Involvement of oligomerization, N-glycosylation and sialylation in the clearance of cholinesterases from the circulation

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The possible role of post-translational modifications such as subunit oligomerization, protein glycosylation and oligosaccharide processing on the circulatory life-time of proteins was studied using recombinant human acetylcholinesterase (rHuAChE). Different preparations of rHuAChE containing various amounts of tetramers, dimers and monomers are cleared at similar rates from the circulation, suggesting that oligomerization does not play an important role in determining the rate of clearance. An engineered rHuAChE mutant containing only one N-glycosylation site was cleared from the circulation more rapidly than the wild-type triglycosylated enzyme. On the other hand, hyperglycosylated mutants containing either four or five occupied N-glycosylation sites, analagous to those present on the slowly cleared fetal bovine serum acetylcholinesterase (FBS-AChE), were also cleared more rapidly from the bloodstream than the wild-type species. Furthermore, the two different tetraglycosylated mutants were cleared at different rates while the pentaglycosylated mutant exhibited the most rapid clearance profile. These results imply that though the number of N-glycosylation sites plays a role in the circulatory life-time of the enzyme, the number of N-glycan units in itself does not determine the rate of clearance. When saturating amounts of asialofetuin were administered together with rHuAChE, the circulatory half-life of the enzyme was dramatically increased (from 80 min to 19 h) and was found to be similar to that displayed by plasma-derived cholinesterases while desialylation of these enzymes caused a sharp decrease in the circulatory half-life to approximately 3–5 min. Determination of the average number of sialic acid residues per enzyme subunit of the five different N-glycosylation species generated, revealed that the rate of clearance is not a function of the absolute number of appended sialic acid moieties but rather of the number of unoccupied sialic acid attachment sites per enzyme molecule. Specifically, we demonstrate an inverse-linear relationship between the number of vacant sialic acid attachment sites and the values of the enzyme residence time within the bloodstream.

INTRODUCTION

Acetylcholinesterase (AChE, EC 3.1.1.7) plays a pivotal role in the cholinergic system where it functions in the rapid termination of nerve impulse transmission. The enzyme has been the subject of intense research due to its focal position in many fields of interest. Modification in the levels of human brain AChE have been reported in various disorders such as Down's syndrome [1] and Alzheimer's disease [2]. Several cholinesterase (ChE) inhibitors have proven to be of value as medicinal agents and are used for the treatment of glaucoma or myasthenia gravis [3]. Previous reports have shown that plasma-derived AChE or butyrylcholinesterase can be used as a prophylactic antidote against organophosphate poisoning in animals [4–7] or for treatment of humans exposed to organophosphate pesticides [8]. Use of ChE as a biological scavenger requires sources for large quantities of purified enzyme and depends on the retention of the enzyme in the circulation for sufficiently long periods of time. Recombinant DNA techniques may provide the method to establish efficient production systems along with the means for introduction of new traits into the enzyme. Production of AChE in a bacterial expression system was reported [9], yet the product accumulated in the cells as inclusion bodies and recovery of the non-glycosylated active enzyme was low. Animal cell lines which are capable of properly performing post-translational modifications may provide an attractive alternative for large-scale production of recombinant protein possessing an extended circulatory life-time [10]. We have previously reported the establishment of recombinant expression systems in which transfected human embryonal kidney (HEK) 293 cell clones produce and secrete high levels of active human AChE (HuAChE) [11,12]. The recombinant enzyme is not O-glycosylated yet contains three sites for N-glycosylation, all of which were utilized [13].

HuAChE displays 90% sequence similarity to fetal bovine serum AChE (FBS-AChE), while both are equally divergent (50% identity) from human serum butyrylcholinesterase (HuS-BChE) [14]. When administered to test animals, plasma-derived HuS-BChE and FBS-AChE displayed clearance profiles characterized by half-life ($t_{\frac{1}{2}}$) values of several hours [7,15]. However, rHuAChE produced in *Escherichia coli* [9] was rapidly cleared from the circulation (M. Fischer, personal communication). Since glycosylation plays a role in determination of the circulatory life-time of proteins (reviewed in [16]), the accelerated elimination of the bacterial product may relate to the absence of post-translation modification processes in bacteria. Indeed, when subjected to sialidase treatment, plasma-derived BChE was rapidly cleared from the rat circulation [15]. In this study, we used various engineered glycoforms of the enzyme which display an N-glycosylation pattern mimicking that of the slowly cleared FBS-AChE, as well as dimerization-impaired AChE, to evaluate the potential contribution of N-glycan addition, oligosaccharide processing and subunit assembly to the residence of ChEs in the circulation.

Abbreviations used: AChE, acetylcholinesterase; ATC, acetyl-thiocholine; FBS-AChE, fetal bovine serum AChE; HEK-293, human embryonal kidney 293 cells; HuAChE, human AChE; HuS-BChE, human serum butyrylcholinesterase; ChE, cholinesterase; rHuAChE, recombinant HuAChE; $t_{\frac{1}{2}}$, half-life; TFPI, tissue-factor pathway inhibitor.

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EXPERIMENTAL

Construction of vectors for expression of hypo- and hyper-glycosylated rHuAChEs

Tripartite vectors expressing the human *ache* cDNA, the chloramphenicol acetyltransferase (*cat*) reporter gene and the selection marker *neo* gene were described previously [12,13]. Mutagenesis was performed by DNA cassette replacement into the wild-type cDNA or into an HuAChE sequence variant [17] which conserves the wild-type amino acids but carries new unique restriction sites based on the degeneracy of the genetic code. The cDNA spanning the codons targeted for mutagenesis was excised by incision at the nearest restriction sites on the appropriate vector and then replaced with a synthetic DNA duplex carrying the mutated codon. Construction of the N-glycosylation-deficient mutant, N350Q/N464Q, was reported previously [13]. The hyperglycosylated D61N mutant was generated by substituting the *Apa*I–*Esp*I fragment of the human *ache* variant Ew7 [13]. The D61N mutation created a fourth N-glycosylation consensus sequence (Asn-Ala-Thr). The S541N mutant was generated by substituting the *Bss*HI–*Sal*I fragment of the human *ache* cDNA. The S541N mutation also generated an additional N-glycosylation site (Asn-Ala-Thr). The HuAChE mutant D61N/S541N, which contains five N-glycosylation sites, was generated by replacing the *Bsu*II–*Spe*I DNA fragment of the D61N-containing construct, with that from the S541N-containing construct. Synthetic DNA oligodeoxynucleotides were prepared using the automated Applied Biosystems DNA synthesizer, and sequences of all the cloned synthetic DNAs were verified by the dideoxy sequencing method (USB sequenase kit).

