The structural basis for the electrophoretic isoforms of normal and variant human platelet arylsulphatase A

Ronald D. PORETZ,*§ Rong Sheng YANG,* Benito CANAS,† Henry LACKLAND,† Stanley STEIN† and Paul MANOWITZ‡

*Department of Molecular Biology and Biochemistry, Rutgers University, Piscataway, NJ 08855,

†Center for Advanced Biotechnology and Medicine, Piscataway, NJ 08855, and ‡Department of Psychiatry,

Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Piscataway, NJ 08816, U.S.A.

Human platelet arylsulphatase A (ASA) exhibits a multiple banding pattern when examined by PAGE. The isoform pattern (IVa) of the enzyme obtained from normal subjects differs from variants (IIIa, IIIb, IVb) which are primarily found in alcoholic patients. Alkaline phosphatase and endo-*N*-acetylglucosaminidase H treatments, as well as ion-exchange chromatography, demonstrate that the isoforms of ASA arise because of charge heterogeneity caused by phosphoglycan moieties. The isoform with the slowest mobility in PAGE lacks phosphate substituents. Based upon mannose-6-phosphate-receptor affinity chromatography it can be concluded that most, if not all, of the enzyme-linked phosphate is in the form of 6-*O*-phospho-D-mannosyl units. Lectin affinity chromatography and peptide-*N*-glycosidase F treatment followed by SDS/PAGE and Western-blot analysis indicate that normal platelet ASA contains two oligomannose and/or hybrid glycan moieties of which one, or both, possess a 6-*O*-L-fucosyl substituent on the asparagine-linked *N*-acetylglucosamine residue. Comparative analysis indicates that the variant IIIa and IIIb types of ASA differ from the IVa ASA with regard to the level of glycan phosphorylation and glycan structure, as well as the polypeptide structure.

INTRODUCTION

Arylsulphatase A (ASA; cerebroside-3-sulphate-3-sulphohydrolase; EC 3.1.6.8) is the primary lysosomal enzyme responsible for the catabolic desulphation of sulphatides in humans [1]. Deficiency of this enzyme leads to the neurological disorder metachromatic leucodystrophy (characterized by abnormally high levels of sulphatides), which results in demyelination [1]. ASA is composed of a glycopolypeptide of molecular mass approx. 62 kDa, and in some tissues also possesses a second glycopolypeptide of 57 kDa [1]. The smaller glycopolypeptide, however, may be a proteolytic product of the 62 kDa glycopolypeptide [2]. A single gene for human ASA has been cloned [3]. The polypeptide produced in fibroblasts [4] and transfected baby hamster kidney 21 cells [5] appears to utilize two of its three potential *N*-glycosylation sites, possessing either oligomannose [4] and/or complex (or hybrid) [6] glycan moieties. Above pH 7.3, the native enzyme is found as a homodimer or heterodimer, depending upon the specific tissue examined. Below pH 5.1, however, the enzyme exists as a multimer composed of two homo- and/or hetero-dimers [7].

PAGE of platelet extracts produces multiple components staining for ASA activity. Manowitz *et al.* [8] have observed a four-band ASA pattern (denoted as IVa) from normal human platelets. Interestingly, alcoholic patients were much more likely than non-alcoholic individuals to exhibit variant ASA banding patterns (IIIa, IIIb, IVb) which differed from the IVa pattern ([9,10], P. Manowitz, unpublished work). Consistent with the possibility that abnormal ASA may contribute to symptoms of alcoholism, both metachromatic leucodystrophic and some alcoholic patients have neuropsychological deficits in spatial abnormalities [11,12] and enlarged cerebral ventricles [11,13–15]. Accordingly, Manowitz *et al.* [9] have suggested that abnormal

ASA banding patterns may reflect physicochemical characteristics of the enzyme indicative of physiologically impaired ASA. It has been documented that ethanol ingestion results in increased levels of sulphatides in mouse brain [16]. Therefore it is hypothesized that the biological consequence of abnormal ASA is manifested, in part, by the coincident impact of chronic alcohol ingestion. The molecular basis giving rise to the electrophoretic banding patterns and the differences in these patterns in platelet ASA from normal and alcoholic individuals is the subject of our studies.

