylation variants of rHuAChE did not reveal any appreciable effect of the number of glycans on the circulatory half-life time of the corresponding forms of the enzyme. However, these recombinant enzymes were both deficient in sialic acid capping, and were composed of a mixed population of monomers, dimers and tetramers, and therefore a possible effect of glycosylation levels on pharmacokinetics may have been masked by the overriding deleterious effect of incomplete glycan sialylation and enzyme subunit assembly.

In the present study, we perform a pharmacokinetic study of sixteen different post-translationally processed recombinant human AChE forms, which define an array of variations with respect to the number of N-glycosylation sites, terminal sialylation and subunit assembly. By exploiting matrix-assisted laser-desorption ionization–time-of-flight (MALDI-TOF)-MS high-resolution analysis, which enables a detailed AChE-associated carbohydrate structural determination, we establish that all the different AChE glycoforms carry N-glycans of similar structures. This allows us to determine the specific contribution of the level of AChE N-glycosylation, and its concerted effect with other post-translational modifications to circulatory longevity.

EXPERIMENTAL

Cell-culture techniques, enzyme production and purification of rHuAChE and its derivatives

The generation of HEK-293 cell lines stably expressing high levels of wild-type (WT) rHuAChE, Asn^{350}→Gln (N350Q) hypoglycosylated rHuAChE and the D61N or D61N/S541N hyperglycosylated variants of rHuACHE have been described previously [11,22,24]. The generation of highly sialylated rHuAChE *in vivo* (WT and glycosylation mutants) was achieved by stably expressing the WT or mutated human AChE genes in the genetically modified 293ST-2D6 cells that express high levels of heterologous 2,6-sialyltransferase [10,14]. Cells were cultured in Dulbecco's modified Eagle's medium ('DMEM') supplemented with 10 % (w/v) fetal bovine serum (FBS). Purification of secreted rHuAChE by affinity chromatography on procainamide columns was as described previously [11,24]. All the mutated versions of rHuAChE under study exhibit catalytic properties that are indistinguishable from those of the WT enzyme.

Enzyme activity

AChE activity was measured by the method of Ellman et al. [25]. Assays were performed in the presence of 0.5 mM acetylthiocholine, 50 mM sodium phosphate buffer, pH 8.0, 0.1 mg/ml BSA and 0.3 mM 5,5′-dithiobis-(2-nitrobenzoic acid). The assay was carried out at 27 °C and monitored by a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.).

Pharmacokinetics

Clearance experiments in mice (three to six ICR male mice per enzyme sample) and analysis of pharmacokinetic profiles were carried out as described previously [11]. The study was approved by the local ethical committee on 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 were fitted to a bi-exponential elimination pharmacokinetic model ($C_t = Ae^{-k_\alpha t} + Be^{-k_\beta t}$), as described previously [11]. This model enables 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. The pharmacokinetic parameters MRT (which reflects the average length of time the administered molecules are retained in the organism) and clearance (which represents the proportionality factor relating the rate of substance elimination to its plasma concentration, where clearance = dose/area under the concentration–time curve; [26]) were independently obtained by analysing the clearance data according to a non-compartmental pharmacokinetic model using the WinNonlin computer program [27]. Circulatory half-life values calculated by non-compartmental analysis were in all cases in good agreement with the $t_{1/2}\beta$ values derived from the bi-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 *N*-glycosidase F (Glyko, Novarto, CA, U.S.A.) treatment, as described previously [24]. Deglycosylated protein was removed by ethanol precipitation, and glycans were recovered and purified from the supernatant as described by Kuster et al. [28]. To increase sensitivity [29–31], purified glycans were fluorescently labelled. Fluorescent labelling of purified glycans with 2-aminobenzamide (2-AB) was performed as described by Bigge et al. [32] using a commercial labelling kit (Glyko, Novato, CA, U.S.A.). During the 2 h labelling incubation, the temperature was kept at 55 °C to prevent heat-induced desialylation of the glycans.

Removal of sialic acid from labelled N-glycans

Agarose-bound sialidase (0.04 unit; Sigma, St. Louis, MO, U.S.A.) was pre-washed five times with water and incubated at room temperature for 16 h with 2AB-labelled N-glycans released from 1.5–2.0 nmol of AChE. Sialidase was removed by Eppendorf centrifugation. Desialylated N-glycans were vacuum-dried, re-suspended in 30 μl of water and stored at −20 °C until use. Glycans prepared in this manner were subjected to MALDI-TOF analysis, or to further glycosidase treatments followed by MALDI-TOF analysis.

Esterification of sialic acids

To allow the concomitant measurement by MALDI-TOF analysis of both neutral and acidic glycans, the carboxylic groups of sialylated 2AB-labelled glycans were converted into their neutral

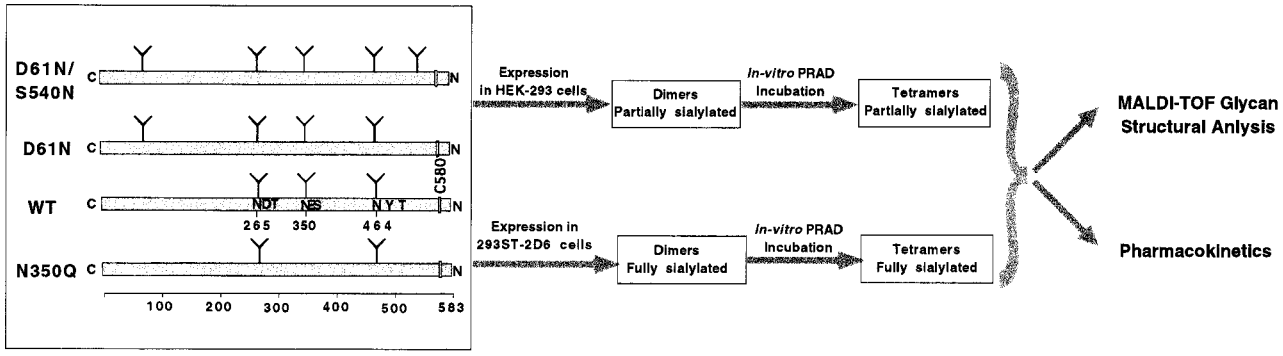


Figure 1 The various forms of rHuAChE under study: experimentation scheme

N350Q, D61N and D61N/S540N are site-directed mutated forms of rHuAChE containing 2, 4 and 5 N-glycosylation sites, respectively, as indicated by schematic biantennary structures. The WT form contains three glycosylation sites at the indicated amino acid sequences. The cysteine residue at position 580, responsible for the generation of dimers, is indicated as a double bar marked C580. The four differently glycosylated AChE forms were expressed in HEK-293 and 293ST-2D6 cells for the generation of partially or fully sialylated dimeric versions of recombinant enzyme respectively. In turn, each version of enzyme was subjected to tetramerization by incubation *in vitro* with the ColQ-derived PRAD peptide. All the forms of rHuAChE were subjected to a detailed pharmacokinetic study paralleled by a quantitative structural analysis of their N-Glycans.

methylated forms by methyl iodide esterification, essentially as described by Kuster et al. [28]. We note that, in this procedure, the 2AB moiety itself undergoes methylation and therefore both neutral and acidic glycans invariably display an increment in molecular mass of 14.015 kDa, in addition to the increase in mass size resulting from sialic acid methylation in the case of acidic glycans. Esterified glycans were purified as described previously [28], and stored at −20 °C until MALDI-TOF analysis.

