Hierarchy of Post-translational Modifications Involved in the Circulatory Longevity of Glycoproteins

DEMONSTRATION OF CONCERTED CONTRIBUTIONS OF GLYCAN SIALYLATION AND SUBUNIT ASSEMBLY TO THE PHARMACOKINETIC BEHAVIOR OF BOVINE ACETYLCHOLINESTERASE

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The tetrameric form of native serum-derived bovine acetylcholinesterase is retained in the circulation for much longer periods (mean residence time, MRT = 1390 min) than recombinant bovine acetylcholinesterase (rBoAChE) produced in the HEK-293 cell system (MRT = 57 min). Extensive matrix-assisted laser desorption ionization-time-of-flight analyses established that the basic structures of the N-glycans associated with the native and recombinant enzymes are similar (the major species (50–60%) are of the biantennary fucosylated type and 20–30% are of the triantennary type), yet the glycan termini of the native enzyme are mostly capped with sialic acid (82%) and α-galactose (12%), whereas glycans of the recombinant enzyme exhibit a high level of exposed β-galactose residues (50%) and a lack of α-galactose. Glycan termini of both fetal bovine serum and rBoAChE were altered in vitro using exoglycosidases and sialyltransferase or in vivo by a HEK-293 cell line developed specifically to allow efficient sialic acid capping of β-galactose-exposed termini. In addition, the dimeric and monocyclic forms of rBoAChE were quantitatively converted to tetramers by complexation with a synthetic peptide representing the human ColQ-derived proline-rich attachment domain. Thus by controlling both the level and nature of N-glycan capping and subunit assembly, we generated and characterized 9 distinct bovine AChE glycoforms displaying a 400-fold difference in their circulatory lifetimes (MRT = 3.5–1390 min). This revealed some general rules and a hierarchy of post-translational factors determining the circulatory profile of glycoproteins. Accordingly, an rBoAChE was generated that displayed a circulatory profile indistinguishable from the native form.

Protein clearance from the bloodstream is known to be a multifactorial process involving different removal pathways. These comprise kidney glomerular filtration, protease degradation, and active removal from the circulation via specific receptors for various determinants on the protein. Various characteristics of the protein, including protein size, subunit assembly, surface charge, hydrophobicity, and carbohydrate contents/structure, may therefore play a role in determining its circulatory longevity (1, 2). In recent years, special attention was focused on proteins such as acetylcholinesterase (AChE)1 and butyrylcholinesterase (BChE), which require long term retention within the circulation to fulfill their therapeutic potential (3–9). Native serum-derived cholinesterases were found to reside in the circulation for long periods, whereas recombinant cholinesterases produced in tissue culture systems were cleared from the circulation rather rapidly (7–9), suggesting that post-translationally related factors determine the circulatory behavior of these enzymes.

In the case of AChE, the contribution of carbohydrates to circulatory residence was demonstrated by the finding that bacterially generated recombinant AChE, as well as N-glycanase-treated cholinesterases of animal cell origin, both devoid of N-glycans, were cleared rapidly from the circulation of experimental animals (7, 10). However, the pharmacokinetic profiles of an array of mutated recombinant human acetylcholinesterases (rHuAChEs), differing by the number of N-glycosylation sites (7), suggested that although N-glycosylation in itself does play a role in determining circulatory residence, the structural features of the N-glycans, rather than their actual number, play a decisive role in circulatory retention of cholinesterases. Among the glycan structural features affecting clearance, variations in the level of terminal sialylation are of particular importance because of the recognition and removal of undersialylated glycoproteins by the liver-specific asialoglycoprotein receptor (11). Indeed, enzymatic removal of sialic acid moieties resulted in a catalytically active form of rHuAChE displaying accelerated elimination from the circulation (7), indicating that sialylation plays a pivotal role in determining the circulatory residence of rHuAChE. The importance of sialylation efficiency was further demonstrated by determining the clearance rates of various derivatives of rHuAChE that differ one from another by their number of N-glycan side chains. Based on direct measurement of the sialic acid content of each one of the examined forms of the enzyme, an inverse linear relationship was found to exist between the number of unoccupied sialic acid attachment sites and the circulatory half-life values of the various enzyme forms (7). This line of studies

1 The abbreviations used are: AChE, acetylcholinesterase; 2-AB, 2-aminobenzamide; BoAChE, bovine acetylcholinesterase; PBS, fetal bovine serum; HEK-293, human embryonal kidney 293 cells; HPAGEC-PAD, high pH anion-exchange chromatography pulsed amperometric detection; HuS-BChE, human serum butyrylcholinesterase, MRT, mean residence time; rBoAChE, recombinant bovine acetylcholinesterase, rHuAChE, recombinant human acetylcholinesterase; α-Gal, α-galactose; β-Gal, β-galactose; MALDI-TOF, matrix-assisted laser desorption ionization-time-of-flight; PRAD, proline rich attachment domain; t-PA, tissue-type plasminogen activator; WAT, tryptophan amphiphilic tail; PBS, phosphate-buffered saline; CL, clearance.

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served as a basis for the conversion of the rHuAChE molecule into its highly sialylated form (8) by the genetic modulation of the glycosylation machinery of AChE producer cell lines. Although highly sialylated rHuAChE was indeed retained in the circulation for extended periods, it was still cleared from the circulation more rapidly than serum-derived FBS-AChE or HuS-BChE (7, 8, 12), suggesting that additional factors participate in determining the circulatory behavior of cholinesterases. In this context, it should be noted that cholinesterases can occur in multiple forms. Cell-bound AChEs consist of tetramers attached to membrane-anchored noncatalytic subunits such as the CoI/Q gene-encoded collagen-like tail, through the proline-rich attachment domain (PRAD (13)), whereas secreted cholinesterases such as those residing in the circulation (e.g., FBS-AChE) are usually composed of soluble homotetramers. In contrast to the tetramereric serum-derived cholinesterases, recombinant cholinesterases produced in tissue culture systems were found to consist mostly of dimers and monomers (7, 12, 14–16).

In a previous report, we documented the cloning of the bovine acetylcholinesterase gene and developed systems for the high level production of recombinant bovine AChE (rBoAChE) in a human cell line (16). This recombinant enzyme displayed a distinct pharmacokinetic profile that was characterized by its rapid removal from the circulation. In contrast, native serum-derived FBS-AChE, with which rBoAChE shares an identical amino acid sequence, was retained in the circulation for extended periods. Thus, two versions of the same protein that differ in their post-translational processing only exhibit markedly different pharmacokinetic behaviors and can therefore serve as a model system for inspection of post-translational factors involved in circulatory residence. This could not be achieved with the human recombinant enzyme due to the lack of an available native long lived form of human AChE.

In the work presented in this report, we determined the effects of the various post-translational features of the recombinant bovine enzyme upon its circulatory behavior. Exhaustive MALDI-TOF analysis of derivatized N-glycans associated with both rBoAChE and native FBS-AChE allowed us to determine the exact structures of the carbohydrates of the respective enzymes. We found that the native and recombinant forms of bovine acetylcholinesterase differ in their glycan-terminal occupancy. The efficient capping of glycans with either sialic acid or with α-galactose, which characterized the native form of bovine AChE, was evaluated for their contribution to circulatory longevity. On the basis of this analysis, a genetic approach for the pharmacokinetic remediation of rBoAChE was designed and implemented. This line of studies was then followed by a series of experiments in which the effect of rBoAChE subunit assembly modulation on circulatory residence was examined. We demonstrate that a series of modulations can convert the rapidly cleared rBoAChE into a circulatory long lived enzyme, exhibiting a pharmacokinetic profile that is indistinguishable from that of the native bovine serum acetylcholinesterase. Finally, by creating a large collection of carefully defined bovine AChE glycoproteins differing in the extent of glycan sialylation and enzyme subunit oligomerization, we demonstrate that a hierarchic pattern of post-translational factors determines circulatory longevity.

MATERIALS AND METHODS

Cell Culture Techniques, Enzyme Production, and Purification of rBoAChE—Generation of HEK-293 cell lines stably expressing high levels of rBoAChE was described previously (16). The generation of in vivo highly sialylated rBoAChE was achieved by transfecting the recombinant sialyltransferase expression HEK-293ST-2D6 cell line (see below) with the pBoAChE-nc vector followed by G418 selection to form rBoAChE stable producer cells. Purification of the secreted rBoAChE and purification of FBS-AChE from calf serum (Biological Industries, Beth Hemeek, Israel) were all described previously (16).