Selection of high producer clones

CsCl-purified plasmid preparations were used to transfect HEK-293 cells by the calcium phosphate method as described before [12]. At least two different clone isolates were tested for each plasmid construct. After 24 h, cells were transferred to medium (2 ml per 100-mm-diam. plate) containing 10% (v/v) AChE-depleted serum [17] and incubated for 48 h. Medium was collected and assayed for AChE; medium of mock-transfected HEK-293 cells served as a control.

Stably transfected isolated clones were generated by G418 selection as previously described [12]. Isolated cell clones were evaluated by measuring secreted AChE activity according to the method of Ellman et al. [18]. Standard assays were performed in the presence of 0.5 mM acetylthiocholine (ATC), 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 using a Thermomax microplate reader (Molecular Devices).

Purification and quantification of ChEs

rHuAChE (wild-type and its glycoform-mutants) and FBS-AChE were purified on procainamide columns as described before [12]. Briefly, cell-culture supernatants were adsorbed to the procainamide–Sepharose 4B columns (4000 units/ml resin) which were then rinsed with 50 mM sodium phosphate buffer, pH 8.0/1 mM EDTA and again with 50 mM sodium phosphate buffer, pH 8.0/0.4 M NaCl/1 mM EDTA. Enzyme elution was performed with decamethonium (0.02 M) in 50 mM sodium phosphate buffer, pH 8.0/1 mM EDTA. HuS-BChE was purified in a similar manner except that the second column rinse was with 50 mM sodium phosphate buffer, pH 8.0/0.12 M NaCl/1 mM EDTA and elution was performed with 0.2 M decamethonium. rHuAChE was quantified by three methods: (a) enzyme activity (assigning a specific activity value of 6300 units/mg of protein [12]), coupled to protein mass determination by the Lowry method [19]; (b) a specific ELISA based on polyclonal antibodies to native rHuAChE [17]; and (c) active-site titration with MEPQ [7-(methylethoxy-phosphonyloxy)-1-methylquinolinium iodide; a gift from Dr. Y. Segall] as described before [11,20].

In vivo clearance experiments

Following extensive dialysis against PBS (pH 7.4) 20 μg of enzyme (about 120 units of HuAChE or 6 units of HuS-BChE, 0.2 ml total volume) was injected into the tail vein of each of 3–6 ICR male mice (25–30 g each). Samples (5 μl) of blood were removed at various periods of time, diluted 1:20 in water and kept on ice until ChE activity was determined using ATC or butyrylthiocholine (BTC) as substrate. ChE activity values in samples removed within 1 min of injection were referred to as input activities and were used for calculation of residual activity throughout the experiment. All values were corrected for background activity determined in blood samples withdrawn 1 h before performing the experiment. Exogenously administered ChE was at least 10-fold higher than the background endogenous ChE activity. Where specified, fetuin or asialofetuin (Sigma; 6 mg/mouse) were injected together with AChE. In some experiments asialofetuin was repeatedly administered at the same dosage, for a period of 8 h at 1 h intervals. When repeated injection coincided with blood sample collection, removal of blood preceded injection. When the experiment was completed, mice were killed with an overdose of anaesthetic. The study was approved by the local ethical committee on animal experiments.

Analysis of pharmacokinetic profiles

The clearance patterns of the enzyme were biphasic and were fitted to a bi-exponential elimination pharmacokinetic model of the general form $C_t = Ae^{-k\alpha t} + Be^{-k\beta t}$, where C_t represented the concentration of enzyme in the circulation at time t and $k\alpha$ and $k\beta$ were the first-order constants of the first and second elimination phases respectively. In all cases, the correlation coefficient was $\geqslant 0.997$.

Desialylation of AChE

Samples (0.5 mg) of rHuAChE or FBS-AChE were incubated for 3 h at 30 °C with 0.25 unit of agarose-bound neuraminidase (Sigma) in 30 mM phosphate buffer, pH 6.8. Neuraminidase was removed by Eppendorf centrifugation and enzyme was dialysed against PBS to remove free sialic acid. Prior to administration, the dialysed AChE was quantified by determination of enzymic activity.

Determination of sialic acid

Determination of sialic acid contents was performed on highly purified enzyme preparations. Sialic acid residues were released by incubation for 1 h at 80 °C in the presence of 0.05 M H_2SO_4. Quantification of sialic acid residues was achieved by the thiobarbituric acid method [21]. Sialic acid extracted into cyclohexanone was quantified at 549 nm alongside a standard curve of *N*-acetylneuraminic acid (Sigma) that was subjected to the same assay conditions.

Sucrose-density-gradient centrifugation

Analytical sucrose-density-gradient centrifugation was performed on 5–25% sucrose gradients containing 1 M NaCl/

50 mM sodium phosphate buffer, pH 8.0. Centrifugation was carried out in an SW41 Ti rotor (Beckman) for 22 h at 160000 *g* (36000 rev./min) at 4 °C. Fractions of 0.3 ml were collected and assayed for AChE activity. Alkaline phosphatase was used as a sedimentation marker.

Enzymic deglycosylation and gel electrophoresis

Purified protein samples were denatured by boiling in the presence of 0.5% SDS/0.06% 2-mercaptoethanol. Where noted, samples were digested with 0.6 unit of N-Glycanase [peptide-*N*4-(*N*-acetyl-β-glucosaminyl) asparagine amidase; Genzyme, U.S.A.] at 37 °C. Digestion buffer included 200 mM sodium phosphate buffer, pH 8.0/0.17% SDS/1.25% Nonidet P-40/30 mM 2-mercaptoethanol.

Purified enzyme was subjected to 0.1% SDS/7.5% polyacrylamide gel analysis [22] in the presence of 2-mercaptoethanol using the Phast System (Pharmacia LKB Biotechnology) with reagents and electrophoresis conditions supplied by the manufacturer. Bands were visualized by silver staining and apparent molecular masses were calculated based on a commercial mixture of polypeptide size markers (Bio-Rad) which were run alongside.

