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Purification and structure of human liver aspartylglucosaminidase

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We have recently diagnosed aspartylglucosaminuria (AGU) in four members of a Canadian family. AGU is a lysosomal storage disease in which asparagine-linked glycopeptides accumulate to particularly high concentrations in liver, spleen and thyroid of affected individuals. A lesser accumulation of these glycopeptides is seen in the kidney and brain, and they are also excreted in the urine. The altered metabolism in AGU results from a deficiency of the enzyme aspartylglucosaminidase (1-aspartamido-β-N-acetylglucosamine amidohydrolase), which hydrolyses the asparagine to *N*-acetylglucosamine linkages of glycoproteins and glycopeptides. We have used human liver as a source of material for the purification of aspartylglucosaminidase. The enzyme has been purified to homogeneity by using heat treatment, $(NH_4)_2SO_4$ fractionation, and chromatography on concanavalin A–Sepharose, DEAE-Sepharose, sulphopropyl-Sephadex, hydroxyapatite, DEAE-cellulose and Sephadex G-100. Enzyme activity was followed by measuring colorimetrically the *N*-acetylglucosamine released from aspartylglucosamine at 56 °C. The purified enzyme protein ran at a 'native' molecular mass of 56 kDa in SDS/12.5%-PAGE gels, and the enzyme activity could be quantitatively recovered at this molecular mass by using gel slices as enzyme source in the assay. After denaturation by boiling in SDS the 56 kDa protein was lost with the corresponding appearance of polypeptides α, β and β_1, lacking enzyme activity, at 24.6, 18.4 and 17.4 kDa respectively. Treatment of heat-denatured enzyme with *N*-glycosidase F resulted in the following decreases in molecular mass; 24.6 to 23 kDa and 18.4 and 17.4 to 15.8 kDa. These studies indicate that human liver aspartylglucosaminidase is composed of two non-identical polypeptides, each of which is glycosylated. The *N*-termini of α, β and β_1 were directly accessible for sequencing, and the first 21, 26 and 22 amino acids respectively were identified.

INTRODUCTION

Aspartylglucosaminuria (AGU) is a lysosomal storage disease caused by a deficiency in the enzyme aspartylglucosaminidase (AGA). The first patients were described by Pollitt *et al.* (1968) in the United Kingdom. AGU patients are rarely encountered outside Finland, where, by 1982, 138 patients from 108 families were diagnosed with the disease (Aula *et al.*, 1974, 1982). The incidence there is 1 in 26000 births, and the carrier frequency in some regions is as high as 1 in 80. Other documented cases include eight of Finnish origin living in Norway (Borud & Torp, 1976; Borud *et al.*, 1978), as well as four English cases (Aula *et al.*, 1982; Jenner & Pollitt, 1967) and six in the United States (Isenberg & Sharp, 1975; Hreidarsson *et al.*, 1983; Chitayat *et al.*, 1988).

The Finnish phenotype is quite consistent; patients are healthy for the first few months, but encounter recurrent infections, diarrhoea and hernias within the first year of life. The coarsening of facial features is barely evident during the first decade, but becomes progressively more obvious thereafter. Mental development to age 5 is quite normal, except for a delay in speech, although adult IQ values are usually below 40. Death occurs in the third to fifth decade of life, the result generally of pneumonia or pulmonary abscess.

Recently it was demonstrated that a $Cys^{113} \rightarrow$ serine mutation is responsible for most, if not all, Finnish cases of AGU (Fisher & Aronson, 1991; Ikonen *et al.*, 1991). Interestingly this mutation has not been found in non-Finnish patients with the disease (Fisher & Aronson, 1991).

The materials stored in the tissues of AGU patients (primarily liver, spleen and thyroid, but also in brain) and excreted in their urine reflect the exact nature of the enzyme deficiency (Beaudet & Thomas, 1989). *N*-acetylglucosaminylasparagine is the primary storage/excretion product, although more complex glycopeptides containing chitobiose are also seen, presumably because terminal asparagine on oligosaccharides interferes with the hydrolysis of the chitobiosyl linkage.

We have recently diagnosed four individuals in a Canadian family with AGU (B. A. Gordon, C. A. Rupar, J. W. Rip, M. D. Haust, E. Scott & G. G. Hinton, unpublished work). The very low level of AGA activity seen in leucocytes and fibroblasts from these patients make it difficult to determine that AGA protein is actually being produced. We have therefore purified AGA from normal human liver and determined its size and subunit composition. This information will enable us to produce antibodies to each of the AGA components and determine the extent to which AGA is being expressed in our four patients. The fractionation procedures used allows co-purification of heparin sulphamidase (the enzyme deficient in Sanfilippo A patients) with AGA up to the DEAE-Sepharose step.