MS

Mass spectra were acquired on a Micromass TofSpec 2E reflectron TOF mass spectrometer [28,33,34]. 2AB-labelled desialylated or 2AB-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 to 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 allowing to redry. Neutral glycans were observed as $[M+Na]^+$ ions. Of the peptide mixture [renin substrate, adrenocorticotropic hormone 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, 1 μl was mixed with freshly prepared α-cyano-4-hydroxycinnamic acid [10 mg/ml in acetonitrile/ethanol/trifluoroacetic acid (49.5:49.5:0.001, by vol.)], 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. The N-glycan structure assignment was confirmed by inspecting the MALDI-TOF spectra of the various preparations following step-wise exoglycosidase trimming [10,28].

In-vitro tetramerization of rHuAChE

Previous studies by Giles et al. [35] demonstrated that rHuAChE can be tetramerized by a PRAD-derived peptide. The synthesis and quality control of the human version of the PRAD peptide of sequence CLLTPPPPPLFPPPFFRG (single-letter amino acid code) was described previously [10]. Preparative tetramerization for the generation of milligram amounts of tetrameric WT and glycosylation variants of rHuAChE for pharmacokinetic studies included 14.4 nmol of rHuAChE (equivalent to 3000 units), which was incubated with 28.8 nmol of PRAD peptide in a final volume of 2 ml. Before administration in mice, *in vitro*-tetramerized rHuAChEs were dialysed extensively against PBS.

Sucrose density gradient centrifugation

Analytical sucrose density gradient centrifugation was performed on 5–25% sucrose gradients containing 0.1 M NaCl/50 mM sodium phosphate buffer, pH 8.0. Centrifugation was performed in an SW41 Ti rotor (Beckman, Fullerton, CA, U.S.A.) for 26 h at 160000 ***g***. Fractions of 0.2 ml were collected and assayed for AChE activity. Alkaline phosphatase, catalase and β-galactosidase served as sedimentation markers.

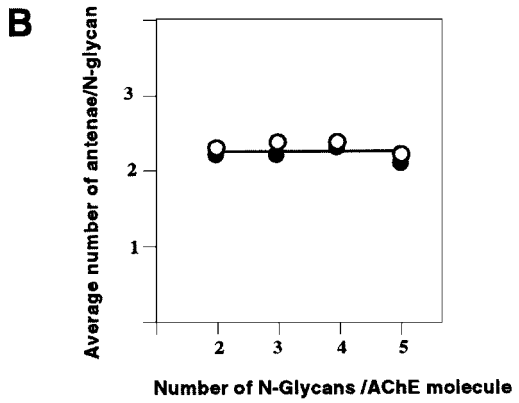
RESULTS

Generation of rHuAChE glycoforms harbouring increasing numbers of N-glycans, and exhibiting various degrees of sialylation and subunit assembly

The general strategy for determining the role of the number of N-glycans on the retention of rHuAChE in the circulation is based in the present report on structural and pharmacokinetic studies involving a series of site-directed glycosylation mutated versions of human AChE (Figure 1; see also [11]). The mutant enzymes N350Q, D61N and D61N/S541N contain 2, 4 and 5 appended N-glycans respectively, as opposed to the WT enzyme, which carries three N-glycans [22,23]. These forms of rHuAChE, as well as the WT enzyme, were produced in HEK-293 cells. In these cells, all the potential native or engineered N-glycosylation sites on rHuAChE, conforming to the consensus Asn-Xaa-Thr/Ser sequence, are utilized [11]. In contrast, disruption of a potential glycosylation site, such as in the case of N350Q, results in the complete abrogation of N-glycan appendage at the corresponding position [22]. We have documented in the past that HEK-293 cells do not contain sufficient amounts of sialyltransferase activity, and therefore the N-glycans of rHuAChE

A

AChE \ Cells	% Sialylated Glycan Termini HEK293	293ST-2D6
D61N/S540N	44±3	95±2
D61N	52±4	96±2
WT	73±6	97±4
N350Q	55±4	94±3



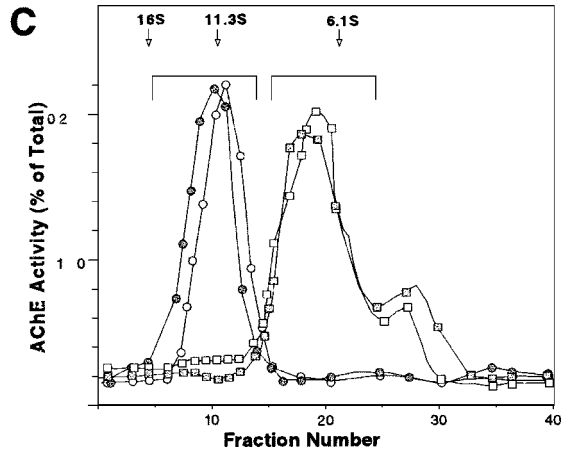


Figure 2 Characterization of glycan capping and branching, and enzyme subunit assembly state of the various forms of rHuAChE under study

(**A**) Percentage of sialylated termini in the glycan pools associated with the various AChE forms, calculated from the frequencies of individual glycan structures detected by the MALDI-TOF analysis (see Table 1 and Figure 4). Note the almost complete sialylation of the glycans associated with the 293ST-2D6 cell-generated AChEs. (**B**) The average number of glycan antennae per glycosylation site calculated for each of the rHuAChE glycoforms under study (based on the data presented in Table 2), were plotted against the number of N-glycan attachment sites of the corresponding enzyme species. Note that in all cases, regardless of the cell-production system, all glycoforms carry a nearly identical average number of glycan antennae. (**C**) Sucrose-gradient sedimentation assay of AChEs before and after PRAD-mediated *in vitro* tetramerization. All mutated rHuAChE forms under study, regardless of their N-glycan terminal sialylation status, tetramerized efficiently in the presence of the PRAD peptide; shown are examples for the diglycosylated N350Q rHuAChE (grey squares = before, grey circles = after PRAD treatment) and pentaglycosylated D61N/S540N rHuAChE (open squares = before, open circles = after PRAD treatment). Arrows denote the elution position of the sedimentation markers alkaline phosphatase (6.1 S), catalase (11.3 S), and β-galactosidase (16 S) included in all samples.

produced in high levels by these cells are inefficiently sialylated [14]. The undersialylated state of HEK-293-cell-produced AChE results in the exposure of N-glycan terminal galactose (Gal) residues, which are efficiently recognized by the liver asialoglycoprotein clearance receptor, thereby negatively affecting the ability of the enzyme to be retained in the circulation for extended periods of time [10–12,14]. To rectify the suboptimal sialylation level of HEK-293-produced rHuAChE, the various mutated forms of rHuAChE, which differ in their numbers of appended N-glycans, were expressed also in the genetically modified 293ST-2D6 cells [10,12], which produce high amounts of recombinant α2,6 sialyltransferase (Figure 1). Indeed, the various glycosylation mutants produced in the modified 293ST-2D6 cells display nearly fully sialylated glycans (Figure 2A), as established by MALDI-TOF structural analysis of the corresponding glycan pools (see below).