RESULTS

Comparison of the clearance profiles of rHuAChE and plasma-derived ChEs

To determine the residence of rHuAChE in the circulation, ICR mice were injected with 120 units of purified enzyme (6300 units/mg of protein). The clearance process of rHuAChE (Figure 1) displays a pattern which fits a bi-exponential elimination pharmacokinetic model (see the Experimental section); the initial decline is characterized by a first-order rate constant ($k\alpha$) of 9.5×10^{-2} min^{-1} ($t_{\frac{1}{2}}\alpha = 7.5$ min), while the second-phase decline exhibited a first-order rate constant ($k\beta$) of 9×10^{-3} min^{-1} ($t_{\frac{1}{2}}\beta =$ 80 min). These values were compared with clearance profiles of equivalent amounts of FBS-AChE and HuS-BChE purified from the respective plasmas. Both of the plasma ChEs showed prolonged residence values in the circulation (FBS-AChE: $k\alpha = 1.7 \times 10^{-2}$ min^{-1}, $k\beta = 7 \times 10^{-4}$ min^{-1}; HuS-BChE: $k\alpha = 7 \times 10^{-3}$ min^{-1}, $k\beta = 4.6 \times 10^{-4}$ min^{-1}) compared with the recombinant

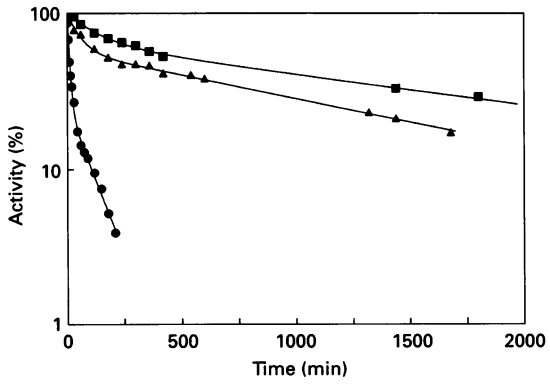

Figure 1 Comparison of clearance rates of recombinant HuAChE and plasma-derived ChEs

Purified enzyme was administered to mice (20 μg/mouse in 0.2 ml). ChE activity values in samples removed immediately after injection were assigned a value of 100% and were used for calculation of residual activity. Values represent average of residual activity determined for 3–6 mice (S.D. < 10%). ●, recombinant HuAChE; ▲, FBS-AChE; ■, HuS-BChE.

product (Figure 1, Table 1). To determine whether the relatively rapid clearance rates of rHuAChE are due to an intrinsic instability of the product, 30 units of rHuAChE were incubated in 0.5 ml of blood samples drawn from ICR mice. After 24 h incubation at 37 °C no decrease in activity was detectable, suggesting that the rHuAChE is stable under these conditions and that the loss of AChE activity in the circulation is mediated by a clearance mechanism and does not reflect some instability/degradation of the recombinant product by blood factors.

Hyperglycosylated rHuAChE does not display extended half-life values

Glycosylation is well known to influence residence values in many biological systems [16]. One prominent feature in which rHuAChE differs from both FBS-AChE and HuS-BChE is the number of N-glycosylation consensus signals present on the enzyme. While FBS-AChE and HuS-BChE contain five and nine such sites respectively [14,23], the rHuAChE sequence [24] displays only three N-glycosylation sites. This difference could have been responsible for the relatively rapid clearance of rHuAChE from the circulation. Indeed, when mice were injected with a mutant rHuAChE which retains only one site for N-glycan attachment (Table 2, N350Q/N464Q, [13]) clearance was substantially enhanced in comparison with wild-type enzyme (N350Q/N464Q: $t_{\frac{1}{2}}\beta = 45$ min; wild type: $t_{\frac{1}{2}}\beta = 80$ min). In line with this observation, we generated rHuAChE molecules containing additional glycan-attachment sites to examine whether these species would be retained in the circulation for longer periods of time than the wild-type enzyme. FBS-AChE shows a high degree of sequence similarity to HuAChE and three of the five FBS-AChE glycosylation sites are identical to those found on recombinant AChE [14]. We therefore used the FBS-AChE template as a guideline for construction of the HuAChE hyperglycosylated forms (Figure 2). Specifically, the Glu and Ser residues at positions 61 and 541 of HuAChE were substituted by Asn to give rise to the Asn-Xaa-Thr/Ser N-glycosylation consensus signals. These two engineered sites are analogous to two natural glycosylation sites present in the FBS-AChE. Analysis of the HuAChE hyperglycosylation mutants by SDS/PAGE (Figure 3, inset) revealed that while the major band of the wild-type species exhibited an apparent molecular mass of 70 kDa, the HuAChE mutants D61N and S541N, which contain four glycosylation sites each, had a higher apparent molecular mass of 72 kDa. The double mutant D61N/S541N species which includes five N-glycan attachment sites, mimicking the potential N-glycosylation attachment site pattern of FBS-AChE, displayed an even higher molecular mass of 74 kDa. Thus in all cases, the added N-glycosylation attachment sites appeared to be utilized. This conclusion is supported by the fact that both stepwise removal and addition of N-glycosylation sites gives rise to mutated enzymes whose apparent molecular masses show a linear dependence on the number of sites present on the molecule (Figure 3). In addition, when subjected to N-Glycanase digestion, which catalyses the hydrolysis of Asn-linked oligosaccharides, the wild-type and each of the mutants co-migrated as a discrete band of approx. 63 kDa (Figure 3), proving that the differences in the molecular mass of the various species is entirely due to differential N-glycosylation. All three hyperglycosylated species displayed catalytic values identical to those manifested by the wild-type enzyme, suggesting that the number of attached N-glycosylation oligosaccharide side chains has no influence on the intrinsic biochemical traits or functional conformation of the enzyme. These results are consistent with previously published data which show that hypoglycosylated AChE retained full

Table 1 Comparison between the pharmacokinetic parameters of rHuAChE and plasma-derived ChEs

ChE	No. of N-glycosyl. sites	Sialic acid/AChE[a]	A[b] (% of total)	$k\alpha$ ($\times 10^{-2}$ min^{-1})	$t_{\frac{1}{2}}\alpha$ (min)	B (% of total)	$k\beta$ ($\times 10^{-3}$ min^{-1})	$t_{\frac{1}{2}}\beta$ (min)
HuAChE	3	4	74±5	9.5±0.8	7.3±0.7	25±1.5	9±0.4	80±4
FBS-AChE	5	9	43±2.2	1.7±0.2	41±5	57±1.7	0.7±0.05	990±67
HuS-BChE	9	16	36±2.2	0.7±0.1	96±13	64±2.3	0.6±0.02	1500±73

[a] Molar ratio of sialic acid residues per cholinesterase catalytic subunit.