EXPERIMENTAL

(a) Assay of AGA

The assay mixture consisted of 75 μl 0.05 M-KH_2PO_4/K_2HPO_4 buffer, pH 6.1, 25 μl of 2-acetamido-1-(β-L-aspartamido)-1,2 dideoxy-β-D-glucose (5 mM), and 50 μl of enzyme (Makino *et al.*, 1966). Incubation was at 56 °C for 1–24 h depending on enzyme concentration, and assays were stopped by addition of 150 μl of cold acetone followed by centrifugation at 16000 *g* for 20 min.

The entire supernatant was transferred to a fresh 1.5 ml Microfuge tube and dried under nitrogen at 50 °C, and the amount of free *N*-acetylglucosamine quantified by a modification of the method of Aminoff, Morgan & Watkins (Keleti & Lederer, 1974).

For the quantification of AGA activity in polyacrylamide gels the appropriate lanes were cut from the gel and sliced into 2 mm

Abbreviations used: AGU, aspartylglucosaminuria; AGA, aspartylglucosaminidase; Con A, concanavalin A; SP, sulphopropyl; HS, heparin sulphamidase.

sections, which were then diced and added directly to the enzyme assay mixture.

AGA activity eluted from concanavalin A (Con A) columns was located indirectly by assaying for heparin sulphamidase (HS), a second lysosomal hydrolase in which we are also interested (Paschke & Kresse, 1979). These two enzymes co-purify up to the DEAE-Sepharose step.

(*b*) Purification of AGA from liver

The fractionation scheme used allows co-purification of AGA and HS to about 500-fold (for AGA). The two activities are then separated on DEAE-Sepharose and can be purified through additional steps independently (only the purification of AGA is reported here).

(i) Extraction. Normal human liver (325 g) was obtained at autopsy and homogenized at 4 °C in extraction buffer (50 mM-Tris-HCl/150 mM-KCl; pH 7.0) at 4 ml/g using a Braun blender (60 s, full speed), followed by a Polytron homogenizer (1–2 min at 75% maximum speed using a 1 cm probe). The crude homogenate was filtered through cheesecloth and centrifuged for 20 min at 4000 *g*.

(ii) Heat treatment. Portions (60 ml) of the supernatant from (i) were heated to 60 °C in a boiling water bath (about 3 min each), cooled, pooled and centrifuged for 20 min at 8000 *g*. Solid $(NH_4)_2SO_4$ was added to the clear red supernatant to 65% saturation at 4 °C. After 4 h the precipitate was collected by centrifugation at 8000 *g* for 45 min, dissolved in extraction buffer and dialysed against 3 × 4 litres extraction buffer over 24 h.

(iii) Con A–Sepharose. The dialysis residue from (ii) above was clarified by centrifugation for 30 min at 8000 *g*, adjusted to contain 2 mM each of $MgCl_2$, $MnCl_2$ and $CaCl_2$, and loaded on a Con A–Sepharose 4B column (2.5 cm × 20 cm; Pharmacia). Fractions (5–6 ml) were collected, and proteins binding to Con A were eluted with a linear (0–0.8 M) gradient of α-methyl mannoside in extraction buffer containing the bivalent metal ions as above. The AGA containing fractions were located by assaying for HS activity and the peak activity fractions were pooled, dialysed against extraction buffer (containing 2 mM-2-mercaptoethanol and adjusted to pH 7.2) and then concentrated by $(NH_4)_2SO_4$ precipitation as described above.

(iv) DEAE-Sepharose. The $(NH_4)_2SO_4$ precipitate was dissolved in DEAE buffer (10 mM-Tris/HCl/25 mM-KCl/2 mM-2-mercaptoethanol, pH 8.2) and dialysed against the same buffer before loading on to a DEAE-Sepharose (1.5 cm × 20 cm) column. The column was washed with DEAE buffer until a total of 40 fractions of 2 ml were obtained when a linear gradient (25 mM–225 mM) of KCl was applied. The column was monitored for AGA/HS and A_{280}.