In addition to efficient terminal sialylation, the subunit assembly state of AChE positively affects its ability to be retained in the circulation [10,12]. We have previously shown that it is possible to generate highly homogenous preparations of rHuAChE tetramers *in vitro*, utilizing a synthetic peptide representing the PRAD of the ColQ protein [10,12,35–37], which represents the membrane-anchored non-catalytic subunit of AChE multimers encountered at the post-synaptic membranes of the neuromuscular junctions [38–41]. The array of rHuAChE glycosylation forms differing in their number of N-glycans were therefore subjected to PRAD-mediated *in vitro* tetramerization (Figure 1). All the glycosylation variants of rHuAChE, regardless of their degree of terminal sialylation (partially sialylated produced in HEK-293 cells, or fully sialylated produced in 293ST-2D6 cells), were equally amenable to tetramerization, and stable tetrameric preparations could be generated with all AChE forms (Figure 2C). Thus, for the present study, sixteen forms of rAChE were generated that differed in: (i) the number of glycosylation sites (2, 3, 4 and 5 N-glycans); (ii) the extent of sialylation (expressed in HEK-293 or 293ST-2D6 cells); and (iii) the subunit-assembly status (mixed populations consisting mostly of dimers, or fully tetrameric preparations generated by incubation with the PRAD peptide).

MALDI-TOF structural analysis of N-glycans released from the various rHuAChE glycoforms

In order to determine the degree of similarity of the N-glycan pools associated with the different versions of rHuAChE glycoforms under study, the structures and relative quantities of the N-glycans derived from the various enzyme forms (Figure 1), were compared by MALDI-TOF analysis. These analyses included the resolution of the basic features of the appended N-glycans, such as the class (complex, hybrid or high-mannose glycans), branching (number of antennae displayed by each glycan) and core or outer-arm substitutions (e.g. GalNAc, fucose, bisecting GlcNAc), as well as determination of their level of terminal glycan sialylation [10,28].

MALDI-TOF analyses following enzymic removal of glycan terminal sialic acid residues (Figure 3 and Table 1) established that the N-glycans associated with all the different rHuAChE forms belong to 11 discrete classes, which vary in their branching and monosaccharide substitutions, ranging from partially galactosylated biantennary to fully galactosylated tetra-antennary complex-type forms. The relative abundance of the different glycan forms are similar in all the species of rHuAChE, regardless of the number of N-glycans appended per enzyme subunit (2, 3, 4 or 5 glycans) and of the recombinant production system (HEK-

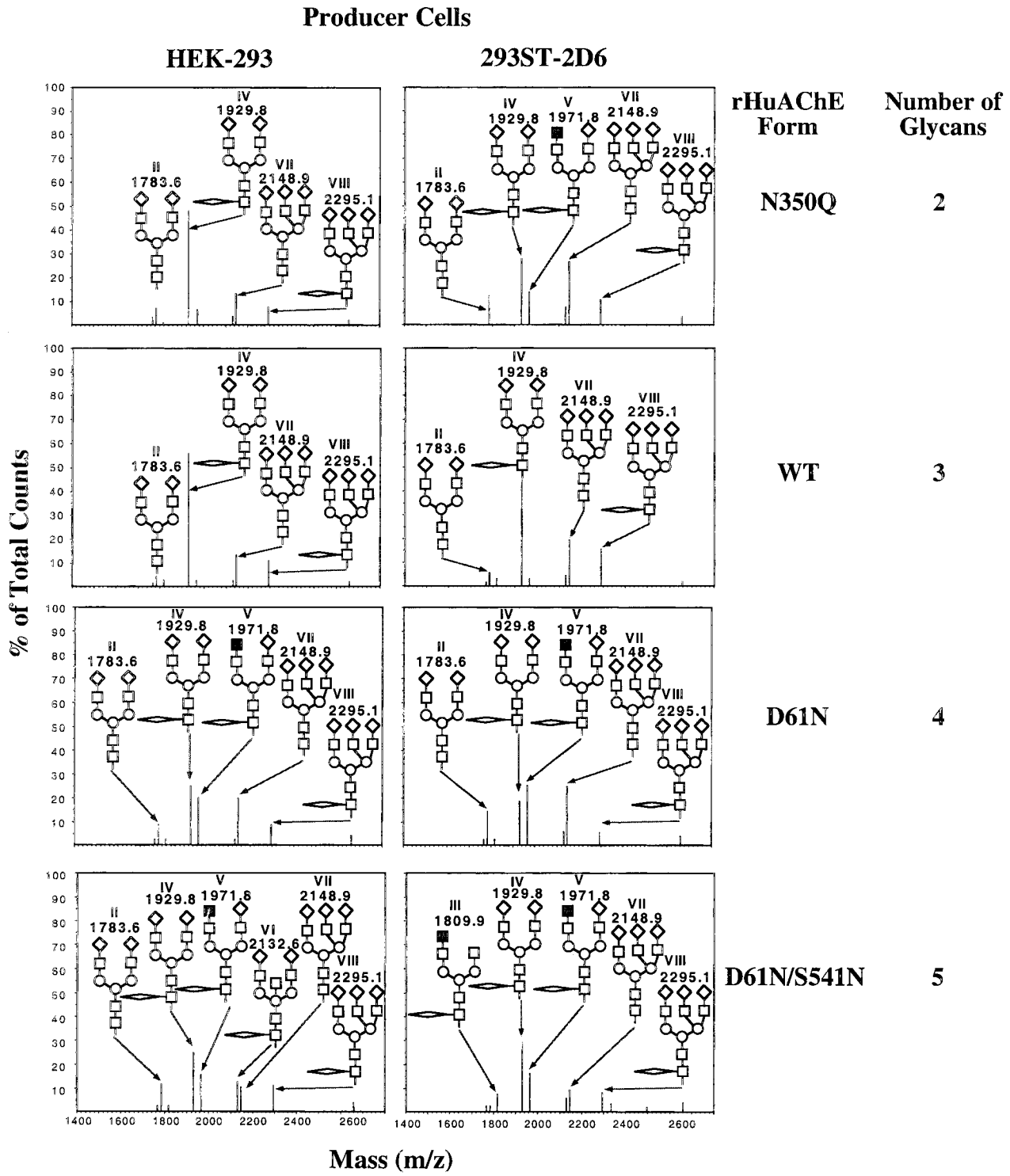


Figure 3 MALDI-TOF mass spectra of desialylated N-glycans released from WT and glycosylation mutant forms of rHuAChE

Purified N-glycans of the indicated various rHuAChEs were subjected to sialidase treatment and 2-AB labelling before MALDI-TOF analysis. Molecular masses and schematic structures are shown for the major glycan forms. Molecular weights represent monoisotopic masses of the respective $[M+Na]^+$ ions of the glycan species. Roman numerals refer to the peak designations ascribed to the various major forms in Table 1. (□), GlcNac; (○), Man; (◇) β-Gal; (—◀), Fuc; (■) α-Gal.