[b] A, B, $k\alpha$ and $k\beta$ are components of the bi-exponential elimination equation ($C_t = Ae^{-k\alpha t} + Be^{-k\beta t}$). A and B are the amounts of enzyme at $t = 0$ (expressed as percentages of total enzyme) which are then cleared in the first and second elimination phases, respectively. $k\alpha$ and $k\beta$ are the first-order constants and $t_{\frac{1}{2}}\alpha$ and $t_{\frac{1}{2}}\beta$ the half-life values of the first and second elimination phases, respectively.

Table 2 Compilation of the pharmacokinetic parameters of rHuAChE and its various mutants

rHuAChE	A (% of total)[a]	$k\alpha$ ($\times 10^{-2}$ min^{-1})	$t_{\frac{1}{2}}\alpha$ (min)	B (% of total)	$k\beta$ ($\times 10^{-3}$ min^{-1})	$t_{\frac{1}{2}}\beta$ (min)
Wild-type	74±5	9.5±0.8	7.3±0.7	25±1.5	9±0.4	80±4
N350Q/N464Q	62±5	6±0.6	11.0±1.2	40±2.2	15±1	45±3
S541N	68±8	10±2	7.1±1.2	32±3	10±0.7	71±5
D61N	69±11	15±4	4.6±0.3	34±4.6	17±1	41±3
S541N/D61N	78±5	18±2	3.9±1	34±5	28±6.4	25±7
C580A	65±3	18±3	3.8±0.7	35±2	13±3	50±8
AsialoWT[b]	100	21±1	3.3±0.15	–[b]	–[b]	–[b]
WT+ASF [c]	63±3	1±0.1	73.0±8	36±3	0.6±0.04	1140±77

[a] A, B, $k\alpha$ and $k\beta$ are components of the bi-exponential elimination equation ($C_t = Ae^{-k\alpha t} + Be^{-k\beta t}$). A and B are the amounts of enzyme at $t = 0$ (expressed as percentages of total enzyme) which are then cleared in the first and second elimination phases, respectively. $k\alpha$ and $k\beta$ are the first-order constants and $t_{\frac{1}{2}}\alpha$ and $t_{\frac{1}{2}}\beta$ the half-life values of the first and second elimination phases, respectively.

[b] Wild-type rHuAChE pretreated with sialidase (see the Experimental section). This species displayed a single-phase clearance profile.

[c] Asialofetuin (ASF) was administered together with the initial bolus of wild-type (WT) rHuAChE and then reinjected at 1 h intervals over an 8 h period.

activity [9,13,25]. Though glycosylation has been shown to influence thermostability of proteins [26], the engineered rHuAChE hyperglycoforms displayed very similar heat-inactivation profiles at 55 °C, as well as comparable stabilities when incubated at 37 °C in mouse serum.

Each of the three hyperglycosylated rHuAChE species was administered to mice and clearance profiles were determined. Unexpectedly, all of the hyperglycosylated forms displayed shorter half-lives in the circulation than the triglycosylated wild-type enzyme (Table 2). Furthermore, the two tetraglycosylated species were found to be markedly different from one another in respect to their clearance rates; while the tetraglycosylated S541N mutant exhibited a slightly reduced half-life in comparison with the wild-type enzyme (S541N: $t_{\frac{1}{2}}\alpha = 7.1$ min, $t_{\frac{1}{2}}\beta = 70$ min), the clearance rate of the D61N species was significantly faster than that of the wild-type species ($t_{\frac{1}{2}}\alpha = 4.6$ min, $t_{\frac{1}{2}}\beta = 40$ min). Moreover the D61N/S541N pentaglycosylated species, which has the highest number of N-attached oligosaccharides and mimics the N-glycosylation occupancy pattern of FBS-AChE, manifested the highest clearance rate ($t_{\frac{1}{2}}\alpha = 3.9$ min, $t_{\frac{1}{2}}\beta = 25$ min). Thus, extension of circulatory life-time was not achieved in the mutated species containing additional N-glycosylation sites, and therefore there is no simple relationship between the number of N-glycan moieties and the circulatory residence of ChEs.

Clearance rates of rHuAChE glycoforms are related to sialic-acid contents

The fact that increased N-glycosylation had an adverse effect on circulatory life-time, raises the possibility that different rates of clearance exhibited by the various rHuAChE glycoforms may be due to differential processing and maturation of the N-glycan units on the various molecular species. N-glycan units of a given protein can have different structures and outer-chain moieties as a result of site-specific glycosylation at multiple sites (reviewed in [10]).

Circulatory half-life values of glycoproteins are influenced by their sialic acid content [27]. To test whether the presence of outer-chain sialic acid residues plays a determinal role in the circulatory life-time of rHuAChE, sialic acid residues were removed from the oligosaccharide chains by treatment with sialidase. Asialylated rHuAChE was administered to mice and clearance of the enzyme from the circulation was monitored. The asialo-rHuAChE indeed exhibited a rapid single-phase clearance pattern with a half-life value of 3.3±0.15 min (Table 2). Furthermore, when FBS-AChE, which exhibits high $t_{\frac{1}{2}}$ values in the circulation (Table 1), was subjected to the same sialidase treatment, the resulting asialo-FBS-AChE was cleared rapidly from the bloodstream at a rate similar to that of asialo-HuAChE ($t_{\frac{1}{2}} = 5.0 \pm 0.4$ min). These results suggest that sialylation is essential for retention of rHuAChE in the bloodstream and that the marked difference in the clearance rates of rHuAChE and FBS-AChE may relate to differences in N-glycan sialylation. To further test this possibility, we examined the ability to retain rHuAChE in the circulation in the presence of saturating amounts of asialofetuin. The latter should allow prolonged residence of undersialylated AChE in the bloodstream [28–32] by saturating the hepatic asialoglycoprotein receptors which comprise the major system for clearance of hyposialylated glycoproteins [27]. When mice were injected with rHuAChE or any of its hyper-