Generation of a HEK-293 Master Cell Line Expressing High Levels of 2,6-Sialyltransferase—Induction of HEK-293 cell clones stably transfected with the pCEP4 α2,6ST plasmid expression vector was performed as described previously (16). Isolation of α2,6-sialyltransferase gene (8) was tested for their ability to generate highly sialylated glycoproteins as follows: 96-well plates (Nunc, Maxisorp) were coated with decreasing amounts of cleared cell extracts (8) of the various clones (5–0.3 μg/ml in carbonate buffer pH 9.6, 4 h at 37 °C). Plates were then rinsed 3 times in 0.9% NaCl. 0.05% Tween 20 and subjected to a consecutive series of incubations (1 h at 37 °C each) in the following: 1) 0.5% Tween 20, 1% gelatin, PBS; 2) biotinylated Sambucus nigra lectin (Sigma, 1 μg/ml in 0.5% Tween 20/PBS); and 3) streptavidin-alkaline phosphatase conjugate (Sigma, 500 milliunits/ml in 0.5% Tween 20/PBS). Following each incubation step, plates were rinsed 3 times in 0.9% NaCl, 0.05% Tween 20. Plates were developed and read in a Thermomax (Mendo Park, CA) as described before (17). Optical density was plotted against bound cellular protein and cell-associated sialic acid levels (measured as mOD/μg protein) of the various clones were calculated. Clone HEK-293ST-2D6 displayed the highest level of sialic acid and subsequently served as the master cell line for expression and production of highly sialylated AChE.

Enzyme Activity—AChE activity was measured according to Ellman et al. (20) and modified in the presence of 0.001% acetylthiocholine iodide, 50 mM sodium phosphate buffer, pH 8.0, 0.1 mg/ml BSA, and 0.3 μM 5,5′-dithiobis-(2-nitrobenzoic acid). The assay was carried out at 27 °C and monitored by a Thermomax microplate reader (Molecular Devices).

Pharmacokinetics—Clearance experiments in mice (3–6 ICR male mice per enzyme sample) and analysis of pharmacokinetic profiles were carried out as described previously (7). 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 fitted to a bi-exponential elimination pharmacokinetic model \( C_t = \text{Ae}^{\text{kt}} + \text{Be}^{\text{bt}} \) as described previously (7). This model enables determination of the parameters \( A \) and \( B \) that represent the fractions of the material removed from the circulation in the first-fast and second-slow elimination phases, respectively, and \( \text{T}_\text{A}a \) and \( \text{T}_\text{b} \) that represent the circulatory half-lives 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 CL (clearance), using the prepotency factor relating the rate of instance elimination to its plasma concentration \( (\text{CL}, \text{dose/area under the concentration time curve}) \) (19) were independently obtained by applying the clearance data according to a noncompartmental pharmacokinetic model using the WinNonlin computer program (20).

Release, Recovery, Purification, and Labeling of N-Glycans—N-Glycans of purified enzyme preparations (100 μg of protein) were released by 2-aminobenzamide (2-AB) was performed according to Bigge et al. (26) with the addition of the prepotency factor relating the rate of instance elimination to its plasma concentration \( (\text{CL}, \text{dose/area under the concentration time curve}) \) (19) were independently obtained by applying the clearance data according to a noncompartmental pharmacokinetic model using the WinNonlin computer program (20).

Role of N-Glycan Structures and Oligomerization

In Vitro Sialylation of rBoAChE—Pure rBoAChE (1.8 nmol) was incubated for 20 h at 37 °C in the presence of 2 milliunits of α2,6ST (Roche Molecular Biochemicals) and 100 nmol of CMP-N-acetyl-neura-
minic acid in 50 mM NaCl (final volume = 800 μl). In vitro sialylated rBoAChE was extensively dialyzed against PBS for pharmacokinetics studies.

**Removal of Sialic Acid from rBoAChE-bound N-Glycans—AChE (100 nmol of enzyme) in PBS was incubated for 16 h with 1.2 units of sialidase (Sigma) at 37 °C, and the solution was then centrifuged at 10,000 × g at room temperature. Sialidase was removed by Eppendorf centrifugation. Desialylated enzyme was dialyzed against PBS to remove free sialic acid.

α-Galactosidase Treatment of FBS-AChE—Highly purified FBS-AChE (6 nmol in PBS) was incubated for 5 h at 37 °C with 8 units of α-galactosidase, followed by extensive dialysis against PBS. To determine whether α-galactose removal proceeded to completion, a portion of the α-galactosidase-treated enzyme was subjected to N-glycosidase F treatment, 2-AB labeling, and desialylation as detailed above, followed by MALDI-TOF analysis. Following AChE treatment with modifying glycosidases (sialidase or α-galactosidase), ChE activity was measured, to verify that enzyme integrity was not compromised by the treatment.

**Esterification of Sialic Acids—**To allow the concomitant measurement by MALDI-TOF analysis of both neutral and acidic glycans, the carboxylic groups of sialylated 2-AB-labeled glycans were converted into their neutral methylated forms by methyl iodide esterification, essentially as described by Kuster et al. (22). We note that in this procedure the 2-AB moiety itself undergoes methylation, and therefore both neutral and acidic glycans invariably display an increment in molecular mass of 14 g/mol in addition to the increase in mass resulting from sialic acid methylation in the case of acidic glycans. Esterified glycans were purified as described (22) and stored at −20 °C until MALDI-TOF analysis.

**Mass Spectrometry—**Mass spectra were acquired on a Micromass TOFSpec 2E reflection time-of-flight (TOF) mass spectrometer. 2-AB-labeled desialylated or 2-AB-labeled esterified glycan samples were mixed with an equal volume of freshly prepared 2,5-dihydroxybenzoic acid (10 mg/ml in 70% acetonitrile) and loaded onto the mass spectrometer target. Routinely, 1 μl of glycan samples diluted 1:10 in water were subjected to analysis. Dried spots were recrystallized by adding 0.5 μl of ethanol and allowed to dry again. Neutral glycans were observed as [M + Na]⁺ ions. 1 μl of peptide mixture (re-nat substrate, ACTH fragment 18–39, 2.5 μg/ml; 2.5 μg/ml all from Sigma), which served as a three-point external calibrant for mass assignment of the ions, was mixed with freshly prepared a-cyano-4-hydroxycinnamic acid (10 mg/ml in 70% acetonitrile; 49.5% ethanol; 0.001% trifluoroacetic acid), loaded on the mass spectrometer target, and allowed to dry. All oligosaccharides were analyzed at 20 kV with a single stage reflectron in the positive ion mode. Between 100 and 200 scans were averaged for each spectrum shown.

**PRAD Peptide Synthesis—**The PRAD peptide CLTTPPPPPLPPPPPPPFRG was synthesized manually in a T-bag from Nε-(9-fluorenyl)me-thoxy carbonyl) chemistry, as described (27). The peptide was dialyzed against 0.05% trifluoroacetic acid for 48 h. Quality control of the peptide was performed by MALDI-TOF-MS, and its concentration was evaluated by its absorbance at 215 nm following reverse-phase high pressure liquid chromatography.

In *Vitro* Tetramerization of rBoAChE—Purified rBoAChE was incubated together with the synthetic PRAD peptide for 12–16 h at room temperature, in the presence of 5 mM phosphate buffer, pH 8.0. In analytical tetramerization experiments designed for sucrose gradient analysis, the tetramer formation was detected in a final concentration of 0.12 nmol of rBoAChE (equivalent to 25 units) that were mixed with different ratios of the PRAD peptide as indicated, in a final volume of 70 μl. Preparative tetramerization for the generation of milligram amounts of tetrameric rBoAChE for pharmacokinetic studies included 14.4 nmol of rBoAChE (equivalent to 3000 units) that were incubated with 28.8 nmol of PRAD peptide in a final volume of 2 ml. Prior to administration to mice, in vitro tetramerized rBoAChE was dialyzed extensively against PBS.