293 cells or 293ST-2D6 cells). In all cases, the overall N-glycan branching was found to be nearly identical (Figure 2B), and the most prevalent form was found to be the biantennary core-fucosylated structure (Table 1; form IV). The N-glycan structural similarity observed for the various rHuAChE forms reflects the N-glycan biosynthetic abilities of the HEK-293 cells, and their propensity for the generation of biantennary complex glycan structures on heterologous recombinant proteins [42,43]. Similar

Table 1 Comparison of the desialylated glycan structures of WT and glycosylation mutants of rHuAChE

Molecular masses, deduced structures and relative abundances of the enzymically desialylated N-glycan structures were determined from the MALDI-TOF spectra represented in Figure 3. Results represent the average ± S.D. obtained from the analysis of at least three independent N-glycan preparations.

	Structure	MW $(M+Na)^+$+2AB	N350Q (2 Glycans)		WT (3 Glycans)		D61N (4 Glycans)		D61N/S541N (5 Glycans)	
			HEK-293	293ST2D6	HEK-293	293ST2D6	HEK-293	293ST2D6	HEK-293	293ST2D6
I		1767.8	2.7±0.4	<0.8	1.68±0.2	1.55±0.4	3.5±0.6	2.4±0.4	3.1±0.4	3.6±0.6
II		1783.6	8.0±0.9	11.6±1.2	4.9±0.8	5.2±0.6	9.9±1.1	14.1±2.0	12.8±1.1	3.4±0.5
III		1809.9	1.4±0.2	<0.8	3.2±1.0	3.6±0.5	1.9±0.3	1.3±0.2	2.9±0.4	8.0±0.6
IV		1929.8	47.7±4	29.5±3.2	56.0±4.1	49.0±4.8	26.4±2.1	19.2±1.8	25.2±1.9	28.7±2.2
V		1971.8	7.4±0.8	12.7±1.1	3.3±0.8	3.9±0.7	19.8±2.1	24.1±2.6	17.7±1.3	18.9±1.1
VI		2132.6	4.1±0.4	7.1±0.9	3.0±0.4	4.6±1.0	3.0±0.5	5.5±0.6	12.9±1.0	5.4±0.5
VII		2148.9	13.9±1.4	24.9±2.9	13.6±1.0	18.8±0.9	21.3±2.2	23.9±2.4	10.5±0.5	9.3±0.7
VIII		2295.1	8.6±0.7	10.7±1.1	11.2±1.6	14.5±1.9	9.4±1.0	5.4±0.7	10.6±0.9	9.6±0.7
IX		2336.5	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8	4.0±0.5
X		2498.5	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8	2.4±0.3
XI		2660.6	2.0±0.3	3.5±0.3	2.2±0.3	2.8±0.8	4.8±0.4	3.9±0.3	4.2±0.4	5.1±0.5
	□GlcNAc ○Man ■GalNAc ◇Gal ◄► Fuc									

N-glycans were found to be associated with recombinant bovine AChE produced in the same cell systems [10].

Although the various AChE forms were mostly found to contain highly similar glycans, some minor structural differences between the glycan repertoires of the various mutated AChEs were observed, such as the unequal occurrence of N-glycans exhibiting outer-arm GalNAc residues (Table 1; form V). These structures were mainly encountered in the mutated D61N and D61N/S541N AChE versions and, to a lesser extent, also in the 293ST-2D6-produced N350Q mutant, which contains only two of the three native glycosylation sites. The relatively low abundance of these GalNAc-terminated glycans, as well as the observation that they are fully capped with sialic acid when expressed in the sialyltransferase 293ST-2D6 co-expressor cell line (see below), suggest that they probably do not play a role in determining pharmacokinetic behaviour differences among the various AChE forms.

Examination of terminal glycan sialylation by MALDI-TOF analysis of iodomethane-mediated esterified glycans (Figure 4, Table 2) clearly established that, although exhibiting glycans of highly similar basic structure, the various AChE glycoforms generated by HEK-293 cells differ one from another in the extent of terminal glycan sialylation (Table 2). A clear relation is observed between the level of non-sialylated glycan termini and the number of N-glycans appended to the various mutants. Thus the HEK-293 cell generated triglycosylated (WT), tetraglycosylated (D61N) and pentaglycosylated (D61N/S541N) AChEs are characterized by 1.8, 4.7 and 6.0 non-sialylated glycan termini per enzyme subunit respectively, reflecting the limited ability of the HEK-293 cell glycosylation machinery to efficiently sialylate an increasing number of glycan termini [11]. In contrast, expression of the various rHuAChE glycoforms in the sialyltransferase-modified 293ST cells resulted in the generation of glycans that display to the same extent almost fully sialylated