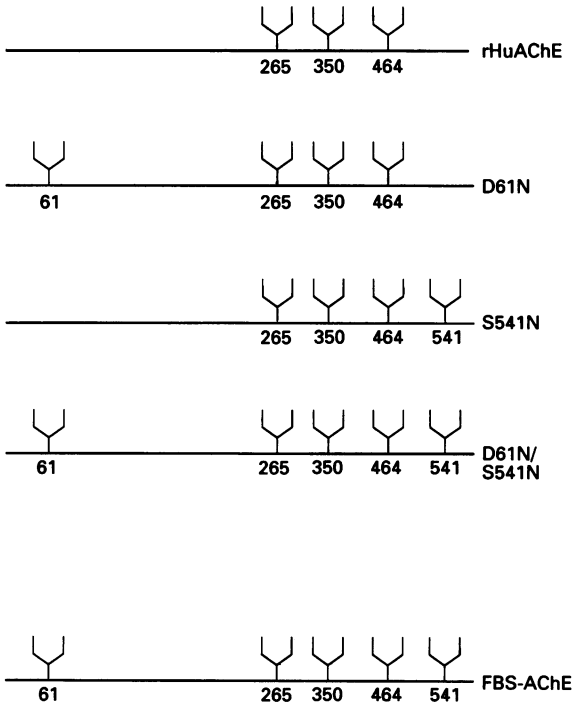

Figure 2 Diagrammatic representation of hyperglycosylation mutant structures

The various hyperglycosylation mutants described in these studies are shown as amino-acid backbones (black line) with their corresponding N-glycans (oligosaccharide projections). Wild-type enzyme (top line) and FBS-AChE (bottom line), which served as a guideline for generation of the hyperglycosylated forms, are shown as well. Numbers refer to the N-glycan attachment sites in the human enzyme amino-acid sequence (top four structures) or the FBS enzyme sequence (bottom structure). These are identical due to the co-linearity of the two proteins [14].

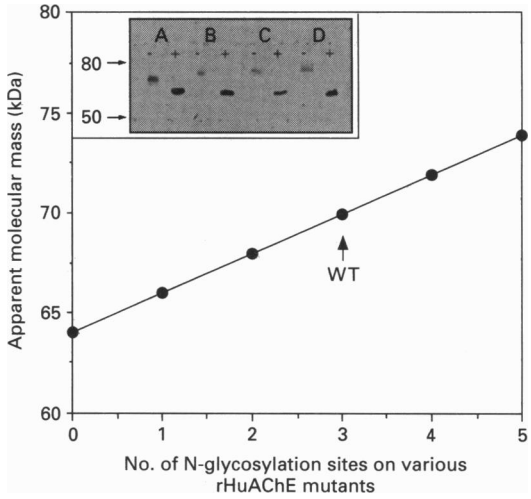

Figure 3 Correlation between apparent molecular masses of rHuAChE derivatives and the number of N-glycosylation sites per enzyme subunit

Purified enzyme preparations (0.2 μg per lane) were subjected to 0.1% SDS/7.5% polyacrylamide gel analysis in the presence of 2-mercaptoethanol. Bands were visualized by silver staining. The apparent molecular masses of the major bands of D61N, S541N, D61N/S541N and wild-type are 72 kDa, 72 kDa, 74 kDa and 70 kDa respectively, based on a commercial mixture of polypeptide size markers (Bio-Rad). A curve displaying the apparent molecular mass of the various rHuAChE glycoforms as a function of the number of N-glycosylation sites is shown. Data referring to hypoglycosylated forms of the enzyme [13] were added. Inset: Silver stained SDS/polyacrylamide gel of wild-type and hyperglycoforms of rHuAChE. (−) and (+) denote untreated and N-Glycanase-treated samples respectively. A, Wild type; B, D61N; C, S541N; D, D61N/S541N. Arrows denote molecular-mass markers in kDa.

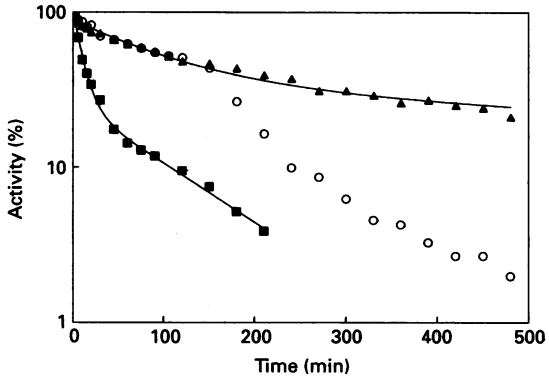

Figure 4 rHuAChE displays prolonged circulatory life-time when administered with asialofetuin

Wild-type rHuAChE was administered to mice without protein additives (■), together with a single bolus of asialofetuin (○), with asialofetuin and thereafter re-injection of asialofetuin at 1 h intervals over a period of 8 h (▲). The clearance profiles were determined as described in the legend to Figure 1.

glycosylated forms together with a single bolus of asialofetuin, the initial decline was markedly slower than that observed for the corresponding enzyme when administered without protein additives (Figure 4) or together with fetuin. The accurate clearance rate was difficult to determine since the clearance process was visibly accelerated after a period of approx. 2 h (Figure 4). Previous reports have shown that the effect of a single bolus of the asialylated protein is limited to a relatively short period of time [32], presumably due to regeneration of the asialoglycoprotein receptors. To overcome this problem AChE was administered to mice simultaneously with asialofetuin; thereafter saturating amounts of asialofetuin were injected into the tail vein at 1 h intervals over a period of 8 h. A dramatic increase in both clearance phases was seen in this case (Figure 4). The calculated half-life values of both the α phase and the β phase displayed approx. a 10-fold increase as compared with the clearance rate of rHuAChE without asialofetuin (Table 2). Similar results were obtained for each of the hyperglycosylated forms of the enzyme when administered together with asialofetuin (results not shown). It is worth noting that in the presence of steady-level asialofetuin, the $t_{\frac{1}{2}}$ values of rHuAChE clearance are similar to those displayed by the plasma-derived enzymes, FBS-AChE and HuS-BChE (Table 1). Taken together, these results indicate that the relative short half-life of rHuAChE stems from inefficient sialylation, and are in line with those reported by Douchet et al. [15] which showed that sialylation plays an important role in retention of BChE in the rat circulation. Most importantly, the results suggest that the primary structure of the rHuAChE can, in principle, allow prolonged residence of the recombinant protein within the bloodstream comparable to natural plasma ChEs.

To determine whether the variation in sialylation levels of the various rHuAChE mutants is related to their differential clearance rates, the average molar ratio of sialic acid per rHuAChE catalytic subunits was calculated. Sialic acid contents of the wild-type and hyperglycosylated rHuAChE species were determined

Table 3 Sialic acid occupancy of rHuAChE and its various mutants

Abbreviation: WT, wild type.

rHuAChE	No. of N-glycosylation sites	Sialic acid/AChE [a]	Calculated vacant sites/AChE[b]
WT	3	4.2±0.3	2
AsialoWT	3	< 0.1	6
C580A	3	2.1±0.2	4
D61N	4	4.1±0.1	4
S541N	4	5.4±0.3	3
D61N/S541N	5	5.3±0.2	5

[a] Molar ratio of sialic acid residues per catalytic subunit.