(v) Sulphopropyl (SP)-Sephadex. A 1 cm × 15 cm column of SP-Sephadex was equilibrated in 10 mM-Tris/maleate, pH 6.0, containing 10 mM-KCl. Fractions 86–92 (from DEAE-Sepharose; Fig. 1 below) were pooled and dialysed against this same buffer and then concentrated to 15.2 ml by ultrafiltration (YM-10 membrane; Amicon, Bio-Rad, Mississauga, ON, Canada). This concentrate, containing 21.5 mg of protein, was loaded and the column was washed with the same buffer (starting at fraction 9) and a linear gradient of KCl (10–200 mM in Tris/maleate, pH 6.0) was started at fraction 24 (Fig. 2 below). Fractions (2.4 ml each) were collected and assayed for their AGA activity and protein content.

(vi) Hydroxyapatite. Pooled fractions from SP-Sephadex with AGA activity were reduced to 8 ml by ultrafiltration (YM-10 membrane) and dialysed overnight against 4 litres of 1 mM-potassium phosphate buffer, pH 7.4. The material was loaded in 1 mM-phosphate on a 1 cm × 9 cm column of hydroxyapatite (Bio-Rad) equilibrated with dialysis buffer and eluted incrementally with increasing concentrations of potassium phosphate buffer, pH 7.4 (3 × 5 ml), at each concentration. Fractions (5 ml each) were collected.

(vii) Sephadex G-100. Fractions with peak AGA activity (from hydroxyapatite) were chromatographed on a Sephadex G-100 column (1.5 cm × 110 cm) after concentration on a YM-10 membrane to 1.0 ml. The column was run in 10 mM-potassium phosphate buffer, pH 7.4, and 1.9 ml fractions were collected for AGA activity and protein measurement. Protein was measured at A_{280} to fraction 35 (Fig. 6 below). The fractions with AGA activity (36–47 inclusive) were pooled and their protein content and distribution was measured on SDS/polyacrylamide gels.

(viii) SDS/PAGE. Gels (12.5%) were run in a mini-gel apparatus (Bio-Rad) using the Laemmli (1970) discontinuous system at 100 V with the Tris/glycine buffer system at pH 8.3. Samples were diluted 1:1 with 2 × treatment buffer [0.125 M Tris/HCl (pH 6.8)/4% (w/v) SDS/20% (v/v) glycerol/10% (v/v) 2-mercaptoethanol] or freeze-dried and then dissolved in the same buffer but at half the strength. Samples were left at room temperature or placed into boiling water for 3 min and loaded on the 5% stacking gel.

(ix) Electrophoresis. In order to minimize *N*-terminal blockage or side-chain modification of peptides which could interfere with peptide sequencing, gels were pre-run in a system containing 50 mM-Tris/HCl, pH 8.0, containing 0.1% SDS and 0.1 mM-sodium thioglycollate for 30 min at 100 V. For electrophoresis the tank and clamp assembly were then rinsed with distilled water and the samples run in the normal Tris/glycine system to which 0.1 mM-sodium thioglycollate was added.

(x) Electroblotting. A mini Trans-Blot electrophoresis transfer cell (Bio-Rad) was used with ProBlott membrane (Applied Biosystems, Foster City, CA, U.S.A.). Gels were removed from the electrophoresis cell and soaked in electroblotting buffer (10 mM-3-cyclohexylamino-1-propanesulphonic acid, pH 11) for 5 min. The transblot sandwich was assembled with a double thickness of ProBlott and proteins transferred at constant voltage (50 V; 100–170 mA) for 30 min.

After removal from the sandwich, membranes were rinsed with distilled water and stained lightly with 0.1% Coomassie Blue R-250 in methanol/acetic acid/water (40:1:59, by vol.). Membranes were destained with 50% methanol, rinsed extensively with distilled water, dried, then stored at −70 °C until *N*-terminal sequencing was carried out.

(xi) *N*-Terminal sequence. Analysis was carried out by using the automated Edman degradation procedure (Matsudaira, 1987).

(xii) Deglycosylation. AGA purified through the second hydroxyapatite step showing only the native ≈ 56 kDa band on silver staining was used. About 2.5 μg of AGA (in 350 μl of 1 mM-potassium phosphate buffer, pH 7.4) was placed in each of two 0.5 ml plastic disposable centrifuge tubes and freeze-dried. Residue was taken up in 25 μl of SDS buffer [40 mM-potassium phosphate/20 mM-Na_4EDTA/0.2% SDS and 1% (v/v) 2-mercaptoethanol, pH 7.4] and heated to 100 °C for 3 min. The samples were cooled to room temperature and 25 μl of 3% (w/v) n-octyl glucoside was added to avoid inactivation of *N*-glycosidase F (Boehringer-Mannheim, Laval, PQ, Canada) by SDS (Haselbeck & Hösel, 1988).