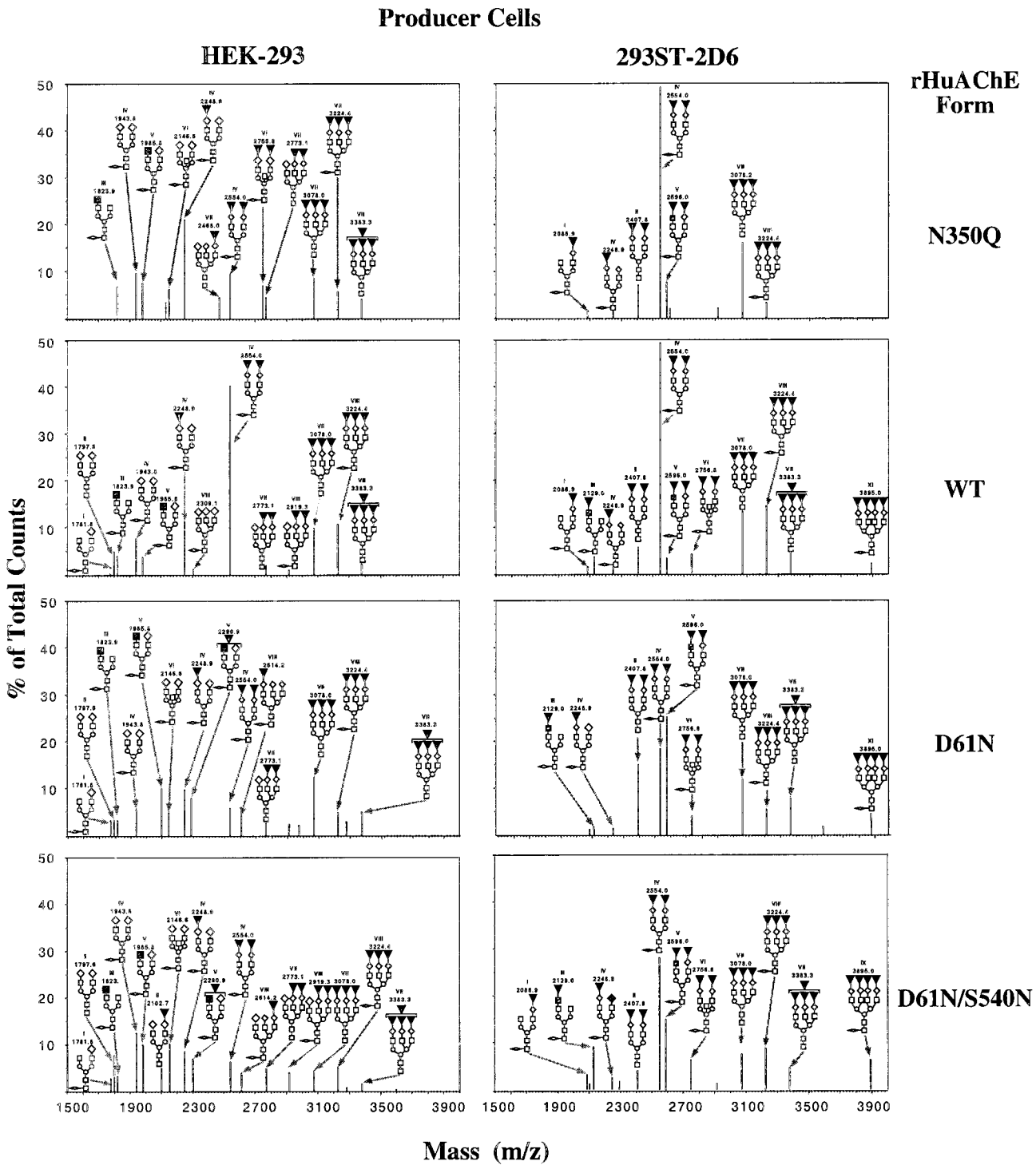


Figure 4 MALDI-TOF mass spectra of the esterified N-glycans pools released from WT and glycosylation mutant forms of rHuAChE

Purified N-glycans of the indicated various rHuAChEs were subjected to 2-AB labelling and iodomethane-mediated esterification prior to MALDI-TOF analysis. The number of glycans were as follows: for the top two panels, 2; upper middle panels, 3; lower middle panels, 4; and bottom panels, 5. Molecular masses represent monoisotopic masses of the respective $[M+Na]^+$ ions of the glycan species. Roman numerals refer to the peak designations ascribed to the various major forms in Table 1. Note that an increase of 14.015 Da of all glycan species occurs due to the methylation of the common 2-AB moiety. (□) GlcNac; (○) Man; (◇) β-Gal; (—◆—), Fuc; (■), α-Gal; (▼) sialic acid.

glycan termini (0.2–0.6 non-sialylated termini/AChE molecule; Table 2), regardless of the number of appended N-glycans. Thus the efficient correction of the sialic acid capping by the expression of the various mutated AChE forms in the genetically modified sialyltransferase 293ST-2D6 cell line resulted in the generation of a set of enzymes that carry highly similar N-glycans with respect to their basic structures and terminal sialic acid capping, and differ one from another only in the actual number of appended

Table 2 Structural branching and sialylation state of N-glycans released from WT and glycosylation-mutant forms of rHuAChEs generated in HEK-293 and 293ST-2D6 cells

The data were calculated on the basis of the frequencies of the individual glycan structures detected by MALDI-TOF analysis (Figures 3 and 4).

Producer cells	AChE form	No. of glycans/AChE molecule	Average no. of antennae/AChE molecule	Sialylated termini/AChE molecule	Non-sialylated termini/AChE molecule
HEK-293	N350Q	2	4.5	2.5	2.0
	WT	3	6.8	5.0	1.8
	D61N	4	9.8	5.1	4.7
	D61N/S541N	5	10.7	4.7	6.0
293ST-2D6	N350Q	2	4.6	4.3	0.3
	WT	3	7.2	7.0	0.2
	D61N	4	9.5	9.1	0.4
	D61N/S541N	5	11.0	10.4	0.6

N-glycans. These can now serve for conducting a meaningful study of the involvement of the number of glycosylation sites in determining the pharmacokinetic behaviour of AChEs.

Pharmacokinetics of rHuAChE glycoforms: the combined effect of N-glycosylation, terminal sialylation and oligomerization in determining the circulatory retention of AChE

The entire array of rHuAChEs differing in their number of N-glycans, both in their partially or fully sialylated configurations (produced by HEK-293 or 293ST-2D6 cells respectively) were subjected to a pharmacokinetic study before or after *in vitro* PRAD-mediated tetramerization. The various forms were administered in mice, and their clearance rates were determined by measuring the residual AChE activity in blood samples withdrawn at different times post-administration. The parameters characterizing the pharmacokinetic performance of each rHuAChE glycoform were calculated according to the biphasic decay and the non-compartmental circulatory elimination models (Table 3 and Figures 5 and 6). Inspection of the MRTs of the various enzyme forms establishes that an increase in the number of appended N-glycans contributes to the ability of the enzyme to be retained in the circulation, yet the manifestation of this contribution clearly depends both on the level of glycan terminal sialylation and the subunit assembly state of the enzyme. Thus, within each of the four categories of rHuAChE enzyme forms under study [**i** = undersialylated/non-assembled; **ii** = undersialylated/tetramerized; **iii** = sialylated/non-assembled; and **iv** = undersialylated/tetramerized; (Table 3)], the presence of increasing numbers of N-glycans on the different mutants appears to affect their pharmacokinetic behaviour according to a different pattern, as detailed below.

Comparison of the pharmacokinetics of the various AChE glycoforms, which are partially sialylated and non-assembled (category **i**; Table 3), revealed that the triglycosylated WT enzyme resides in the circulation for significantly longer periods of time [MRT for WT (undersialylated/non-assembled) = 102 min] than the diglycosylated N350Q form [MRT for N350Q (undersialylated/nonassembled) = 67 min]. However, a further increase in the number of appended N-glycans resulted in a decrease in their circulatory residence in a progressive manner, with the triglycosylated WT, tetraglycosylated D61N and pentaglycosylated D61N/S541N versions of rHuAChE displaying MRTs of 102 min, 46 min and 25 min respectively. This decline in circulatory residence, which accompanies the increase in the number

Table 3 Pharmacokinetic parameters of WT and mutated versions of rHuAChE differing in their number of appended N-glycans, level of terminal sialylation and subunit oligomerization state

Category	rHuAChE status	AChE glycoform	No. of glycans	A (% of total)	$T_{1/2}\alpha$ (min)	B (% of total)	$T_{1/2}\beta$ (min)	Clearance (ml/h per kg)	MRT (min)
i	Undersialylated nonassembled*	N350Q	2	55±3	8.4±1	33±3	54±4	176	67
		WT	3	74±5	7.3±0.7	25±1.5	80±4	124	102
		D61N	4	76±11	4.6±0.3	34±4	44±3	227	46
		D61N/S541N	5	78±5	3.9±1	34±5	25±7	370	25
ii	Undersialylated tetramerized†	N350Q	2	45±7.2	6.7±2.6	56±8.5	60±10	107	87
		WT	3	55±6	6.9±2	46±4	129±13	83	194
		D61N	4	77±2.5	4.5±0.5	25±3	71±4	232	85
		D61N/S541N	5	72±7	4.0±0.6	29±3	75±6	228	82
iii	Sialylated, nonassembled‡	N350Q	2	67±9	21±4	26±7.2	120±22	88	132
		WT	3	58±4	14±1	42±2	133±9	93	195
		D61N	4	52±5.5	11.8±3	46±3.6	129±13	65.5	190
		D61N/S541N	5	60±7	13±2	38±7	133±15	68	201
iv	Sialylated, tetramerized§	N350Q	2	52±10	29±8	49±10	158±22	44	201
		WT	3	59±4	30±5	40±4	595±40	13	740
		D61N	4	58±9	40±8	43±4.5	760±50	11	1055
		D61N/S541N	5	40±3.4	22±7	58±4	713±85	9.8	1020

* Produced in non-modified HEK-293 cells.
† Produced in non-modified HEK-293 cells and subjected to *in-vitro* PRAD-mediated tetramerization.
‡ Produced in sialyltransferase co-expressor 293ST-2D6 cells.
§ Produced in sialyltransferase co-expressor 293ST-2D6 cells and subjected to *in-vitro* PRAD-mediated tetramerization.