[b] No. of unoccupied sialic acid attachment sites per catalytic subunit was calculated as [(number of N-glycosylation sites) × 2 − (number of sialic acid residues)].

by the thiobarbituric acid method [21]. Purified enzyme was quantified by three independent methods: (1) enzymic activity and protein determination; (2) immunoassay (quantitative ELISA); and (3) active-site titration. Results obtained by all the quantification methods yielded similar values for rHuAChE (±7%), indicating that the enzyme preparations were virtually free from impurities which can distort interpretation of the sialic acid quantification.The wild-type rHuAChE species was found to contain four sialic acid residues per enzyme subunit (Table 3). This is lower than the value expected for an enzyme with three N-glycosylation sites, each of which can be expected to contain a bi-antennary N-glycan unit with two sites for sialic acid attachment (see below). The two tetraglycosylated rHuAChE species were found to differ in their sialic acid contents; while the S541N species carries five sialic acid moieties per molecule, the D61N species displayed the same sialylation level as in the wild-type enzyme; namely, four sialic acid residues per molecule. Thus, the engineered N-glycan located on amino acid 61 is differently processed so that no sialic acid is terminally appended at this site. Indeed, the pentaglycosylated D61N/S541N species was found to contain only five sialic acid residues per enzyme subunit, confirming that the additional 541 but not the 61 N-glycan moiety is sialylated. The varying levels of sialylation displayed by the different rHuAChE glycoforms may possibly play a role in determining their different rates of clearance. In contrast to the various rHuAChE glycoforms which exhibit incomplete sialylation, the plasma-derived ChEs are efficiently sialylated. FBS-AChE and HuS-BChE, which contain five and nine N-glycosylation sites respectively, exhibit a molar ratio of sialic acid:enzyme of (9±2)/1 and (16±3)/1 respectively (Table 1).

Effect of rHuAChE subunit assembly on the rate of clearance

Plasma-derived ChEs are usually in the form of tetramers [33,34]. Wild-type rHuAChE, as well as the hyperglycosylated species, retains the ability to assemble into homo-oligomers consisting of dimers and tetramers, yet the relative proportions of the various assembled forms constituting the enzyme preparations may vary under different growth or storage conditions ([35,36]; C. Kronman, B. Velan, D. Marcus, A. Ordentlich, S. Reuveny and A. Shafferman, unpublished work). Tertiary structure as well as molecular mass has been shown to contribute to the *in vivo* circulatory life-time of proteins [37]. To examine the possible influence of the assembled forms on clearance rates, two preparations of the wild-type enzyme with an equal ratio of sialic acid per subunit (4:1), but differing one from another in the ratio of assembled forms (as determined by sucrose gradients), were introduced into mice. Examination of enzyme removed from the bloodstream 2 h after injection revealed that no significant alteration of subunit organization occurs in the circulation. In both cases, the time-course of enzyme elimination was found to be similar (results not shown). These findings suggest that the number of assembled subunits in itself does not play an important role in determining the clearance rate of the enzyme from the circulation.

To assess further the possible contribution of subunit assembly on circulatory retention, we tested the assembly-deficient mutant (C580A) described previously [11]. The replacement of cysteine at position 580 by alanine, generated monomeric forms of rHuAChE that, like the wild-type species, utilize all three N-glycosylation sites and are also catalytically indistinguishable from the wild-type [11]. This monomeric variant of HuAChE was administered to mice and clearance profiles were determined. The $t_{\frac{1}{2}}\alpha$ and $t_{\frac{1}{2}}\beta$ values were 4.0±0.7 min and 50±11 minutes respectively (Table 2), considerably shorter than the values exhibited by the wild-type enzyme. Incubation of the C580A HuAChE mutant in plasma at 37 °C for over 6 h had no effect on its activity, demonstrating that the difference in clearance rates of the wild-type and C580A mutant can not be explained in terms of a greater lability of the monomeric form relative to wild type [11,38]. On the other hand, when subjected to sialic acid analysis, the dimerization-impaired species was found to contain only two sialic acid residues per molecule as compared to the 4:1 molar ratio found in the wild-type enzyme (Table 3). Thus, the relatively rapid clearance of the monomeric form can be explained in terms of sialic acid content. Previous studies [38,39] indicated that transit of monomers through the *trans*-Golgi apparatus and *trans*-Golgi network en-route to secretion is more rapid than that of the assembled wild-type AChE. This rapid transport may provide an explanation for the lower efficiency of monomer sialylation compared with wild-type sialylation.

DISCUSSION

Comparison of the rates of clearance of rHuAChE and serum-derived ChEs reveals that the former is eliminated from the bloodstream faster than either FBS-AChE or HuS-BChE, both of which are cleared from the circulation at comparable rates. rHuAChE is highly homologous to FBS-AChE (90% sequence similarity) while both are equally divergent from HuS-BChE (50% sequence similarity), suggesting that the relatively rapid rate of clearance of rHuAChE does not stem from its primary sequence but rather from some post-translational process which differentiates it from the serum-derived ChEs. One salient feature which distinguishes rHuAChE from FBS-AChE and which may account for their differential clearance rates is their order of assembly. While FBS-AChE preparations consist entirely of tetramers, various amounts of dimers, tetramers and monomers comprise different rHuAChE preparations. Since the filtration rate through kidney glomerular tubules is sensitive to the molecular mass of proteins, allowing continuous removal of proteins with molecular masses of less than 70 kDa from the circulation [37], one can speculate that subunit assembly may play a role in retention of exogenous ChEs from the circulation. Yet, the nearly identical clearance rates of rHuAChE preparations containing differently assembled forms of enzyme, contradicts the alleged importance of ChE assembly in circulatory longevity.