**Sucrose Density Gradient Centrifugation—**Analytical sucrose density gradient centrifugation was performed on 5–25% sucrose gradients containing 0.1 mM NaCl, 50 mM sodium phosphate buffer, pH 8.0. Centrifugation was carried out in an SW41 Ti rotor (Beckman) for 26 h at 160,000. Fractions of 0.2 ml were collected and assayed for AChE activity. Alkaline phosphatase, catalase, and β-galactosidase were used as sedimentation markers.

**RESULTS**

**Characterization of the Basic N-Glycan Structures Associated with Recombinant and Native Bovine AChE**

To determine whether the differential pharmacokinetic patterns of FBS-AChE and its recombinant version expressed in a mammalian cell line (16) can be correlated with the presence/absence of specific glycan structures, the N-glycans associated with the two enzymes were subjected to a detailed structural analysis by MALDI-TOF mass spectroscopy in conjunction with specific exoglycosidase digestions.

In a first series of studies, the glycans derived from rBoAChE and FBS-AChE were deacetylated by sialidase treatment and extensively analyzed in their nonsialylated form. This treatment which removes terminal sialic acids enables easy detection of the glycans by overcoming the inherent difficulty of analyzing negatively charged oligosaccharides by MALDI-TOF (22, 28, 29). To increase sensitivity further, N-glycans released by the N-glycosidase F digestion were fluorescently labeled with 2-AB (see “Materials and Methods”). The analysis of N-glycans generated by this procedure provided the complete basic structure, branching/antennary typing and specific monosaccharide substitutions (e.g. fucosylation, presence of GalNAc) regardless of the terminal sialic acid occupancy status.

The MALDI-TOF spectra obtained for rBoAChE and FBS-AChE (Fig. 1A and Table I) revealed that the oligosaccharide species associated with both enzymes are comprised of a large variety of 8–11 basic glycan structures, differing both quantitatively in their relative abundance and qualitatively with respect to branching and monosaccharide substitutions. Based on molecular weight matching, the basic structural identity of most of the glycans could be deduced. In some cases, an ambiguity remained due to equivalent masses of isomeric or anomer monosaccharides. To resolve these ambiguities, desialylated glycan pools were subjected to a series of monosaccharide-specific exoglycosidase (α-galactosidase, β-galactosidase, and fucosidase) treatments, and the resulting glycan products were subjected to MALDI-TOF analysis. Based on the molecular weight shift of the trimmed glycans (Fig. 1B–D) the structures of the entire spectrum of glycans could be deciphered unequivocally (Table I). The relative abundance of the various oligosaccharide structures could thereby be determined, provided that these comprise more than 1% of the total glycan pool.

The analyses established that although the glycan pools of serum-derived FBS-AChE and 293 cell-generated recombinant AChE display a complex array of diverse structures, some general similarities between the two profiles can be found. In both enzymes, the vast majority of the oligosaccharides is of the complex type, the one notable exception being a hybrid glycan form (Table I, peak I) which is present in FBS-AChE at relatively low frequencies. The most prevalent glycan structure in both rBoAChE and FBS-AChE is the biantennary fucosylated form, (Man)₇-GlcNAc₂-(β-gal),Fuc (Table I, peak V), which constitutes approximately 40–50% of the total glycans and is present at approximately 7–10-fold higher levels than its nonfucosylated counterpart (Table I, peak III). In both enzymes, 20–32% of the glycans are triantennary (Table I, peaks VIII–X), whereas tetraantennary glycans are present at very low levels (Table I, peak XI). Interestingly, the predilection toward fucosylated forms is apparent in biantennary but not triantennary glycans. Low levels of glycans (4–5%) display masses (Table I, peaks IV and VI) that allow their structures to be interpreted as biantennary forms that contain either a bisecting GlcNAc residue or an outer arm Gal to GalNAc substitution. Some of our MALDI structural data (not shown) suggest that most of these forms are of the latter type, although the presence of very low amounts (<1%) of GlcNAc-bisected forms cannot be excluded.

Comparison of the various desialylated glycan structures and the abundance associated with the two forms of bovine AChE revealed that the most significant difference with regard to the basic glycan structures is the presence of high levels of...
(25–30% of total glycans) of α-gal-containing oligosaccharides in the native FBS form of the enzyme and not in recombinant BoAChE. Although most of these forms are of the biantennary type (Fig. 1A and Table I, peak VII), low levels (~2%) of triantennary glycans that carry α-gal are also present exclusively in the native enzyme (Fig. 1A and Table I, peak X).
Determination of the Sialylated Glycan Forms Associated with Recombinant and Native Bovine AChE

In view of the fact that the sialylation level of AChE was established to be important in determining its pharmacokinetic behavior (7, 8), the glycans were subjected to a thorough MALDI-mediated determination of their terminal sialic acid occupancy. As mentioned earlier, MALDI-TOF analysis of sialylated oligosaccharides is inherently complicated by the fact that the sialic acid residues are frequently converted into charged ions that form salts with alkali metal ions, resulting in a multiple peak spectrum that cannot be easily interpreted (28). Moreover, scoring of sialic acid levels is difficult due to fragmentation of sialic acid-containing glycans in some cases (22). Although sialylated glycans can be monitored in the MALDI-TOF negative mode (29, 30), the spectral data gathered in this manner does not include neutral oligosaccharide forms, precluding determination of the relative distribution of the various charged and noncharged glycans comprising the entire glycan pool. Therefore, we quantitatively determined the sialic acid terminal occupancy of the bovine AChEs-derived glycans by a set of MALDI analyses carried out with glycans in which the negative charges of their sialic acid residues were neutralized by iodomethane-mediated esterification (22). Although sialylated glycans can be monitored in the MALDI-TOF negative mode (29, 30), the spectral data gathered in this manner does not include neutral oligosaccharide forms, precluding determination of the relative distribution of the various charged and noncharged glycans comprising the entire glycan pool. Therefore, we quantitatively determined the sialic acid terminal occupancy of the bovine AChEs-derived glycans by a set of MALDI analyses carried out with glycans in which the negative charges of their sialic acid residues were neutralized by iodomethane-mediated esterification (22). The peaks of the neutralized glycans can be readily detected in the MALDI-TOF-positive mode and assigned accurate masses, taking into account that the equimolar introduction of methyl groups into sialic acid residues results in a MALDI-recognizable constant increase of the molecular weight. Since conversion of acidic glycans into neutral forms allows visualization of the entire range of N-glycans in the positive mode, the relative abundance of all the different glycan forms (sialylated as well as non-sialylated) comprising the glycan pool can be determined.

Inspection of MALDI-TOF spectra established that the major glycan forms and their relative abundances (Fig. 2A) are in excellent agreement with those detected by analysis of the desialylated glycans (Fig. 1). This indicates that the esterification process did not introduce any bias that may cause disproportionate detection of specific glycan structures. Whereas the basic structures of the major glycans associated with FBS-AChE and rBoAChE are similar (with the notable exception of glycans containing terminal α-gal in FBS-AChE only, as mentioned above), the two preparations differ significantly in their sialic acid content. In the glycans of native FBS-derived protein, almost all of the β-gal moieties serve as acceptors for sialic acid (Fig. 2A1), in contrast to the glycans associated with the recombinant enzyme, of which a significant fraction terminates in uncapped β-gal (Fig. 2A2).

Considering the relative MALDI-scored abundances of the glycan forms, and based on the fact that bovine AChE possesses four utilized N-glycosylation sites (16), it is possible to score the molar ratio of the various glycan forms with respect to their antennary terminal groups (Table II). Thus, FBS AChE-associated glycans exhibit (per enzyme subunit) 7.3 sialylated termini, 1 terminal α-gal, and only 0.3 termini ending in β-gal. In contrast, the recombinant version of the protein produced in HEK-293 cells is heavily undersialylated, exhibiting a molar ratio of 4.1 sialylated termini and 4.5 termini ending in β-gal, per enzyme subunit.