To one tube *N*-glycosidase F (5 μl, 1 unit) was added, whereas the second tube received 5 μl of water. Both were incubated for 15 h at 37 °C, after which aliquots were analysed by SDS/PAGE.

RESULTS

Early in these studies we noted that AGA activity could be increased by incubating samples at 56 °C rather than 37 °C. At

Table 1. Purification of aspartylglucosaminidase

A unit of AGA activity results in a ΔA_{585} of 1.0/2 h in the standard assay.

Step	Protein (mg)	Activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude homogenate	27950	4570	0.1635	1	100
Supernatant after heating	15040	4290	0.2852	1.7	94
Dialysed $(NH_4)_2SO_4$ cut	5890	4160	0.7063	4.3	91
Con A pooled	160	3590	22.44	137	78
DEAE (86–92) pooled	22	1800	81.82	513	40
SP-Sephadex	11	1600	145.5	890	35
Hydroxyapatite	1.0	880	880	5380	19
Sephadex G-100	0.18	720	4000	24500	15
Hydroxyapatite*	0.16	680	4250	26000	14.9

* Only part of sample was run; the value is corrected for total activity.

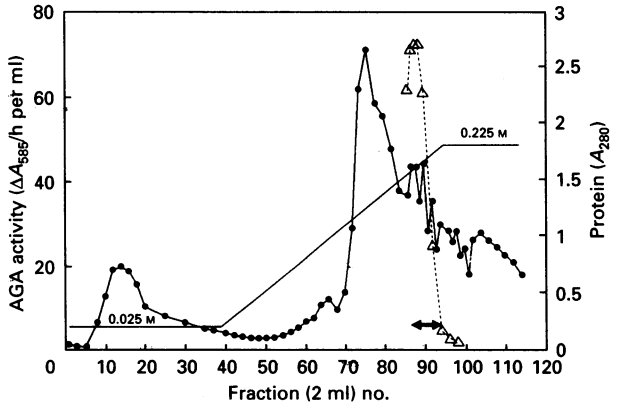

Fig. 1. DEAE-Sepharose chromatography of HS and AGA obtained after Con A–Sepharose chromatography

●, A_{280}; △, AGA activity; ——, KCl concentration; ↔, fractions pooled for SP-Sephadex chromatography.

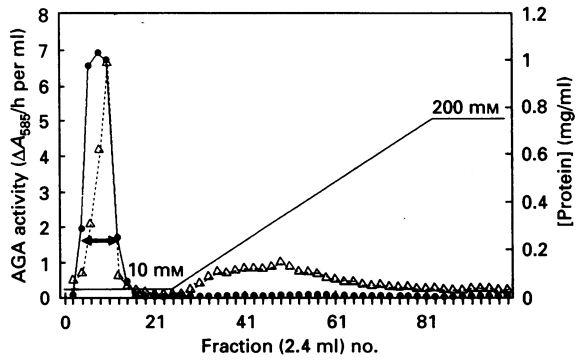

Fig. 2. SP-Sephadex chromatography of AGA obtained after DEAE-Sepharose chromatography

△, [Protein] (mg/ml); ●, AGA activity; ——, KCl concentration; ↔, fractions pooled for hydroxyapatite chromatography.

this temperature linear kinetics were seen for at least 8 h. In some cases assays were run overnight at 56 °C where 'peak finding' rather than exact quantification of AGA activity was the primary

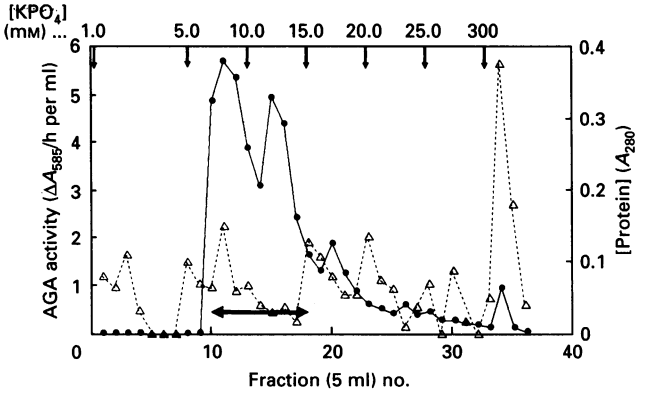

Fig. 3. Hydroxyapatite chromatography of aspartylglucosaminidase purified up to (and including) the SP-Sephadex step

△, A_{280}; ●, AGA activity, ↔, fractions pooled for further purification (G-100 Sephadex). The vertical arrows indicate the concentrations of potassium phosphate (KPO_4) buffer, pH 7.4, used to step-elute the column.

objective. Once AGA could be identified on SDS-containing gels purification was in some instances measured on the basis of protein distribution in the gels.