Comparison of the amino-acid sequences reveals that while FBS-AChE and HuS-BuChE contain five and nine sites for appendage of N-glycosylation side-chains respectively, HuAChE contains only three, all of which are utilized [13] in established

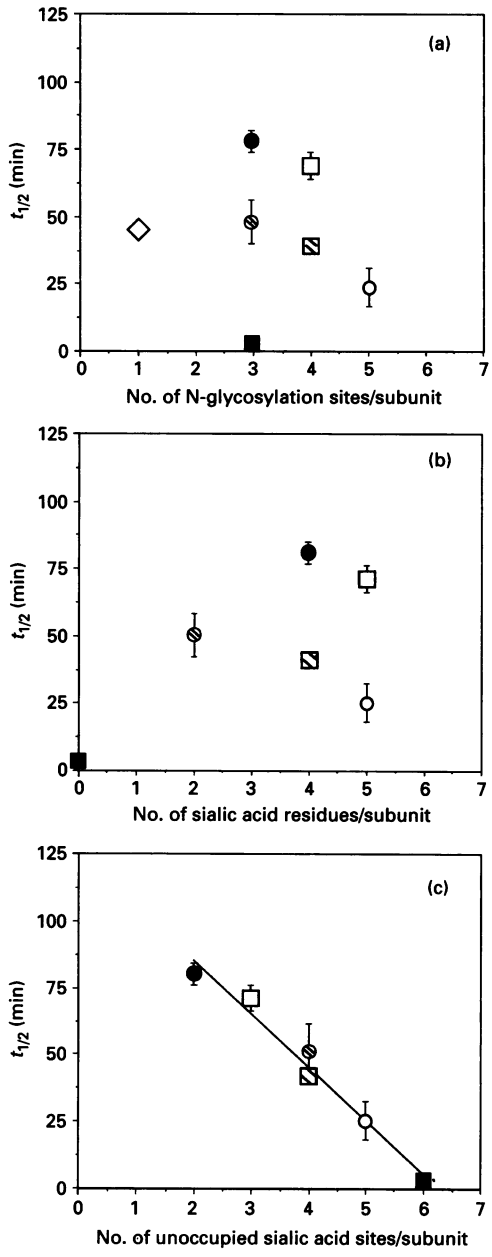

Figure 5 Dependence of clearance rates of rHuAChE and its various mutants on the number of N-glycosylation side-chains, sialic acid residues, and unoccupied sialic acid attachment sites

The half-life values (second phase, $t_{\frac{1}{2}}\beta$) of the various rHuAChEs were plotted against the number of N-glycosylation sites/subunit (**a**), the molar ratio of appended sialic acid residues/subunit (**b**) and the number of unoccupied sialic acid attachment sites/subunit (**c**) for each of the different rHuAChE species. The number of unoccupied sialic acid attachment sites was calculated as described in Table 3. Key to symbols: diamond, N350Q/N464Q monoglycosylated mutant; closed circle, wild-type enzyme; open square, S541N tetraglycosylated mutant; striped circle, dimerization-impaired C580A mutant; striped square, D61N tetraglycosylated mutant; open circle, D61N/S541N pentaglycosylated mutant; closed square, sialidase-treated wild-type rHuAChE (asialoWT).

recombinant HEK-293 cell clones which efficiently produce and secrete active enzyme [12,36]. N-linked side chains can control a wide variety of functions including resistance to thermal inactivation and proteolytic attack, signal transduction, receptor activation, intracellular folding and activity, as well as playing a pivotal role in determining plasma clearance. Thus, it may be possible that the relatively rapid removal of rHuAChE from the circulation stems from the low level of N-glycosylation. In the present study we found that an rHuAChE mutant which contains only one N-glycosylation site was indeed cleared more rapidly from the circulation of mice than the wild-type AChE expressed in the same HEK-293 cell type (Table 2), suggesting that N-glycan moieties are indeed essential for the prolonged circulatory life-time of the enzyme. In accordance with these results, we examined whether the circulatory life-time of ChEs can be extended by increasing the number of N-glycosylation sites per enzyme. To this end, we generated a series of hyperglycosylated rHuAChE forms which contain either one or two additional sites for N-glycosylation at sites homologous to those present in FBS-AChE. This allows formation of catalytic subunits which bear the basic amino-acid backbone of human AChE yet which mimic the N-glycan occupancy pattern of the slowly cleared FBS-AChE (see Figure 2). Unexpectedly, all these hyperglycosylated mutants of rHuAChE were eliminated more rapidly than the wild-type enzyme (Table 2), even though all the additional sites are utilized (Figure 3), demonstrating that the circulatory life-time does not correlate exclusively to the number of N-glycosylation side-chains (Figure 5a) and that increasing the number of N-glycosylation side-chains in itself can not ensure sustained residence in the bloodstream but on the contrary, may have an adverse effect on enzyme residence in the bloodstream.

Enzymically desialylated rHuAChE displayed an extremely short life-time in the circulation (Table 2), suggesting an essential role for sialic acid in retention of AChE in the bloodstream. Comparison of the two tetraglycosylated species of rHuAChE (D61N and S541N, Table 2) which contain the same number of N-glycan moieties, demonstrates that the difference in rates of clearance is directly related to the sialic acid contents. Finally, when enzyme was administered together with excess asialofetuin to saturate the hepatic asialoglycoprotein clearance system, the circulatory residence of rHuAChE was dramatically increased (Figure 4). In this case, both the α and β elimination phases are similarly extended (approx. 10-fold increase) and exhibit $t_{\frac{1}{2}}$ values comparable with those of the plasma-derived FBS-AChE and HuS-BChE (see Tables 1 and 2). Similar results were obtained for each of the hyperglycosylated forms of the enzyme when administered with asialofetuin (results not shown). Extended circulatory residence was not observed when any form of the enzyme was administered together with fetuin. Taken together, these results argue against the possibility that the hyperglycosylated forms are more rapidly cleared due to the possible presence of immature high-mannose structures, but rather suggest that the clearance rate of rHuAChE and its hyperglycosylated forms is determined by the sialylation efficiency of the N-glycan units. However, the sialic acid content in itself can not provide an explanation for the residential half-life values of the various species (Figure 5b) since FBS-AChE and HuS-BChE contain significantly different amounts of sialic acid residues per subunit (Table 1), yet are similarly retained in the bloodstream. Moreover, the D61N tetraglycosylated species both contain the same proportion (4:1) of sialic acid residues per enzyme subunit yet they display strikingly different half-life values in the circulation. Likewise, the tetraglycosylated S541N mutant and the pentaglycosylated D61N/S541N mutant both contain 5:1 sialic acid per enzyme, yet their elimination rates from the bloodstream are different and faster than that of the wild type (Table 2).