### Table I

| Peak Designation | Composition | MW (M+Na)⁺2AB | N-Glycan Abundance (% of Total) |
|------------------|-------------|----------------|----------------------------------|
|                  |             |                | rBoAChE (HEK-293) | FBS-AChE Native |
| I                | (Man)3GalNAcβ(Fuc) | 1726.0 | 0.8 | 6.6±0.3 |
| II               | (Man)3GalNAcβ[BGal]4(Fuc) | 1757.8 | 2.5±0.5 | 1.8±0.4 |
| III              | (Man)3GalNAcβ[BGal]3 | 1782.6 | 6.9±1 | 5.5±0.8 |
| IV               | (Man)3GalNAcβ[GalNAc]2(Fuc) | 1809.9 | 2.1±0.4 | 2.6±0.4 |
| V                | (Man)3GalNAcβ[BGal]4(Fuc) | 1929.8 | 52.4±4 | 38.3±3.2 |
| VI               | (Man)3GalNAcβ[GalNAc]4[BGal]2(Fuc) | 1972.8 | 2.4±0.4 | 1.7±0.4 |
| VII              | (Man)3GalNAcβ[BGal]4[αGal]4(Fuc) | 2091.5 | <0.8 | 24.8±1.4 |
| VIII             | (Man)3GalNAcβ[BGal]3[αGal]3 | 2149.9 | 25.3±2 | 15.8±2.1 |
| IX               | (Man)3GalNAcβ[BGal]4(Fuc) | 2295.1 | 5.9±2 | 3.3±0.6 |
| X                | (Man)3GalNAcβ[BGal]4[αGal]4(Fuc) | 2475.5 | <0.8 | 2.2±0.4 |
| XI               | (Man)3GalNAcβ[BGal]4(Fuc) | 2690.6 | 1.5±0.4 | 1.6±0.2 |

GalNac  ◯ αGal  ◯ βGal  ◯ Mur  ◯ GlcNac  → Fuc

### Role of N-Glycan Structures and Oligomerization

Comparison of the basic glycan structures of desialylated rBoAChE and FBS-AChE

Molecular weights, deduced structures, and relative abundances of enzymatically desialylated N-glycan structures associated with rBoAChE and FBS-AChE were determined from the MALDI-TOF spectra represented in Fig. 1.
Taken together, the following conclusions can be drawn from the structural profiles of the carbohydrates released from the recombinant and native forms of bovine AChE: (i) complex-type N-glycans are associated with both recombinant and native BoAChE. The basic structures of the glycans and their relative abundances in the two enzyme preparations are similar. (ii) The prevalent glycan structure in both cases is that of the biantennary type. (iii) Most of the glycans are core-fucosylated. (iv) A minority of the glycans carry a GalNAc residue, whereas little or none contain bisecting GlcNAc. (v) A significant fraction of the biantennary glycans derived from the native FBS-AChE display terminal α-gal residues. This structure is not detected in the recombinant enzyme. (vi) FBS-AChE and rBoAChE differ sharply in their extent of terminal sialic acid capping; although native FBS-AChE enzyme exhibits almost fully sialylated termini, the recombinant enzyme is markedly undersialylated.

Assessment of the Role of Terminal α-Galactosylation and Sialylation on Circulatory Longevity

Since the two major differences between the N-glycans derived from native long lived AChE and from the rapidly cleared recombinant enzyme are related to their terminal capping, we examined the effect of specific monosaccharide trimming of the intact FBS-AChE enzyme on its circulatory residence.

To examine the effect of sialic acid removal on the pharmacokinetic behavior of FBS-AChE, the intact enzyme was subjected to extensive incubation in the presence of a large excess of sialidase (see “Materials and Methods”). A portion of the sialidase-treated glycoprotein was processed to allow MALDI-TOF inspection of the modified glycans, and the rest of the enzyme sample was tested for its pharmacokinetic behavior (Fig. 3A and Table III). The MALDI-TOF spectrum of the N-glycans released and purified after sialidase treatment of FBS-AChE (Fig. 3A2) was found to be identical to the spectrum...
The Effect of rBoAChE Oversialylation on Its Pharmacokinetic Behavior

HEK-293 cells, which serve as a production system for rBoAChE, are characterized by limited levels of sialyltransferase activity (8). This paucity is most pronounced for recombinant glycoproteins overexpressed at unusually high levels (8), in which case the limitation in sialyltransferase activity of the cells precludes the production of recombinant proteins exhibiting fully sialylated glycans (37). We have shown that this could be remedied by high level sialyltransferase production resulting from the coexpression of a 2,6ST gene together with the gene encoding for the protein of interest (8). The finding that rBoAChE is pronouncedly undersialylated in comparison to FBS-AChE, and the fact that removal of the terminal sialic acid residues had a dramatic impact on clearance, prompted us to test the possibility of preventing rapid clearance of the recombinant bovine protein by expressing it in cell clones that display high levels of sialyltransferase activity.

Generation of Highly Sialylated rBoAChE—The generation of a versatile cell line producing high levels of 2,6ST, which may serve for the production of fully sialylated glycoproteins, would require a reliable and rapid method for screening of cells that express high levels of sialyltransferase activity. The enzymatic method for detection of sialyltransferase activity by monitoring incorporation of radiolabeled sialic acid in the presence of soluble cell extract fractions (38) is both cumbersome and is not amenable with the simultaneous processing of many cell samples. Moreover, the measurement of high levels of cell-associated sialyltransferase activity does not in itself demonstrate that glycans associated with these cells are indeed more efficiently sialylated. We therefore developed a solid-phase detection system based on a S. nigra agglutinin lectin-binding assay which scores the relative amounts of sialylated glycoprotein in cell extracts (see “Materials and Methods”). By utilizing this method, we could observe that individual cell clones isolated from a HEK-293 cell pool stably transfected with the rat 2,6ST gene exhibited differing levels of glycoprotein-associated sialic acid (Fig. 4A). Extracts prepared from cells that were not transfected with the rat 2,6ST gene failed to display significant S. nigra agglutinin lectin affinity, in accordance with the low level sialyltransferase activity associated with these cells. Of all transfecant clones tested, clone 293ST-2D6 exhibited the highest levels of sialylated glycoproteins, commensurate with those scored in extracts of HuH7 cells (which, by virtue of their hepatic origin, possess very high levels of sialyltransferase activity). Clone 293ST-2D6 subsequently served as a host cell system for production of rBoAChE. Specific lectin probing of electrophoretic recombinant AChE purified from HEK-293 and 293ST-2D6 cells demonstrated (data not shown) that the enzyme products of the two cell lines indeed differed markedly with respect to terminal occupancy of their glycans.

Characterization of the Glycan Structures of Oversialylated rBoAChE—MALDI-TOF structural analysis of N-glycans recovered from recombinant BoAChE expressed in the 293ST-2D6 cells (Fig. 2B) established that the basic backbone structure of the N-glycans was not affected by the overexpression of the heterologous sialyltransferase gene. Close inspection of the glycan populations of the rBoAChE produced in the nonmodified and sialyltransferase-modified cells could give the impression that some minor species are associated with the products of one cell line but not of the other (Fig. 4B). For instance, the tetraantennary form (peak XI) is represented as 2% of the total glycans in the enzyme of 293ST-2D6 cells only. However, the tetraantennary form actually is present in the nonmodified HEK-293 cell product as well and can be detected if the glycan pool was subjected to sialidase treatment (Table I, peak XI). It

### Table II: Terminal occupancy of N-glycans associated with rBoAChE and FBS-AChE

| Structure          | Molar ratio (structure/AChE subunit) | rBoAChE | FBS-AChE |
|--------------------|--------------------------------------|---------|----------|
| Terminal sialic acid | 4.1/7.3                             | 7.3     | 7.3      |
| Terminal β-gal     | 4.5/0.28                             | 0.28    | 0.28     |
| Terminal α-gal     | 0/1.0                               | 1.0     | 1.0      |
| N-Glycans          | 4                                    | 4       | 4        |
| N-Glycan termini   | 9.1/8.9                             | 8.9     | 8.9      |

a The molar ratio of total sialic acid (mol/mol AChE subunit) calculated from the MALDI-TOF spectra of FBS-AChE and rBoAChE N-glycans (Fig. 2A) was 7.7 and 4.2, respectively. Note that one of the sialylated glycan forms (m/z = 3383.3 Da) contained 4 sialic acid residues per 3 glycan termini, and therefore this structure includes a nonterminal sialic acid residue. The molar ratio of terminal sialic acids per AChE subunit was calculated as (total sialic acid (mol/mol AChE subunit)) – (nonterminal sialic acid (mol/mol AChE subunit)) = (terminal sialic acid (mol/mol AChE subunit)).

b Calculated from the MALDI-TOF spectra of FBS-AChE and rBoAChE (Fig. 2A).

c Bovine AChE contains 4 utilized N-glycosylation sites (16).

d Calculated from the frequencies of the various glycan forms in Table I.