EXPERIMENTAL

Preparation of platelet ASA

Partially purified ASA was prepared from either pooled, outdated normal human platelet concentrates (New Jersey Blood Services) or platelets from fresh whole blood (50 ml) obtained from individual donors. The platelet concentrates were washed and freeze-thawed as described previously [17] to release ASA, with the exception that a single iso-osmotic wash was followed by two hypo-osmotic saline washes to ensure lysis of erythrocytes. Following centrifugation of the lysate at 20000 *g* for 20 min to remove cell debris, ammonium sulphate was added to a concentration of 20% saturation. After precipitation and centrifugation, the supernatant was brought to 50% saturation with additional ammonium sulphate. The pellet obtained after centrifugation was resuspended in 0.1 M-sodium acetate, pH 5.0. For those preparations used in the lectin affinity experiments, a single ammonium sulphate precipitation was performed by bringing the 20000 *g* supernatant directly to 50% saturation. After centrifugation, this pellet was resuspended in 10 mM-Tris/HCl buffer, pH 7.4, containing 0.15 M-NaCl, 1 mM-MnCl₂, 1 mM-CaCl₂, 1 mM-MgCl₂, and 0.02% NaN₃ (buffer A). When neces-

Abbreviations used: ASA, arylsulphatase A; TBS, Tris-buffered saline; KLH, keyhole limpet haemocyanin; conA, concanavalin A; LCA, *Lens culinaris* agglutinin; PHA-E, *Phaseolus vulgaris* erythroagglutinin; PHA-L, *Phaseolus vulgaris* leucoagglutinin; RCA I and RCA II, *Ricinus communis* agglutinins; SBA, soybean agglutinin; WGA, wheat germ agglutinin; MPR, mannose-6-phosphate receptor; endo-H, endo-*N*-acetylglucosaminidase H.

§ To whom correspondence should be addressed.

sary, concentration of ASA activity was accomplished with a Centricon-30 (Amicon) unit. ASA was stored at -20°C for further analysis.

Determination of ASA activity and protein concentration

ASA activity was determined by the method described by Manowitz *et al.* [18]. One unit of activity is defined as the amount of enzyme which produces 1 μmol of *p*-nitrocatechol/h. Protein concentration was measured by the Lowry assay [19].

PAGE

Electrophoresis was performed by the method of Manowitz *et al.* [8] except that 8% (w/v) precast Novex gels (Novel Experimental Technology, San Diego, CA, U.S.A.) were employed with the Tris/glycine/HCl buffer system of Davis [20]. The gels were stained for ASA-active components by the method described previously [8]. Reducing SDS/PAGE was performed with 10% (w/v) precast Tris/glycine Novex gels. The running buffer was Tris/glycine, pH 8.3 (2.9 g of Tris base and 14.4 g of glycine/l) containing 0.1% SDS. Following electrophoresis, transfer of components to nitrocellulose was performed with Tris/glycine buffer, pH 8.3, containing 10% (v/v) methanol at 20 V for 40 min. Both electrophoresis and transfer were accomplished with Novex equipment. The nitrocellulose membrane was blocked with 10 mM-Tris/HCl buffer, pH 7.5, containing 0.15 M-NaCl (TBS buffer), 0.1% Tween-20 and 0.5% BSA. After incubation for 1 h at room temperature, the membrane was washed with TBS containing 0.1% Tween-20. Subsequently, the membrane was incubated with rabbit anti-(ASA peptide) serum at room temperature for 1 h. The washed membrane was incubated with alkaline phosphatase-conjugated IgG from goat anti-(rabbit IgG) serum (Promega) prepared in TBS. Following washing of the membrane, the bromochloroindolyl phosphate/Nitro Blue Tetrazolium substrate solution (Promega) was used for colour development.

Treatment of ASA with hydrolases

Recombinant *Streptomyces plicatus* endo-*N*-acetylglucosaminidase H (endo-H) isolated from *Escherichia coli* (Boehringer Mannheim), recombinant *Flavobacterium meningosepticum* peptide *N*-glycosidase F produced in *E. coli* (Genzyme), bacterial alkaline phosphatase type III-N (Sigma) and *Clostridium perfringens* neuraminidase type X (Sigma) were utilized.