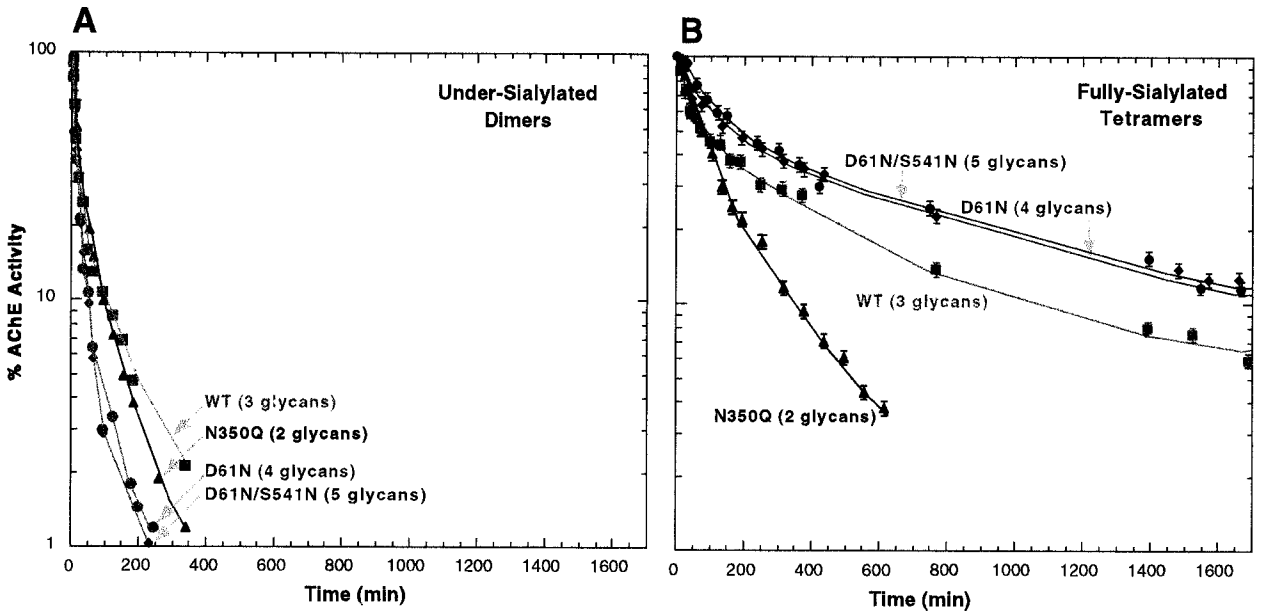


Figure 5 Comparison of the circulatory elimination profiles of WT and mutated forms of rHuAChE

Purified preparations (100 units) of WT and mutated forms of rHuAChE, generated in HEK-293 cells (exhibiting undersialylated N-glycans) or 293ST-2D6 cells (exhibiting fully sialylated glycans), were administered to 3–6 ICR male mice (per enzyme sample) before (dimeric) or after (tetrameric) *in vitro* PRAD-mediated tetramerization. The residual AChE activity in the circulation was assayed at the indicated time points post-administration; circulatory removal curves were determined for all the AChE forms and the derived pharmacokinetic parameters are presented in Table 3. (**A**) HEK-293 generated AChEs; (**B**) 293ST-2D6 cell-generated AChEs following PRAD treatment. (▲) N350Q; (■) WT; (●) D61N; (◆) D61N/S541N. Error bars represent the standard deviation.

of appended glycans, is fully compatible with the relatively high number of N-glycans terminating with exposed Gal residues in the tetraglycosylated and pentaglycosylated HEK-293-produced preparations (Table 2).

All of the various rHuAChE glycoforms under investigation, including those which carry additional N-glycans, were efficiently converted into enzyme tetramers (Figure 2C), corroborating that the appended N-glycans do not obstruct contact-zone interactions required for the efficient generation of stable tetrameric forms [44], and in line with the reported spatial architecture of AChE tetramers determined by X-ray crystallography [45]. All the enzyme forms belonging to this category (undersialylated/tetramerized) exhibited prolonged circulatory residence in comparison with their non-assembled counterparts (compare MRTs of the corresponding rHuAChE glycoforms of categories **i** and **ii**; Table 3). Pharmacokinetic profiling of this group of enzyme forms (category **ii**; Table 3), demonstrated that, as in the previous category (category **i**), an increase in the MRT value is promoted by the addition of a third glycan [MRTs for N350Q (undersialylated/tetramerized) and WT (undersialylated/tetramerized) = 87 and 194 min respectively], and this increase is not improved further by the presence of a fourth or fifth glycan appendage. However, unlike in the case of the non-assembled enzyme forms, the MRTs of the two hyperglycosylated forms of the enzyme are similar [MRTs for D61N (undersialylated/tetramerized) and D61N/S541N (undersialylated/tetramerized) = 85 and 82 min respectively]. In this case, the positive pharmacokinetic effect of tetramerization mitigates the deleterious effect of asialoglycoprotein receptor-mediated circulatory removal, resulting in the equal residence of the tetra- and penta-glycosylated AChEs.

The pharmacokinetic influence of the number of appended N-glycans is most apparent in the case of fully sialylated forms of AChEs. Fully sialylated tri-, tetra- and penta-glycosylated AChEs in their non-assembled form (category **iii**; Table 3), are retained equally as well in the circulation for considerably longer periods of time than diglycosylated N350Q rHuAChE [MRT (sialylated/non-assembled) for WT, D61N and D61N/S541N = 195, 190 and 201 min respectively; MRT for N350Q (sialylated/non-assembled) = 132 min]. Following their tetramerization, a dramatic pharmacokinetic effect was observed for the fully sialylated rHuAChE glycoforms (category **iv**; Table 3). The fully sialylated tetramers display MRT values that increase proportionally with the number of N-glycans within the 2–4 glycan/subunit range (MRT for sialylated/tetramerized N350Q, WT and D61N = 201, 740 and 1055 min respectively). The high MRT exhibited by the mutated tetraglycosylated form represents a 10-fold increase in the circulatory residence as compared with the unmodified WT enzyme [category **i**, MRT for WT (undersialylated/non-assembled) = 102 min] and a 40% increase over the WT enzyme in its fully modified state. This latter effect is only observed upon full sialylation and tetramerization, emphasizing the requirement of optimal sialylation and subunit assembly for assessment of the pharmacokinetic role of N-glycan appendage. Addition of a fifth glycan at amino-acid residue 541, such as in the case of the D61N/S541N mutant, does not improve the pharmacokinetics further [category **iv**, MRT for WT (sialylated/tetramerized) = 1020 min], suggesting that the four glycans in the D61N mutant are sufficient to confer this high, unprecedented circulatory residence to rHuAChE.