"The apparent difference between the number of N-glycan termini and the sum of terminal sialic acid, β-gal, and α-gal is accounted for by the presence of the structure corresponding to peak 1 in Table I (m/z = 1726.0). This hybrid structure possesses none of these terminal moieties, yet contributes 6.8% glycan ends."
Role of N-Glycan Structures and Oligomerization

Therefore follows that the failure to observe this form in the glycan pool of the nonmodified cell product, without prior treatment with sialidase (Fig. 4B, upper panel), is not due to its absence but rather to its distribution among multiple sialoforms (combinations of non-, mono-, di-, tri, and/or tetrasialylated), each of which may be below detection levels. Such careful inspection of the MALDI-TOF spectra allows us to conclude that expression of the heterogeneous 2,6-sialyltransferase gene in the 293ST-2D6 cells did not introduce any alterations in the basic structure of the oligosaccharides comprising the glycan pool of rBoAChE.

MALDI-TOF analysis (Figs. 2B and 4B) established that the N-glycans of rBoAChE expressed in 293ST-2D6 cells are quantitatively capped with sialic acid residues (approximately 8.7 sialylated termini/enzyme subunit and only 0.25 nonsialylated β-gal termini/enzyme subunit). This high extent of sialylation of glycan termini is similar to that of FBS AChE (compare Fig. 2B to Fig. 2A1). Alignment of the different forms comprising the glycan pools of the HEK-293- and 293ST-2D6-produced enzymes (Fig. 4B) revealed that whereas the ratio of (nonsialylated) (partially sialylated):(fully-sialylated) glycans of the HEK-293 product was approximately 43:27:25, the corresponding values for the 293ST-2D6 product was 0:9:89. Notably, 92% of the most prevalent glycan species (Fig. 4B, peak V) was not fully sialylated in the HEK-293 product, whereas in the 293ST-2D6 product over 89% of this glycan form is in its fully sialylated form. It is worth noting that the level of expression of rBoAChE in these cells is quite high (>50 mg/liter). Taken together, these results clearly demonstrated that the corexpression at high levels of both sialyltransferase and roBoAChE allows highly efficient sialylation of the various glycan forms associated with rBoAChE.

**Pharmacokinetic Behavior of Oversialylated rBoAChE** — When subjected to pharmacokinetic studies the genetically modified-fully sialylated roBoAChE produced by the 293ST-2D6 cells demonstrated a significant increase in circulatory residence that is manifested by an increase in $T_{1/2}\beta$ from 29 to 120 min and a greater than 3-fold increase in MRT from 57 to 197 min (Table III). This increase was similar to that exhibited by HEK-293-produced rBoAChE that was sialylated in vitro by commercial rat liver α2,6ST (Table III). Although the extent of glycan sialylation of roBoAChE from 293ST-2D6 cells is at least as high as that of FBS-AChE, the residence time of the native enzyme (MRT = 1390 min) is still higher than that of the
Role of N-Glycan Structures and Oligomerization

The circulatory clearance profiles of the various AChEs were determined as described (see "Materials and Methods"). The biphasic pharmacokinetic curves were fitted to an equation of the form $C_t = Ae^{-k_1t} + Be^{-k_2t}$. A and B represent the fractions of the material removed from the circulation in the first (fast) and second (slow) decay phases, respectively. $T_{1/2a}$ and $T_{1/2b}$ represent the circulatory half-life values of the enzyme in the fast and slow phases, respectively. In all cases, the correlation coefficient was $>0.987$. The clearance curves were also analyzed by fitting to a noncompartmental pharmacokinetic model for the calculation of MRT and Clearance using a Window-based program. Elimination half-life values calculated by noncompartmental analysis coincided with the $T_{1/2}$ values obtained by the biexponential elimination pharmacokinetic model.

### Table III
Pharmacokinetic parameters of BoAChEs differing in their state of glycan sialylation and oligomerization

| Bovine AChE, source/type | Treatment | $T_{1/2a}$ | $T_{1/2b}$ | Clearance | MRT |
|-------------------------|-----------|------------|------------|-----------|-----|
| HEK-293/recombinant     | Sialidase | 100        | 3.5 ± 0.4  | 1190      | 3.2 |
| HEK-293/recombinant     | In vitro sialylation | 56 ± 7 | 7.5 ± 2 | 123 ± 20 | 70  |
| 293ST-2D6/recombinant   | In vitro sialylation | 48 ± 4 | 11 ± 3 | 50 ± 5 | 120 ± 10 | 59  |
| HEK-293/recombinant     | In vitro tetramerization | 68 ± 3 | 9 ± 1 | 33 ± 4 | 118 ± 14 | 92  |
| 293ST-2D6/recombinant   | In vitro tetramerization | 35 ± 2 | 30 ± 10 | 67 ± 2 | 965 ± 40 | 6.5 |
| FBS/native              | α-Galactosidase | 43 ± 2 | 41 ± 5 | 57 ± 2 | 960 ± 67 | 6.5 |
| FBS/native              | Sialidase | 100 * | 3.5 ± 0.3 | 1180 | 3.6 |

*a* Sialidase-treated rBoAChE and FBS-AChE displayed a single phase clearance profile.

*b* Recombinant BoAChE produced by the sialyltransferase expression master cell line (see "Results").

**Fig. 4.** Generation of HEK-293 cell lines that allow expression of recombinant glycoproteins with high levels of sialylation. HEK-293 cells were stably transfected with the rat β-galactosidase α,2,6-sialyltransferase (2,6ST) gene, and sialic acid levels associated with individual cell clones were measured by S. nigro agglutinin detection as described under “Materials and Methods.” A, relative sialylation levels associated with the 2,6ST stably transfected cell pool (striped) and of individual cell clones (shaded). The sialylation level determined for the HuH7 hepatic cell line was assigned a value of 100. The sialylation level of nontransfected HEK-293 cells is also shown. Cell clone 2D6 (dark shading) subsequently served as a cell host (assigned as 293ST-2D6) for transfection of rBoAChE expression vectors and recombinant protein production.

**B.** Glycan structures of rBoAChE produced by HEK-293 or 293ST-2D6 cells. Glycan structures were deduced from the mass spectral data shown (see "Materials and Methods"). The circulatory clearance profiles of the various AChEs were determined as described (see "Materials and Methods").

**Conversion of rBoAChE into Stable Tetrameric Forms**

Sucrose gradient analyses of the fully sialylated rBoAChE produced by 293ST-2D6 cells (Fig. 5B1) established that the enzyme consists mainly of dimeric and monomeric forms of AChE catalytic subunits; the relative proportions of monomers, dimers, and tetramers (G1:G2:G4) was approximately 40:50:10. This subunit assembly profile is similar to that of HEK-293 cell produced rBoAChE (Fig. 5A1) and is in sharp contrast to the native serum-derived BoAChE that consists of tetramers only (see Refs. 16 and 39 and see also Fig. 3). Since enzyme subunit assembly was not affected by glycan modification, it is clear that the partial improvement in circulating residence exhibited by the 293ST-2D6 cell product is a direct outcome of its improved sialylation.

To assess the contribution of the oligomerization status of BoAChE to its circulatory retention, an in vitro system for the
efficient generation of stable rBoAChE multimers was developed. To this end, we generated a synthetic peptide containing the ColQ PRAD, a region that was shown in the elegant studies of Bon and co-workers (13, 15, 40, see also Ref. 41) to be necessary and sufficient for efficient assembly of AChE tetramers \textit{in vivo}, via the C-terminal tryptophan amphiphilic tail (WAT) domain of AChE (42). The synthetic human PRAD peptide was synthesized on the basis of the published sequence of the human ColQ protein (43) and in accordance with the defined boundaries of the attachment domains as established by deletion and site-directed mutagenesis studies (40). The identity and purity of the synthetic peptide were confirmed by MALDI-TOF analysis (Fig. 6A, inset).

Incubation of the PRAD peptide with purified preparations of rBoAChE resulted in a dose-dependent oligomerization as detected by sucrose gradient sedimentation assays (Fig. 6B).