Hydrolase treatment of ASA (100 m-units) in buffer A examined by non-denaturing PAGE employed 50 m-units of neuraminidase or alkaline phosphatase in 100 μl . Before treatment with endo-H (15 m-units), ASA (100 m-units) solutions were adjusted to pH 5.6 with 0.5 M-sodium acetate, pH 5.6. Peptide *N*-glycosidase F treatment required that the ASA sample was adjusted to pH 8.6 with 0.5 M-glycine/NaOH buffer, pH 8.6, and conditions recommended by Genzyme were employed. The assay mixtures were incubated at 37°C for 20 h except where noted. When necessary, lyophilization of the reaction mixture was performed to concentrate samples before PAGE.

SDS/PAGE and Western-blot analysis of hydrolase-treated samples employed 50 m-units of highly enriched ASA. This enzyme preparation was obtained from the 20–50% saturated ammonium sulphate fraction of the lysed platelet supernatant by first performing DEAE-Sepharose chromatography (10 mg of protein/ml of gel) in a 5 cm-long column. The sample was applied in 10 mM-Tris/HCl buffer, pH 7.5, followed by two column volumes of the same buffer. Enzyme-containing protein was eluted with the same buffer incorporating 0.5 M-NaCl. The ASA was further purified by the use of two sequential gel filtrations through Sephacryl S-300 (Pharmacia Fine Chemicals), initially at pH 7.5 and then at pH 5.0. Endo-H (10 m-units)

digestion was performed in 0.2 M-sodium acetate buffer, pH 5.6, at 37°C for 20 h. Before treatment with peptide *N*-glycosidase F, the ASA sample was denatured by boiling for 5 min in 15 μl of 0.2 M-sodium phosphate buffer, pH 8.6, containing 0.5% SDS and 50 mM- β -mercaptoethanol. Following the addition of 10% (v/v) Nonidet P-40 (5 μl) and 10 μl of hydrolase (375 m-units), reactions were incubated at 37°C for 24 h. A more complete digestion was accomplished by the addition of a second aliquot of enzyme after 24 h, and continuation of the incubation for a total of 48 h.

Anti-(ASA peptide) serum

Based upon the deduced amino acid sequence of human ASA cDNA [5], a peptide was synthesized composed of α -aminobutyric acid, cysteine and alanine attached to the *N*-terminus of a 14-mer peptide corresponding to amino acid residues 424–437 of ASA. The peptide was conjugated to the carrier proteins, keyhole limpet haemocyanin (KLH) and ovalbumin, as described elsewhere [21]. Rabbits challenged initially with the KLH conjugate and boosted with the ovalbumin conjugate produced antisera capable of recognizing denatured human ASA.

Lectin-binding ability of ASA

ASA preparations were dialysed overnight at 4°C against buffer A. The eight lectins examined, as either Sepharose 4B or agarose conjugates (approx. 3 mg of protein/ml of gel), were concanavalin A (conA), *Lens culinaris* agglutinin (LCA), *Phaseolus vulgaris* erythroagglutinin (PHA-E), *Phaseolus vulgaris* leucoagglutinin (PHA-L), *Ricinus communis* agglutinins (RCA I and RCA II), soybean agglutinin (SBA) and wheat germ agglutinin (WGA). The first five lectins were purchased from Sigma Chemical Co.; the others were from E. Y. Laboratories (San Mateo, CA, U.S.A.). All lectin gels were washed and prepared as 20% (v/v) suspensions in buffer A. ASA (90 m-units) was added to 50 μl of each gel suspension, mixed and incubated at 4°C for 20 h. The supernatants and pellets were separated by centrifugation at 3000 *g* at 4°C for 2 min. After washing three times (by suspension of the beads in 100 μl of cold buffer A followed by centrifugation as before), the pellets were incubated at room temperature with shaking for 30 min in 100 μl of eluting buffer to release bound glycoprotein. These eluting buffers were buffer A containing the appropriate competitive inhibitor (see [22]) (0.5 M-methyl- α -D-mannopyranoside for conA and LCA; 0.2 M-*N*-acetylgalactosamine for PHA-E, PHA-L, and SBA; 0.2 M-lactose for RCA I and RCA II; and 0.2 M-*N*-acetylglucosamine for WGA). The eluant and gel were separated by centrifugation at 15000 *g* and 4°C for 2 min. The first supernatant containing unbound protein, the sugar eluant, and control supernatants (an equivalent amount of ASA treated with the appropriate adsorbant in the presence of the specific saccharide inhibitor, followed by centrifugation) were analysed separately for ASA activity.