The pioneering studies of Ashwell and co-workers have well established the fact that the hepatic asialoglycoprotein receptor provides a major system for the removal of undersialylated glycoproteins by binding and uptake of galactose-terminal glyco-

proteins (reviewed in [27,40]). The latter is mediated by receptor recognition of sites on the N-linked oligosaccharide which display non-sialylated galactose residues. In accordance with these findings our results indeed demonstrate that the decisive factor in determining the variance in clearance rates is related to the number of N-glycan termini which are not occupied by sialic acid residues, rather than to the absolute number of sialic acid residues present on the oligosaccharide chain. Estimation of the number of sites which are vacant of sialic acid depends upon the branching of the N-glycan unit. Experimental determination of branching by quantification of terminal galactose residues present on desialylated enzyme is not possible due to the very large amounts of highly purified enzyme required [41,42]. Yet, we can expect rHuAChE produced in HEK-293 cells to be mostly bi-antennary since bisecting GlcNAc is detected in the sugar chains of enzymes expressed in kidney cells due to strong expression of GlcNAc transferase III [43]; this in turn should lead to commitment of the oligosaccharide to a processing pathway leading to a bisected bi-antennary complex-type structure [16]. In addition, studies with recombinant tissue-factor pathway inhibitor (TFPI) have shown that in HEK-293 cells the recombinant TFPI is of the bi-antennary type, unlike that expressed in Chinese hamster ovary cells where approx. 60% of the N-glycan units have tri- and tetra-antennary structures [44]. Similarly, the most abundant species of oligosaccharides found on recombinant human protein C expressed in HEK-293 cells are of the bi-antennary type [45]. Based on these facts, it is reasonable to assume that the N-glycan structures of rHuAChE expressed in HEK-293 cells contain primarily bi-antennary projections. Accordingly, we calculated (Table 3) the number of non-sialylated sites per enzyme subunit for each of the rHuAChE species as follows: unoccupied sialic acid sites = (the number of N-glycosylation sites × 2) − the number of sialic acid residues (O-glycosylation of rHuAChE does not occur [12] and therefore cannot contribute to the sialic acid content of the enzyme). When the clearance rates are plotted against the calculated unoccupied sites an inverse linear relationship is observed. Though these results are more pronounced for the β phase in which a more accurate measurement is possible (Figure 5c), the general trend is clearly seen in the α phase as well (results not shown). This linear correlation was retained also when the calculated number of antennary projections per glycosylation site was not precisely 2.0 but anywhere within the range of 1.5 to 2.5, suggesting that even if the rHuAChE N-oligosaccharides were to consist of equimolar amounts of bi- and tri-antennary moieties, the linear dependence of clearance on the number of unoccupied sialic-acid attachment sites is observed. This mode of presentation of the data provides a rationale for the unexplained differences observed in the rates of clearance of the two tetraglycosylated species, or the rapid elimination of the pentaglycosylated form as well as the accelerated clearance of the assembly-impaired form (monomer) of the enzyme (C580A mutant). On the other hand, neither the number of N-glycosylation sites nor the number of appended sialic acid residues provided a linear dependence for the different rates of clearance observed for the various rHuAChE species (compare Figures 5a and 5b to Figure 5c). Moreover, enzyme molecules which display a higher content of sialic acid moieties concomitant with a greater number of vacant sialic acid attachment sites are removed from the circulation more rapidly than glycoforms which contain less sialic acid residues but which display higher sialic acid occupancy (see Tables 2 and 3). It therefore appears that the number of unoccupied or vacant sialic acid attachment sites is the predominant factor in determining the rates of clearance, overriding the possible contribution of increased N-glycosylation or sialic acid contents to circulatory longevity. The fact that the clearance rate of monomeric AChE correlates with the sialic acid occupancy in a similar manner to that of the wild-type multimeric species (Figure 5c) and that when monomeric rHuAChE was administered together with asialofetuin the enzyme was retained in the circulation to the same extent as the wild-type enzyme under the same conditions (results not shown) supports our conclusion that oligomerization in itself is not an important factor in determining the circulatory half-life.

While the linear relationship between non-occupied sialylation sites and *in-vivo* circulatory half-life can be demonstrated for all the examined rHuAChE species in which two to six potential sites are indeed vacant, extrapolation to species with less or more vacant sites should be approached with caution. Ashwell and Morell [27] have determined two as the minimal requirement of exposed galactose residues for clearance by the hepatocyte receptor. Thus, the linear relationship shown in the present study may probably be valid for those species which have at least two vacant sialic acid attachment sites. To our knowledge, our results are the first to show a strictly linear relationship between vacant sialic acid attachment sites and circulatory life-time of exogenous glycoprotein, and to provide the means to assess the potential contribution of varied factors such as state of assembly, number of N-oligosaccharides and sialic-acid contents to circulatory longevity, utilizing an array of engineered proteins of virtually identical primary sequences.

Fully sialylated ChE species should display extended circulatory residence as suggested by the experiments in which rHuAChE was administered together with asialofetuin. In this case, circulatory half-lives are comparable with those of plasma ChEs. Glycoproteins purified from plasma in which they normally reside e.g. α_1-acid glycoprotein [29], human choriogonadotropin [31], carcinoembryonic antigen [30], rat transcortin [28] and prothrombin [32] usually do not serve as ligands for the asialoglycoprotein receptor, presumably due to protective N-glycan processing. Indeed, plasma-derived FBS-AChE and HuS-BChE, which exhibit extended circulatory life-times, were found to be practically fully sialylated (Table 1). The undersialylation of the rHuAChE molecule may be due to some limitation of the biosynthetic pathway in HEK-293 cells. Indeed, when expressed in HEK-293 cells, recombinant human protein C contained a 2-fold lower content of sialic acid than its plasma-derived counterpart [45]. On the other hand, undersialylation of rHuAChE in HEK-293 cells may depend on an intrinsic property of the polypeptide. Structure-related variations in processing of N-glycans of other proteins was reported previously [46] as is indeed reflected by the difference in the sialic acid contents observed for the D61N and S541N tetraglycosylated mutants (Table 3).

To date, we have generated a panoply of mutagenized rHuAChE species which differ in their kinetic properties and affinity to various inhibitors [17,20,47–49]. These may be usefully incorporated in a designed *in vivo* bioscavenger of pernicious neurotoxins. The present study provides some clues regarding the design of such a bioscavenger with extended circulatory longevity.

This work was supported by the U.S. Army Research and Development Command, Contract DAMD17-93-C-3042 (to A.S.). We thank Mrs. L. Raveh for helpful discussions and Levana Motola, Nehama Zeliger and Tamar Sery for their excellent technical assistance.

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Received 9 February 1995/30 June 1995; accepted 6 July 1995