Assembled rBoAChE cosedimented at 11.3 S with the catalase sedimentation marker, indicating that the oligomerized rBoAChE is in tetrameric form (13). The PRAD-mediated \textit{in vitro} tetramerization process indeed followed faithfully the

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**FIG. 5.** Pharmacokinetic profiles and subunit assembly status of rBoAChE produced by HEK-293 and 293ST-2D6 cells. Left panels, purified rBoAChE from HEK-293 cells before (A1) or after (A2) \textit{in vitro} tetramerization in the presence of a synthetic peptide, representing the human ColQ-derived PRAD or purified rBoAChE from 293ST-2D6 cells, before (B1) or after (B2) \textit{in vitro} PRAD-mediated tetramerization, were administered to mice for determination of their circulatory clearance profiles and pharmacokinetic parameters as described in legend to Fig. 3 (see also Table III). Elimination curves of the various rBoAChE preparations (black circles) are shown alongside the elimination curve of FBS-AChE (gray curve). Right panel, sucrose gradient sedimentation profiles of the corresponding rBoAChE preparations. G1, G2, and G4 annotate the monomeric, dimeric, and tetrameric BoAChE peaks, respectively. Arrows denote the elution position of the sedimentation markers alkaline phosphatase (6.1 S), catalase (11.3 S), and \(\beta\)-galactosidase (16 S) included in all samples.

**FIG. 6.** \textit{In vitro} PRAD-mediated tetramerization of rBoAChE. A, schematic representation of a membranal asymmetric AChE tetramer which is composed of the noncatalytic ColQ subunit and 4 catalytic AChE monomers. The amino acid sequence of human PRAD and its localization within the ColQ protein are shown. Inset, MALDI-TOF analysis of the pure synthetic human PRAD. B, recombinant BoAChE was incubated in the presence of PRAD, as described under “Materials and Methods” and subjected to sucrose gradient analysis. The molar ratio of AChE subunits and PRAD peptides included in each of the \textit{in vitro} tetramerization reaction mixtures is shown (right). G1, G2, and G4 annotate the monomeric, dimeric, and tetrameric BoAChE peaks, respectively. Arrows denote the elution position of the sedimentation markers alkaline phosphatase (6.1 S), catalase (11.3 S), and \(\beta\)-galactosidase (16 S) included in all samples.
known stoichiometry of the ColQ/AChE assembly into hetero-
meric complexes of four catalytic and one noncatalytic subunit.
Incubation of rBoAChE in the presence of PRAD at a lower
molar ratio (PRAD:AChE = 1:8) resulted in tetramerization of
only part of the rBoAChE molecules. Incubation of rBoAChE in
the presence of a large excess of PRAD peptide (PRAD:AChE =
10:1) resulted in the formation of tetramers only; no other
multimeric forms could be detected. Notably, the PRAD-medi-
at oligomerization process allowed the full conversion of both
dimeric (G2) and monomeric (G1) forms into tetramers. No
tetramerization could be detected when rBoAChE was incu-
bated with an unrelated peptide comprising a commensurate
number of amino acids. Furthermore, tetramerization via
PRAD incubation did not occur with an AChE version lacking
the C-terminal tail (not shown). Taken together, these results
suggest that the tetramerization process faithfully reflects the
physiological interaction between PRAD and WAT responsi-
ble for the generation of highly stable tetrameric forms of AChE
(44).

The in vitro generated tetramers exhibited a high degree of
stability, withstanding prolonged storage, several cycles of
freezing-thawing, and extensive dialysis. This was further sub-
stantiated by our finding that following administration to mice,
the enzyme retained its tetrameric configuration over long
periods (not shown). Indeed, recently, the structure of an Elec-
rophorus AChE tetramer has been resolved by x-ray crystal-
lography (45) and suggested that tetrameric forms of AChE are
highly stable.

Tetramerization of either Torpedo or rat AChE by direct
contact of proline-rich domains and AChE subunits in a cell-
free milieu was attempted in the past using polyproline rather
than PRAD peptides. Efficient tetramerization occurred in the
presence of cellular extracts that possibly contributed factors
required for oligomeric assembly or in cells producing AChE
which were cultured in the presence of polyproline (40). The in
vitro tetramerization in the presence of a PRAD synthetic
peptide and in the absence of additional assisting factors (such
as cell extracts), reported here and in previous studies by Giles
et al. (41), may indicate that polyproline and bona fide PRAD
domains are not equivalent in their ability to promote tet-
ramerization, the latter being a much more efficient oligomer-
ization mediator. One should note, however, that the present
study was conducted with the bovine version of AChE rather
than the rat or Torpedo enzymes. The possibility that the
bovine AChE is more prone for tetramerization than other
AChE counterparts cannot be ruled out, yet there are no amino
acid sequence differences between the rat and bovine genes in
their amphiphilic C-terminal tails that encompass the WAT
domain interacting with PRAD (16, 46).

Pharmacokinetics of rBoAChE Tetramers

In vitro PRAD-mediated tetramerization of the recombinant
BoAChE produced by non-modified HEK-293 cells (which pro-

tegrate limited levels of glycan sialylation only) displayed a
significant pharmacokinetic improvement compared with the non-
tetramerized enzyme (Fig. 5A2 and Table III). Specifically, the
biexponential $T_{1/2}$ value increased from 29 to 118 min, and the
MRT increased from 75 to 190 min. However, when fully sia-
lylated 293ST-2D6 cell-produced rBoAChE was subjected to in
vitro PRAD-mediated tetramerization, circulatory residence was
increased in a much more pronounced manner, the $T_{1/2}$
and mean residence time values for the tetramerized enzyme
was 965 and 1340 min, respectively (Fig. 5B2 and Table III).
Most notably, following PRAD-mediated tetramerization, the
fully sialylated rBoAChE displays a pharmacokinetic profile
which was very similar to that observed for the long lived
native serum-derived BoAChE ($T_{1/2} = 990$ min; MRT = 1390
min). Thus, although enzyme oligomerization in itself can in-
crease the circulatory lifetime of partially sialylated rBoAChE,
its effect on circulatory longevity can be fully appreciated only
when the enzyme is efficiently sialylated.

We have noted above that in contrast to removal of terminal
$\alpha$-galactose residues from the FBS-AChE, which practically did
not affect its circulatory lifetime ($T_{1/2} = 930$ min; MRT = 1150
min, see Table III), desialylation of the tetrameric FBS-AChE
promoted its rapid clearance from the circulation ($T_{1/2} = 3.5$
min; MRT = 3.6 min). In view of the fact that protein oligomer-
ization contributes to circulatory retention, one could have
argued that the rapid clearance of desialylated FBS-AChE was
actually caused by tetramer disassembly during the prolonged
incubation in the presence of sialidase, required for complete
removal of sialic acid from the intact protein. That this is not
the case is clearly demonstrated by sucrose gradient analyses
(Fig. 3, A1 and B1, insets) which show that the glycosidase-
treated enzymes retained their tetrameric form, indicating that
the differential effects of $\alpha$-galactose and sialic acid removal
cannot be attributed to tetramer dissociation but are rather a
direct outcome of the glycan modifications brought about by the
respective enzymatic treatments. In line with the dramatic
decrease in circulatory residence caused by desialylation of
tetrameric FBS-AChE, we note that recombinant AChE tet-
ramers also displayed a rapid removal rate following sialidase
treatment, indistinguishable from that exhibited by the desia-
lylated native enzyme. In fact, the pharmacokinetic profile of
the desialylated tetrameric AChEs was identical to that ob-
tained following desialylation of non-tetrameric AChEs such as
the recombinant versions produced by either HEK-293 cells or
293ST-2D6 cells.

DISCUSSION

Similar Basic Glycan Structures Are Associated with Recom-
binant HEK-293-produced and Native Serum-derived Bovine
Acetylcholinesterase—Previous studies (7–9) that were based
on chemical determination of sialic acid, monosaccharide com-
position, charge, and size distribution analyses and HPAEC-
PAD profiling of oligosaccharides allowed partial characteriza-
tion of some features of the glycans associated with cholinesterases. For example, HPAEC-PAD analyses revealed that a complex array of glycan types, which are differentially sia-
ylated, are associated with recombinant acetylcholinester-
ase (8). In another study, exoglycosidase sequencing in conjunc-
tion with size distribution chromatography allowed glycan
structural analysis that was confined to the major species of
the multiple forms associated with serum-derived AChE (9).
Due to their limited power of resolution, these methodologies
could not provide a comprehensive structural analysis of the
total spectrum of glycans, and therefore the biological signif-
icance of the findings was not entirely clear.