Lectin-Sepharose affinity chromatography of ASA

ASA (500 m-units) in buffer A was applied to a column containing 1.0 ml of LCA-Sepharose 4B gel. A flow rate of 300 $\mu\text{l}/\text{min}$ was employed for application and elution, and 1.0 ml fractions were collected. A stepwise elution protocol was used consisting of the sequential addition of: 10 ml of buffer A (4°C); 10 ml of buffer A containing 0.05 M-methyl α -D-mannopyranoside and 0.01% BSA (23°C); and 10 ml of buffer A containing 0.5 M-methyl α -D-mannopyranoside and 0.01% BSA (23°C). The BSA was included as a carrier protein to help recover the enzyme from solutions containing low concentrations of protein. Affinity chromatography with WGA and RCA I adsorbants was performed in a similar manner. The same buffers

were employed except that the eluting buffers contained 0.2 M-*N*-acetylglucosamine or lactose respectively, instead of methyl α -D-mannopyranoside. All fractions collected were assayed for ASA activity.

Mannose-6-phosphate-receptor (MPR) affinity chromatography of ASA

A highly enriched preparation of ASA (400 m-units) was applied to a column packed with 0.5 ml of MPR conjugated to agarose (0.9 mg of protein/ml of gel, a gift from Dr. P. Lobel, Center for Advanced Biotechnology and Medicine, Piscataway, NJ, U.S.A.) equilibrated in normal saline containing 0.02 % sodium azide. The MPR was purified from bovine serum by the procedure of Hoflack *et al.* [23]. A flow rate of 6 ml/h was employed for application and elution, and 0.5 ml fractions were collected. ASA activity in the eluate was determined by the *p*-nitrocatechol sulphate assay [18]. Following elution of protein with the application buffer, bound ASA was eluted with normal saline containing 5 mM-D-mannose 6-phosphate. Pooled fractions were analysed by PAGE.

RESULTS

The characteristic IVa PAGE pattern of normal platelet ASA is shown in Fig. 1, compared with the IIIa and IIIb patterns of the variant enzymes from alcoholic patients. Both the number of components and the relative mobility of the isoforms differ between the two patterns. A simplification of each pattern is achieved by prior treatment of the samples with alkaline phosphatase. In all the cases the isoforms are converted into forms which have mobilities corresponding to only the slowest-moving isoform (isoform 1) of the appropriate untreated sample. The

Fig. 1. Polyacrylamide electrophoretograms, stained for enzyme activity, of platelet ASA preparations from pooled normal individuals and two individuals possessing variant forms of the enzyme

Lanes 1–3 and 10, normal type IVa enzyme; lanes 4–6, variant type IIIa enzyme; lanes 7–9, variant type IIIb enzyme. Samples in lanes 2, 5, and 8 were pretreated with alkaline phosphatase. Samples in lanes 3, 6, and 9 were pretreated with endo-H.

Fig. 2. SDS/PAGE Western-blot analysis of ASA samples from pooled platelets of normal individuals

(a) and (b) represent two separate experiments differing only in the pretreatment of ASA with peptide *N*-glycosidase F. Lane 1, untreated ASA; lane 2, ASA pretreated with endo-H; lane 3, ASA pretreated with peptide *N*-glycosidase F; (a), 375 m-units of enzyme added at zero time for a 24 h incubation period; (b), 375 m-units of enzyme added at both zero time and 24 h for a total incubation time of 48 h).