DISCUSSION

Examination of an array of fully processed rHuAChE enzyme forms, which are efficiently sialylated and assembled into their tetrameric forms, revealed that the number of appended glycans influences their ability to reside in the circulation over extended periods of time (see the fully sialylated tetramers in Figure 6). Thus, di- tri and tetra-glycosylated rHuAChE exhibit MRT

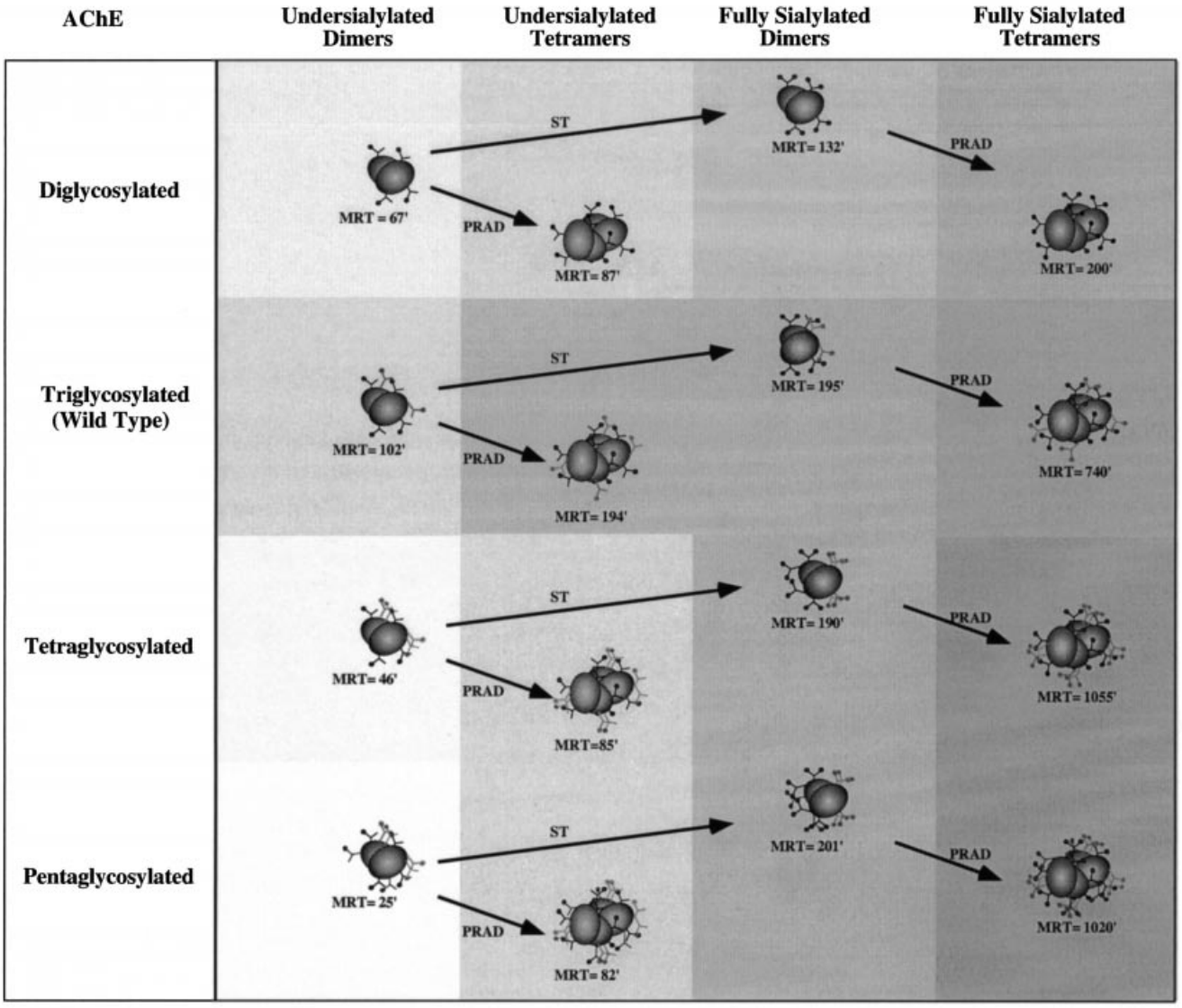


Figure 6 Schematic representation of the combined effect of the number of appended N-glycans, N-glycan sialic acid capping and oligomerization state on the circulatory retention of rHuAChE

All 16 forms of AChE under study are depicted as globular spheres representing the AChE subunit. Bifurcated extensions protruding from the spheres represent N-glycans. The circles terminating some of these bifurcated extensions represent sialic acid moieties. Diglycosylated, Tetraglycosylated and Pentaglycosylated, refer to the N350Q, D61N and D61N/S541N rHuAChE mutants respectively. Undersialylated dimers are generated by expression in HEK-293 cells, whereas fully sialylated dimers are generated in the sialyltransferase expressor 293ST 2D6 cell system, and are denoted by ST. The *in vitro* quantitative tetramerization is denoted by PRAD. A decrease or an increase in MRT is indicated by a change to lighter or darker backgrounds, respectively (the darker the background, the higher the MRT).

values of 200, 740 and 1055 min respectively. The MRT of the fully processed tetraglycosylated D61N rHuAChE (1055 min) is of the same magnitude as that of FBS-AChE (MRT = 1340 min; [10]), with which it shares the same number of appended glycans at corresponding locations [13]. Structural examination of FBS-AChE carried out previously [10] demonstrated that this native enzyme, which physiologically resides in the circulation, occurs entirely in a fully sialylated tetrameric form. In contrast, the WT human AChE, which possesses only three N-glycans, is not encountered as a native circulatory enzyme, but rather as a post-synaptic or erythrocyte-associated, membrane-anchored complex.

The manner in which the N-glycosylation load, terminal N-glycan sialylation and subunit oligomerization act together in determining the residence time of the enzyme (Figure 6) clearly suggests that a multifactorial mechanism is involved in the removal of AChE from the circulation and that these multiple factors exert their influence in a hierarchical manner. Terminal N-glycan sialylation is the governing factor in this hierarchy, since totally desialylated forms of AChE are cleared rapidly, and equally as well, from the circulation within minutes, regardless of their oligomerization state and their number of appended N-glycans ([10,12], and results not shown). The dominant position of sialylation is reflected also by the observation that, under

partial sialylation conditions, increasing the number of N-glycans both on dimers and tetramers has a deleterious rather than advantageous effect on AChE residence time (Figure 6, undersialylated dimers and undersialylated tetramers). In this case, increasing the number of N-glycans on the enzyme surface results in a quantitative increase in the number of terminal Gal residues, which serve as highly potent clearance epitopes. This situation was responsible for our inability in the past to assign a positive pharmacokinetic role for the rHuAChE N-glycan number [11], since in that study, rHuAChE forms were prepared in HEK-293 cells, which possess only limited sialyltransferase activity, and therefore suffered from pronounced undersialylation. Inefficient enzyme assembly, which represents a second factor in the hierarchical AChE multifactorial elimination pathways [12], may be remedied by PRAD-mediated tetramerization, yet, in itself, is not sufficient for enabling the increased number of glycans to fully exert their pharmacokinetic effect (Figure 6, undersialylated tetramers). Rather, the full manifestation of the circulatory longevity promoted by quantitative N-glycosylation requires a pre-existing state of highly efficient tetramerization and sialylation of the enzyme.