Here by MALDI-TOF profiling in conjunction with exoglyco-
sidase mapping, we were able to determine the actual struc-
tures of essentially all the oligosaccharides comprising the
glycan pools of both HEK-293-produced rBoAChE and serum-
derived native bovine AChE. Though both enzyme species dis-
played an intricate array of glycan forms, common features of
the two glycan pools could be established as follows. (i) The
rBoAChE and FBS-AChE-associated glycans are of the com-
xplex type, displaying either biantennary (major species =
(Man)$_5$(GlcNAc)$_b$(Gal)$_a$(Fuc)$_a$) or triantennary structures (major
species = (Man)$_b$(GlcNAc)$_b$(Gal)$_a$)$_b$), the most prevalent
form being the biantennary-fucosylated type. Whereas most
of the glycan structures contained fucose, glycans exhibiting
GalNAc were identified at very low levels (Table IV). (ii) With
the exception of terminal monosaccharide occupancy, the basic


discussion
structures of the oligosaccharides comprising both glycan pools were virtually identical (Fig. 1D). (iii) The relative abundances of the different basic structures of the glycans of both enzymes were similar (Table I).

The extent of similarity shared by the recombinant and native forms of AChE with respect to their basic N-glycan structures was remarkable considering the fact that they are pharmacokinetically distinct and that they markedly differ in their origin. The recombinant enzyme originates from a human embryonic kidney cell line selected for its high expression of heterologous product. The native AChE is generated by fetal bovine cells of unknown origin. Since the probability that FBS-AChE is generated by fetal bovine kidney cells is low, this degree of glycan resemblance suggests that as long as the producer cells are equipped with sufficient amounts of necessary and appropriate elements of the glycosylation machinery, the glycan structures appended to a particular protein may be dictated to a considerable extent by the protein per se.

Oligosaccharides Associated with the Rapidly Cleared Recombinant and the Long Lived Native Bovine Acetylcholinesterase Diverge in Their Glycan-terminal Occupancy—Comparison of the N-glycans derived from rBoAChE and FBS-AChE (Tables II and IV) revealed that the rBoAChE is pronouncedly undersialylated as compared with the FBS-AChE (in rBoAChE approximately 50% of the glycan termini exhibited an exposed β-gal, and in FBS-AChE only 3.2% of the glycan termini exhibited an exposed β-gal). These results are not in agreement with those reported previously (9), where on the basis of carbohydrate composition analysis of monosaccharides released from the glycan pools associated with FBS-AChE, it has been suggested that the long lived FBS-AChE is heavily undersialylated and that half of the glycan terminal associated with this enzyme are not capped by sialic acid. This discrepancy may be partly explained by the fact that in this study (9) the number of potential glycosylation sites used to calculate termini occupancy was erroneously considered to be 5, rather than 4 (16).

Inspection of the various glycans of rBoAChE revealed that undersialylation is not random. We note a preferential sialylation of higher branched (triantenary) glycans (Fig. 4B, peaks VII and VIII), as has been reported in other recombinant expression systems, as well (47). Nevertheless, since the triantennary forms comprise a relatively low sub-population of the total glycans of rBoAChE, there is an overall state of undersialylation of the glycans associated with this enzyme.

Glycans containing α-gal are not present on rBoAChE yet in the native FBS-AChE enzyme constitute 25–30% of the total glycan pool. These are mostly of the biantenary form, although triantennary glycans exhibiting α-gal were also detected (Fig. 1A, peaks VII and X). The lack of α-gal-containing glycans in the recombinant enzyme produced by cells of human origin (HEK-293) is in full accordance with the well-documented fact that humans, Old World primates, and anthropoid apes lack the glycosyltransferase activity required for α-gal appendage (48).

The Role of Cell and Protein-specific Factors in Determining Glycan Forms of BoAChE—The marked difference in the composition of the glycan termini suggests that cellular factors are decisive in determining the nature of terminal glycosylation, as indeed manifested by the correlation between the low abundance of sialyltransferases in HEK-293 cells (8) and the poor sialylation of rHuAChE (7–8) or of rBoAChE produced in these cells (16). Nevertheless, that terminal glycosylation is not determined solely by cell-specific elements but also by protein-related factors is substantiated by the findings that other recombinant proteins expressed in HEK-293 cells show a glycosylation pattern distinctly different from that found for rBoAChE. For example, the major glycan forms of recombinant protein C produced in HEK-293 cells carry an outer-arm fucose in addition to the core fucose and contain high levels of Gal to GalNAc substitutions (49). In the case of rBoAChE, glycan structures carrying more than one fucose were undetected, whereas Gal to GalNAc substitutions were identified on less than 5% of the glycanse (Table IV). The low level of GalNAc-terminating glycans in HEK-293-produced rBoAChE is significant with respect to circulatory longevity, since terminal GalNAc may serve as an acceptor for the appendage of sulfate groups. SO₄-terminating glycans were described for pituitary hormones such as luteinizing hormone and thyrotropin, where they ensure the necessary oscillatory nature of the pituitary endocrine effect by the rapid removal of the hormones from the bloodstream via a specific hepatic clearance receptor (50, 51). The lack of high levels of GalNAc in rBoAChE precludes a significant role for this epitope in the removal of rBoAChE.

Do α-Galactosylated Glycans Affect Circulatory Longevity of Glycoproteins?—The possible effect of α-gal resides on the circulatory residence of therapeutic glycoproteins is a matter of debate (52–55). Previous studies concerning the pharmacokinetic behavior of tissue-type plasminogen activator (t-PA) have indicated that recombinant glycoforms that contained α-galactose moieties in their carbohydrate chains exhibited a significantly longer mean residence time in the circulation of chimpanzees than those that lacked such structures (56). However, the effect of α-gal removal on clearance of t-PA was not examined experimentally, and therefore the correlation of α-gal-containing glycans with long lived protein species may be no more than circumstantial. Here we find that quantitative removal of terminal α-gal did not significantly alter FBS-AChE clearance (Fig. 3B1), indicating that α-gal does not provide pharmacokinetic advantage to the administered enzyme.

In the case of t-PA, the authors (56) attributed the protective effect of α-gal to the presence of high levels of anti-α-gal antibodies in the circulation of some primates (apes, Old World monkeys, and humans). It was suggested that these antibodies may interact with the glycoprotein in such a manner so that recognition sites in the liver may be obstructed, leading to reduction in hepatic uptake rates. One should note that the pharmacokinetic behavior of FBS-AChE in rodents (Fig. 3, A1 and B1) which lack antibodies against α-gal is similar to that found in primates such as rhesus monkeys (5), suggesting that at least in the case of FBS-AChE, antibodies against α-gal do not appear to play any role in determining circulatory lifetime.

Increasing Cellular Sialyltransferase Levels Results in the Generation of Highly Sialylated rBoAChE Glycans with Improved Pharmacokinetic Performance—The generation of a master cell line, 293ST-2D6, expressing stable high levels of recombinant sialyltransferase (Fig. 4A), enabled us to examine
whether increased sialylation of rBoAChE enhanced its circulatory retention to levels that are comparable to those of FBS-AChE. Inspection of the glycans associated with the sialyltransferase-modified cell product (Fig. 4B, lower panel) established that these are shifted to higher molecular weights, as compared with the glycans from rBoAChE produced by nonmodified HEK-293 cells and that only 8.5% of the glycans are undersialylated. This fraction is composed of the monosialylated biantennary fucosylated glycan (peak Vp) and its immature precursor form lacking a galactose (peak Ibp). The latter is refractive to further sialylation due to its immature nature. This glycan species may correspond to the sialylation refractive glycan forms observed by us in human rAChE using the HPAEC-PAD detection system (8), but that could not then be accurately quantified nor assigned a definitive structure, due to the limitations of the detection system employed.