dephosphorylated enzyme obtained from the IVa sample yields a single component of mobility significantly slower than the mobility of the dephosphorylated product of the IIIa and IIIb samples. Consistent with these results, anion-exchange h.p.l.c. at pH 7.5 of IVa ASA resulted in the partial resolution of the individual charged isoforms (results not shown). ASA activity, applied to a PL-SAX column (5 cm $\times$ 2 cm; Polymer Laboratories) in 10 mM-Tris/HCl, pH 7.5, was eluted with a salt gradient of 0–0.5 M-NaCl in the Tris/HCl buffer. Enzymic staining of PAGE gels containing individual fractions from the column showed the sequential, though overlapping, elution of the electrophoretic isoforms. The slower-moving electrophoretic isoforms were eluted before the faster-moving electrophoretic isoforms. In addition, neuraminidase treatment is without effect on the electrophoretic mobility of the enzyme IIIa and IVa isoforms (results not shown), suggesting that sialic acid is not responsible for the heterogeneity observed in electrophoresis and ion-exchange chromatography. Apparently, the appearance of electrophoretic isoforms is caused by the charge heterogeneity of phosphate groups on the enzyme. Given that discrete electrophoretic forms with quantum-like differences in mobility are detected, the simplest explanation is that the IVa type contains non-, mono-, di- and tri-phosphorylated forms, and the IIIa and IIIb types are mixtures of non-, mono- and di-phosphorylated forms.

Evidence that such phosphate groups are carbohydrate-linked is demonstrated by the electrophoretic mobility of enzymes pretreated with endo-H. Fig. 1 shows that endo-H treatment of each enzyme produces a single electrophoretic component staining for enzymic activity with a mobility consistent with not only the loss of phosphate groups, but also a mass of neutral carbohydrate. Again, the mobility of the enzyme from the IVa sample, which lacks the glycan-linked phosphate as well as the oligomannosyl moieties, differs from the analogous products from the IIIa and IIIb samples, which in turn differ from each other. In contrast with the large difference in mobilities of the alkaline phosphatase-treated variants as compared with the analogous IVa sample, the differences in mobilities of the endo-H products are relatively small.

Confirmation that all of the *N*-linked carbohydrate on the IVa sample is endo-H sensitive, and presumably composed of oligomannose or hybrid units, is provided by the evidence that treatment of the denatured enzyme with either endo-H or peptide *N*-glycosidase F produces a product with a mobility in SDS/PAGE corresponding to the loss of 4 kDa when compared with the untreated enzyme. In Fig. 2, the Western-blot analysis of the enzyme after SDS/PAGE shows that the enzyme yields a single band with a mobility corresponding to a molecule of 62 kDa. Endo-H treatment results in a single product of 58 kDa. Limited peptide *N*-glycosidase F treatment (375 m-units of enzyme for a 24 h incubation time) produces two components of 60 and 58 kDa (Fig. 2a, lane 3), and extensive peptide *N*-glycosidase F digestion (375 m-units of enzyme added at both zero time and 24 h for a total incubation time of 48 h) yields a single component of 58 kDa (Fig. 2b, lane 3). Accordingly, ASA from the IVa sample appears to be composed of a single glycopolypeptide chain which possesses two *N*-linked oligomannose and/or hybrid moieties.

As expected for a lysosomal enzyme, some, if not all, of the phosphate is present as 6-*O*-D-mannosylphosphate groups. MPR for targeting of lysosomal enzymes recognizes the D-mannose 6-phosphate ligand as a component of the glycan moiety of lysosomal enzymes [24]. As shown in Fig. 3, affinity chromatography of a partially purified preparation of platelet ASA through an MPR-agarose column results in the separation of unbound ASA from bound ASA, eluted with 5 mM-D-mannose

Fig. 3. MPR-agarose affinity chromatography of ASA from pooled platelets of normal individuals, as monitored for enzyme activity (A_{515}).

D-Mannose 6-phosphate (5 mM) was added to the normal saline eluant where indicated. Untreated ASA preparation (Δ); alkaline phosphatase-treated preparation of ASA ($\blacklozenge$).

Fig. 4. LCA-Sepharose affinity chromatography of ASA from pooled platelets of normal individuals as monitored for enzyme activity (A_{515}).