The role of glycosylation in determining the circulatory residence of glycoproteins has been examined in many systems [46–48]. Clearance of erythropoietin, tissue plasminogen activator, interferon and various hormones [1,49–51] depends on removal pathways that involve glycan recognition. In the case of erythropoietin, tissue plasminogen activator and sex-hormone-binding globulin, removal of glycan side chains enhanced protein residence in the circulation of experimental animals [1,49,52]. However, in most, if not all, of these cases, the appended glycans were either suboptimally sialylated or consisted of high-mannose forms, and consequently served as excellent substrates for highly potent hepatic removal systems. The removal of glycans, as investigated in such studies, may serve therefore to evaluate the contribution of the specific glycan processing of these proteins to circulatory residence, rather than allow any definite conclusion regarding the role of glycan loading in itself on circulatory retention. In this respect, these reported results are similar to those documented for glycosylation mutants of rHuAChE produced in the HEK-293 cell system, where addition of glycans had a clear adverse pharmacokinetic effect, owing to the increase of pharmacokinetically unfavourable uncapped glycan termini (the present study, and [11]). In the present study, by ensuring optimal processing of the N-glycans and by maximizing other post-translational modifications, rHuAChE enabled us to assess directly the effect of the actual number of N-glycans on its pharmacokinetic performance.

The improved pharmacokinetic performance exhibited by AChE carrying an increased number of appended N-glycans might be a consequence of one or more of the following phenomena: (i) interference with glomerular filtration; (ii) decreased susceptibility to plasma-residing proteolytic enzymes and/or improved thermostability of the molecule conferred by surface-bound glycans; and (iii) a clearance-epitope masking effect exerted by glycan-promoted shielding.

All the forms of rHuAChE under study possess a molecular mass greater than the kidney filtration cut-off threshold (approx. 70 kDa; [53]), and therefore it is unlikely that N-glycosylation exerts its effect via prevention of kidney glomerular filtration. Furthermore, the addition of a single glycan (average molecular mass = 2.5 kDa) per enzyme subunit contributes insignificantly to the overall mass of the tetrameric molecule (mass < 280 kDa). N-glycosylation introduces into the AChE molecule surface an increase in negative charge, resulting from the presence of the terminal sialic acid residues. This increase in the negative charge might also affect elimination by the kidneys, yet we noted that a mutated version of rHuAChE in which seven negatively charged surface amino acids have been replaced by neutral amino acid moieties [54] did not exhibit any variation in its pharmacokinetic performance (results not shown), strongly suggesting that the surface charge does not play a significant role in the circulatory residence of AChE.

Table 4 Stability of rHuAChE glycoforms in murine plasma

Purified rHuAChEs (0.25–0.3 unit/ml) were incubated in the presence of murine plasma at 37 °C. At the indicated time points, samples were examined for residual enzyme activity. Enzyme activity is expressed as percentages of input activity measured immediately following enzyme dilution in plasma. Note that within the time range of 0.5–6 h, enzyme activity remained constant within experimental error, at levels similar to input values. The decrease in activity at 24 h was similar for all rHuAChE glycoforms.

		Residual AChE activity (%)						
AChE form	Time (h)...	0.5	1.0	2.0	3.0	4.0	6.0	24.0
N350Q		95±3	96±3	95±4	97±3	95±4	97±3	70±8
WT		94±5	96±4	94±5	97±2	95±4	96±3	74±9
D61N		95±4	97±3	95±3	97±2	96±3	94±5	71±6
D61N/S541N		96±2	94±5	96±4	95±2	97±3	95±4	73±5

It is also unlikely that the observed effects of N-glycosylation reported here are due to protection of the AChE molecule against deleterious proteolytic blood-borne activities, since incubation in murine plasma at 37 °C of all the AChE glycoforms under study did not result in any detectable loss of enzyme activity within the time-frame relevant to pharmacokinetic profiling (Table 4). Moreover, even when a prolonged incubation *in vitro* in the presence of murine serum ($\geqslant 24$ h) did have some effect on enzyme activity, it affected equally all the various AChE glycoforms.

The most likely interpretation of the observed pharmacokinetic improvement upon N-glycan addition is therefore the masking of clearance epitopes. Such epitopes are exposed when glycosylation sites are removed, as in the case of the N350Q mutant, which exhibits a pharmacokinetic behaviour inferior to that of the WT enzyme regardless of its sialylation and oligomerization state (Figure 6). It is worth noting that a possible involvement of amino acid epitopes in the clearance rate of AChE has been suggested by the observation that fully sialylated and tetramerized human and bovine AChE, both of which carry four glycans per enzyme subunit, differ in their circulatory retention rate (MRT for rHuAChE = 1055 min; MRT for bovine recombinant AChE = 1340 min; [11,12] and this report). These two versions of AChE differ in their primary amino acid sequence only by 34 residues [13]; yet these residues are clustered in three divergence patches that are all located on the surface of the molecule, and therefore may form unique surface-related epitopes, which might serve as ligands for protein removal. Furthermore, the existence of surface-related clearance epitopes was substantiated by the observation that a pharmacokinetically beneficial epitope-sheathing effect is promoted by poly(ethylene glycol) conjugation to the widely dispersed lysine residues on the AChE surface [55].

It may therefore follow that a further increase in the circulatory retention of the human version of AChE may be achieved by introducing additional N-glycosylation consensus signals to the tetraglycosylated rHuAChE at appropriate positions, which will mask the surface-related epitopes that promote clearance. It should be noted that, in the present study, addition of a fifth glycosylation site at position 541 (D61N/S541N rHuAChE)

failed to provide any detectable pharmacokinetic advantage over the tetraglycosylated D61N rHuAChE (Table 3). It is possible that this position is situated at too far a distance from any circulatory elimination epitope, and therefore cannot provide effective shielding. Further studies, which will examine the pharmacokinetic effect of introducing additional glycosylation sites in proximity with the human/bovine divergent amino-acid surface clusters, are presently being carried out at our laboratory.

The findings reported here have practical biotechnological implications with regard to the development of a prophylactic or post-exposure bioscavenger of organophosphate poisons. The series of studies documented here and in previous reports [10–12,14] establish that at least three post-translational modifications, N-glycosylation, glycan sialylation and subunit assembly state, are involved in a hierarchical manner in determining the clearance rate of exogenously administered recombinant glycoproteins. The ability to modulate these three parameters by recombinant DNA-engineering manipulations might allow us to favourably tailor the circulatory residence times not only of AChEs, but also of other biomolecules with therapeutic potential.

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