Examination of the pharmacokinetic behavior of the highly sialylated rBoAChE revealed that this glycoprotein resided in the circulation for considerably longer periods ($T_{1/2} = 120$ min, MRT = 197 min) than the undersialylated enzyme produced in nonmodified HEK-293 cells ($T_{1/2} = 29$ min, MRT = 57 min). However, the extended residence time exhibited by this glycoprotein still falls short of the corresponding retention values exhibited by the native FBS-AChE ($T_{1/2} = 990$ min, MRT = 1390 min) indicating that factors other than sialic acid sialylation are also important for the extension of the circulatory life-time.

In Vitro Tetramerization of BoAChE and Its Effect on Circulatory Longevity—In contrast to the rBoAChE that was characterized by the predominant presence of dimeric and monomeric forms, naturally occurring forms of AChE, whether circulatory or membrane-bound, are arranged in multimeric complexes (16, 39, 57–60). Dimerization of catalytic subunits of AChE occurs invariably by covalent disulfide bridging involving cysteine residues situated in the C-terminal domains of the enzyme (14). Dimers further assemble into stable quaternary complexes that represent the main form of circulatory acetylcholinesterase, as is the case of serum-derived fetal bovine AChE. In several occasions monomeric subunits of AChE have been reported (59, 61), but their origin as breakdown products of multimeric forms could not be ruled out (62–65). The nonphysiological dimeric and monomeric configurations of cell culture-generated AChE has been reported to characterize other versions of recombinant cholinesterases as well. For example, recombinant preparations of rat or Torpedo AChE produced in COS cells (13, 40), human butyrylcholinesterase from Chinese hamster ovary cells (12), and human AChE from HEK-293 cells (7, 14), all consist of mainly dimers of catalytic units and only of negligible fractions of tetramers. The subunit assembly state of AChE does not affect the catalytic ability of the enzyme nor does it influence directly the production rates of recombinant versions of the enzymes produced in various cell culture systems (14). Recombinant forms of AChE are consistently characterized by a short circulatory residence time when administered to experimental animals (7, 8, 12, 16), whereas the naturally occurring forms of serum-derived tetrameric AChEs possess a long circulatory residence time. These observations suggested that the oligomerization status of AChE may play an important role in the pharmacokinetic properties of the corresponding enzymes. Yet, the prevailing pharmacokinetically deleterious effect of glycan undersialylation and the inability to control the oligomerization state of AChE precluded until now the full appreciation of the role of oligomerization in circulatory retention. For example, Saxena et al. (12) conducted a comparative pharmacokinetic study between various acetylcholinesterases differing in the extent of their tetramer/dimer content, but this study was not conclusive since the various AChE forms inspected differed not only in their oligomerization status. Similarly, in an earlier study in our laboratory (7), no clear-cut correlation between the oligomerization state of various human recombinant AChEs produced in HEK-293 cells and their circulatory clearance rate could be shown. It was therefore important to develop a system that allows one to control both the degree of glycan sialylation and the extent of enzyme subunit oligomerization. This could now be achieved thanks to the pioneering studies of Bon et al. (13, 40) that have established that coexpression of AChE and the ColQ PRAD in COS cells resulted in the generation of stable secreted AChE tetramers.

Here, we show that in vitro tetramerization by purified PRAD is sufficient for the process of tetramer assembly. This is an extension of the original system developed by Giles et al. (41). Most notably the stoichiometry of the PRAD-AChE association (Fig. 6D) reflects faithfully the known molar ratio of $1 \times$ ColQ:4×AChE within the membranal quaternary complexes of AChE, confirming their tetrameric nature and the expected presence of a single PRAD peptide in the asymmetric complex (42). The strict 4:1 ratio for tetramerization of AChE was also indirectly suggested by coexpression experiments in COS cells (13) in which it was found that a 4:1 ratio of plasmid DNA in the cotransfection mixture is required for generation of tetramers. It should be noted that in vitro oligomerization in the presence of PRAD led to efficient tetramerization not only of rBoAChE dimers (G2) but also of monomeric (G1) forms (Fig. 6B).

The studies presented here clearly show that tetramerization prevents the fast removal of recombinant BoAChE from the circulation (Fig. 5 and Table III). Most notably, the pharmacokinetic values of the tetramerized enzyme were improved to the extent that the efficiently sialylated recombinant AChE displays a pharmacokinetic profile that is virtually indistinguishable from that of its native serum-derived counterpart. Inspection of the state of assembly in the circulation following administration of fully tetramerized enzyme failed to reveal the presence of dimers or monomers, even after long periods (not shown), suggesting that removal from the circulation does not involve an intermediate stage in which the tetramer is dissociated into dimers or monomers.

The observation that the oligomerization status contributes to the serum residence time of AChE suggests a simple explanation for the scarcity of naturally occurring dimeric forms of circulating AChE, since the only population of AChE molecules that survive in the circulation over long periods are those arranged into tetrameric complexes. Alternatively, the relatively low amounts of tetramerized AChEs in recombinant cell production systems may reflect the lack of a selection process which allows the preferential accumulation of the tetramerized enzyme population.

Why are tetramers more stable in the circulation? This may reflect a simple size-exclusion phenomenon; high molecular weight complexes are not amenable to glomerular filtration, whereas dimers and monomers of relatively low hydraulic volume are removed efficiently from the circulation by the kidneys. Alternatively, the assembly of enzyme subunits into tetrameric forms may mask some epitopes that contribute to the clearance of the enzyme subunit. The possible involvement of such protein-related elements in cholinesterase removal from the circulation is at present a subject of study in our laboratory.

What Is the Interplay between Sialylation and Tetramerization in Determining Circulatory Longevity?—In Fig. 7A we depict in a schematic manner the contribution of both glycan sialylation and enzyme tetramerization to the pharmacokinetic behavior of rBoAChE. Conversion of the partially sialylated non-assembled enzyme (Fig. 7A-b) into either its fully
sialylated form (Fig. 7A-c) or into its tetramer configuration (Fig. 7A-c) results in a significant increase in the circulatory lifetime of the enzyme, yet these enzyme forms still are cleared more rapidly than the native serum-derived form of bovine acetylcholinesterase. Only when the partially sialylated non-assembled recombinant enzyme has been subjected to both improvement in the level of terminal sialylation as well as tetramerization (Fig. 7A-f) does the modified product remain in the circulation for periods that are comparable to the native enzyme (Fig. 7B). However, these two factors do not operate by simple rules of additivity in their contribution to protein longevity. This is exemplified by the fact that both assembled and non-assembled recombinant enzyme forms that are totally devoid of sialic acid still bear α-galactose. This is exemplified by the fact that removal of terminal α-gal moieties that we show result in the exposure of β-gal residues did not significantly affect circulatory residence (Fig. 7B-h). Since following α-galactosidase treatment of FBS-AChE, 1 glycan terminus per enzyme subunit (~12% of the total glycan termini) carries an un capped β-gal, one must assume that elimination from the circulation of rBoAChE by the hepatic asialoglycoprotein receptor requires more than one exposed β-gal residue per AChE subunit. This is in accordance with the observations of Ashwell and Morell (66) that clearance via the asialoglycoprotein receptor is strongly dependent upon the density of the appended terminal-galactose residues. One should, however, keep in mind that the sharply lower MRT value exhibited by the α-galactosidase-treated enzyme may indeed reflect the fact that this enzyme is approaching the threshold of circulatory tolerance for asialoglycoproteins.

Concluding Remarks—AChE possesses a valuable therapeutically potential by virtue of its high affinity to organophosphorus poisons. Its use as an efficient bioscavenger has been advanced by the development of recombinant production systems (10, 21) and the identification of catalytically favorable mutations (67–71). Yet the short circulatory residence time of the various recombinant AChE preparations represents one of the factors that preclude the development of an efficient acetylcholinesterase-based recombinant bioscavenger, and the formulation of a method for the pharmacokinetic improvement of the enzyme represents a major biotechnological challenge. In the present study we show that a delicate hierarchy of post-translation-related components can indeed determine circulatory residence time and that when these factors are combined judiciously in their optimized configurations, one can reconstitute into the rBoAChE mold all the elements that would promote its circulatory longevity. This study serves as a basis for the genetic modification of producer cell lines toward the generation of diverse pharmacokinetically long lived biomolecules and suggests a mode of operation for unraveling cellular processes and biochemical factors involved in circulatory longevity.

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