The protein was eluted with buffer A, followed by buffer A containing 0.05 M-methyl α -D-mannopyranoside and 0.01 % BSA or 0.5 M-methyl α -D-mannopyranoside and 0.01 % BSA, where indicated.

6-phosphate in normal saline. PAGE of the two ASA populations demonstrates that the unbound enzyme corresponds to isoform 1 (the slowest-moving electrophoretic isoform) of the IVa pattern, and the bound and eluted material is composed of the three remaining isoforms (results not shown). Consistent with these results, alkaline phosphatase treatment of IVa ASA, which results in a product with the mobility of isoform 1, also converts the enzyme into a form unable to bind to the MPR. These results indicate that isoform 1 lacks D-mannose 6-phosphate and the remaining three isoforms possess this moiety.

Interestingly, the difference in the level of glycan phosphorylation or in the ability to bind to MPR is not related to the nature of the glycan moiety itself. Analytical batch adsorption experiments or affinity chromatography employing RCA I, RCA II, WGA, SBA, PHA-E and PHA-L adsorbents demonstrated that all forms of platelet ASA lack the complex glycan moieties which allow binding to these lectins. Con A-Sepharose and LCA-Sepharose do bind ASA. Fig. 4 shows the elution profile of a partially purified preparation of type IVa platelet ASA from an LCA-Sepharose column. All isoforms of the ASA bind to the

lectin adsorbant. Both the more tightly bound (requiring > 0.05 M-methyl α -D-mannopyranoside for elution) and the weakly bound (requiring only 0.05 M-methyl α -D-mannopyranoside for elution) enzyme pools produced the original type IVa isoform pattern upon PAGE (results not shown). Further evidence that the glycan phosphate groups do not affect the binding of ASA isoforms to LCA is inferred from the elution pattern of alkaline phosphatase-treated ASA, which is virtually identical to that of the untreated enzyme (results not shown). Apparently, all isoforms behave as would be expected for glycoproteins containing L-fucosylated oligomannose moieties as the only N-linked glycans [22], and the different isoforms cannot be distinguished by their abilities to bind to specific lectins. However, approx. 10 % of each isoform does differ from the bulk of the enzyme, perhaps reflecting the presence of a non-L-fucosylated population (see [22]).

DISCUSSION

Molecular indicators of potential alcoholism and the basis for the genetic predisposition to alcoholism are actively being pursued [25]. The association of variants of platelet ASA in a significant number of diagnosed alcoholics and their relative absence in the non-alcoholic population is intriguing. To learn about the molecular basis of the behaviour of the variants detected by PAGE, the cause of isoform electrophoretic heterogeneity must be approached first. Based upon the results of the action of alkaline phosphatase and endo-H on normal human platelet ASA, it is clear that the total electrophoretic heterogeneity is caused by the presence of carbohydrate-linked phosphate esters on the enzyme. It is well documented that the major pathway of lysosomal enzyme targeting during biosynthesis involves the recognition of enzyme-linked D-mannose 6-phosphate units by one or more related MPRs [24]. Analysis of ASA by MPR affinity chromatography confirms that the isoform not affected by alkaline phosphatase does not interact with the MPR, and the phosphorylated forms of the enzyme possess all or some of these esters as D-mannose 6-phosphate moieties. Interestingly, the retention of the glycan-bound phosphate ester units by ASA, following presumed targeting of the enzyme to the lysosome, appears to be tissue-dependent. The results reported here demonstrate that more than 75 % of platelet ASA is phosphorylated. In contrast, liver appears to contain ASA with virtually undetectable alkaline phosphatase-susceptible populations [26]. Such differences in the behaviour of ASA may reflect factors associated with ASA half-life and/or the phosphatase activity within lysosomes of different tissues.

Type IVa platelet ASA appears to differ from the variant IIIa and IIIb enzymes with regard to both their carbohydrate and polypeptide moieties. This is inferred from the behaviour of the two forms of ASA following treatment with alkaline phosphatase or endo-H. Though all three ASAs yield a single component, the enzymes are converted into products having different electrophoretic mobilities. Comparison of the changes in mobilities of the dephosphorylated forms of each ASA with those of the corresponding type following endo-H treatment demonstrates that removal of carbohydrate from the type IVa form has a greater effect on the mobility of the enzyme than removal of N-glycan from the IIIa or IIIb enzymes. Nevertheless, the difference in the electrophoretic mobilities following endo-H treatment of all three enzyme types also suggests that the ASAs possess differences in domains other than those of the endo-H-susceptible glycans. Such differences between the normal and variant forms, as well as between the variant enzymes, may reside in the primary amino acid sequence or post-translational modifications not associated with the N-linked glycans, and may be related to

sulphation, phosphorylation, acylation and/or *O*-linked glycosylation of the polypeptide. Because of the limited quantities of enriched preparations of variant enzymes, we have not yet determined the apparent molecular mass of the deglycosylated forms of the IIIa and IIIb enzymes by SDS/PAGE.

The structure of the *N*-linked carbohydrate moieties of ASA are not fully elucidated. Both superficial structural studies on human lung ASA [27] and biosynthetic studies [6] on fibroblast ASA indicate that the enzyme possesses complex-type glycans. However, the endo-H susceptibility of human fibroblast ASA is consistent with the existence of either oligomannose or hybrid glycan units. Our finding that normal platelet ASA has two endo-H-susceptible glycans of approx. 2 kDa each, indicates that the only peptide *N*-glycosidase F-cleavable glycans of the enzyme are oligomannose and/or hybrid structures. The probability that the glycans are of the oligomannose type is further supported by the apparent absence of sialic acid, and the inability of the enzyme to bind to a number of lectins other than those which recognize α -D-mannosyl units. The possibility exists, however, for the presence of phosphorylated hybrid structures. Endo-H is capable of acting on the phosphorylated hybrid structures of lysosomal enzymes [28]. Further structural information may be obtained from the behaviour of the enzyme during LCA affinity chromatography. The relatively strong interaction between the platelet ASA and LCA suggests that the asparagine-linked *N*-acetylglucosamine residue possesses a 6-*O*- α -L-fucosyl residue on at least one of the two *N*-linked structures of at least 90% of the ASA isolated.

It is clear from this work that differences in both the glycan and polypeptide moieties exist between the type IVa form of ASA from normal individuals and the variants detected predominantly in alcoholic patients, as well as between the variants themselves. As with other phosphorylated lysosomal enzymes, ASA is believed to be directed to lysosomes during biosynthesis by way of the MPR pathway [24]. Evidence exists, however, that an MPR-independent pathway is also involved in ASA intracellular targeting [29]. A growing body of literature is accumulating linking alcohol with abnormal protein glycosylation [30,31], dolichol metabolism [32] and glycoprotein trafficking [33,34] in the liver. Accordingly, it may be hypothesized that the structural abnormalities of the ASA variants may interfere with the ability of these enzymes to be correctly targeted to lysosomes. A consequence of such mistargeting may be the subclinical alteration of sulphatide metabolism. Ethanol may exacerbate, by a direct or indirect effect, the abnormal sulphatide metabolism, thereby inducing the clinical sequelae.

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REFERENCES

1. Kolodny, E. H. (1989) in *The Metabolic Basis of Inherited Disease*, (Scriver, C. R., Beaudet, A. L., Sly, W. S. & Valle, D., eds.), pp. 1721–1750, McGraw-Hill, New York

2. Steckel, F., Hasilik, A. & von Figura, K. (1985) *Eur. J. Biochem.* **151**, 141–145
3. Kreysing, J., von Figura, K. & Gieselmann, V. (1990) *Eur. J. Biochem.* **191**, 627–631
4. Ameen, M., Lazzarino, D. A., Kelly, B. M., Gabel, C. A. & Chang, P. L. (1990) *Mol. Cell. Biochem.* **92**, 117–127
5. Stein, C., Gieselmann, V., Kreysing, J., Schmidt, B., Pohlmann, R., Waheed, A., Meyer, H. E., O'Brien, J. S. & von Figura, K. (1989) *J. Biol. Chem.* **264**, 1252–1259
6. Bräulke, T., Hille, A., Hüttner, W. B., Hasilik, A. & von Figura, K. (1987) *Biochem. Biophys. Res. Commun.* **143**, 178–185
7. James, G. T., Thach, A. B., Klassen, L. & Austin, J. H. (1985) *Life Sci.* **37**, 2365–2371
8. Manowitz, P., Goldstein, L. & Bellomo, F. (1978) *Anal. Biochem.* **89**, 423–429
9. Manowitz, P., Villano-Fine, L., Nora, R., Pernicano, K., Nathan, P. & Chokroverty, S. (1987) *Ann. N.Y. Acad. Sci.* **492**, 341–343
10. Hulyalkar, A. R., Nora, R. & Manowitz, P. (1984) *Alcohol. Clin. Exp. Res.* **8**, 337–341
11. Manowitz, P., Kling, A. & Kohn, H. (1978) *J. Nerv. Ment. Dis.* **166**, 500–506
12. McCrady, B. S. & Smith, D. E. (1986) *Alcohol.* **10**, 145–149
13. Skomer, C., Stears, J. & Austin, J. (1983) *Arch. Neurol.* **40**, 354–355
14. Wilkinson, D. A. & Carlen, P. L. (1980) in *Advances in Experimental Medicine and Biology. The Biological Effects of Alcoholism* (Begleiter, H., ed.), vol. 126, pp. 683–699, Plenum, New York
15. Bergman, H., Borg, S., Hindmarsh, T., Idestrom, C. M. & Mutzell, S. (1980) in *Advances in Experimental Medicine and Biology. The Biological Effects of Alcoholism* (Begleiter, H., ed.), vol. 126, pp. 771–786, Plenum, New York
16. Rawat, A. K. (1974) *Res. Commun. Chem. Pathol. Pharm.* **8**, 461–469
17. Georgopoulos, D. E. & Manowitz, P. (1982) *Biochem. Genet.* **20**, 907–918
18. Manowitz, P., Goldstein, L. & Nora, R. (1981) *Biol. Psychiat.* **16**, 1107–1113
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
20. Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
21. Tsao, J., Lin, X., Lackland, H., Tous, G., Wu, Y. & Stein, S. (1991) *Anal. Biochem.* **197**, 137–142
22. Goldstein, I. J. & Poretz, R. D. (1986) in *The Lectins. Properties, Functions and Applications in Biology and Medicine* (Liener, I. E., Sharon, N. & Goldstein, I. J., eds.), pp. 35–250, Academic Press, New York
23. Hoflack, B., Fujimoto, K. & Kornfeld, S. (1987) *J. Biol. Chem.* **262**, 123–129
24. Kornfeld, S. & Mellman, I. (1989) *Annu. Rev. Cell Biol.* **5**, 483–525
25. Devor, E. J. & Cloninger, C. R. (1989) *Annu. Rev. Genet.* **23**, 19–36
26. Sarafian, T. A., Fluharty, A. L. & Kihara, H. (1984) *Biochem. Med.* **31**, 80–86
27. Nakamura, M., Gasa, S. & Makita, A. J. (1984) *J. Biochem. (Tokyo)* **96**, 207–213
28. Varki, A. & Kornfeld, S. (1983) *J. Biol. Chem.* **258**, 2806–2818
29. Waheed, A., Van Etten, R. L., Koob, R. & Drenckhahn, D. (1987) *Eur. J. Cell Biol.* **45**, 262–267
30. Stilber, H. & Borg, S. (1991) *Scand. J. Clin. Lab. Invest.* **51**, 43–51
31. Renau-Piqueras, J., Sancho-Tello, M., Cervellera, R. B. & Guerri, C. (1989) *Alcohol. Clin. Exp. Res.* **13**, 817–823
32. Kril, J. J., Pullarkat, R. K., Pullarkat, P. S. & Harper, C. G. (1990) *Alcohol. Clin. Exp. Res.* **14**, 528–530
33. Tuma, D. J., Casey, C. A. & Sorrell, M. F. (1991) *Alcohol. Clin. Exp. Res.* **15**, 808–813
34. Tuma, D. J. & Sorrell, M. F. (1988) *Semin. Liver Dis.* **8**, 69–80

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