The purification of AGA up to and including the second hydroxyapatite step is shown in Table 1. HS and AGA behave similarly on Con A–Sepharose, and fractions enriched in HS are also enriched in AGA. About 80% of the AGA activity initially present in homogenates was present in HS preparations and a purification of 32-fold over the previous [$(NH_4)_2SO_4$] step was obtained.

HS and AGA separated nearly completely on DEAE-Sepharose (Fig. 1). HS activity was present in the large A_{280} peak (fractions 70–85) that was eluted just before AGA (HS activity not shown on Fig. 1). On these columns, 15–20% of the AGA peak is lost to the HS preparation. Despite this, the activity recovered in the AGA peak as selected accounts for 40% of the activity in the crude homogenate.

AGA did not bind to SP-Sephadex (Fig. 2), although about 50% of the protein left in the AGA preparation did. The procedure gives a rapid near doubling of specific activity to 890-fold with little loss.

Preliminary experiments (not shown) indicated that AGA bound weakly to hydroxyapatite. Preparations purified through the SP-Sephadex step were dialysed against 1 mM-potassium phosphate, pH 7.4, and loaded on hydroxyapatite columns equilibrated in the same buffer. After washing with 1 mM-potassium phosphate, pH 7.4, bound proteins were eluted with 3 × 5 ml aliquots of increasing molarities of phosphate buffer at pH 7.4 (5, 10, 15, 20, 25 and 300 mM; Fig. 3). All AGA activity bound to the column, and about 90% of it could be eluted in the 5 and 10 mM steps combined. The amount of enzyme activity eluted with 5 and 10 mM-phosphate (Fig. 3) is considerably greater than shown, owing to the loss of linearity of the AGA assay at high enzyme concentrations.

Purification was monitored by SDS/PAGE. Pooled hydroxyapatite material (enriched 5380-fold in AGA over homogenates) contained a noticeable enrichment of a 'barbell'-shaped band at ≈ 56 kDa (the presumed molecular mass of AGA before denaturation) when preparations were diluted 2-fold with treatment buffer and run on SDS-containing gels without heating (lane 2, Fig. 4).

This band shape seems unique to the 56 kDa protein, since other polypeptides in the same lane of the gel are unaffected.

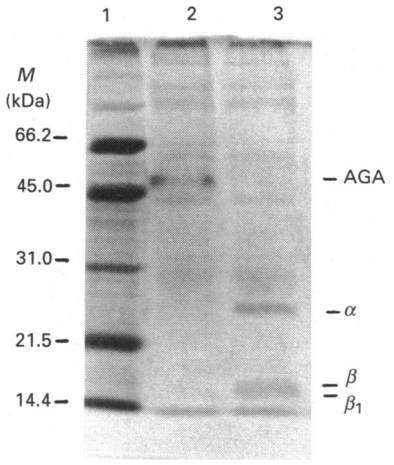

Fig. 4. SDS/PAGE of AGA purified through the hydroxyapatite step and pooled as indicated in Fig. 3

The lanes are: 1, molecular-mass (*M*) markers; 2, AGA from pooled hydroxyapatite material run directly, i.e. 'native'; and 3, same as lane 2, but after heating 3 min at 100 °C in denaturation buffer.

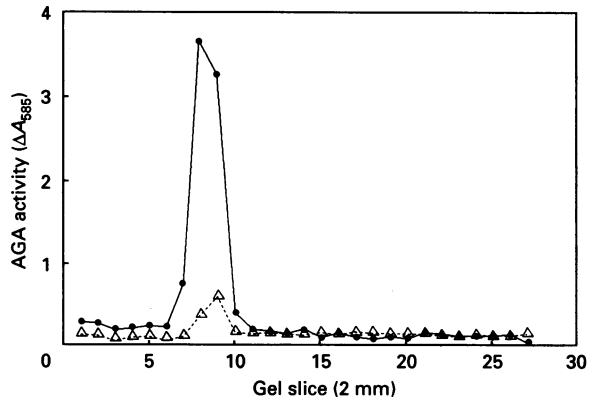

Fig. 5. SDS/PAGE of AGA purified through the hydroxyapatite step

Lanes (exact duplicates of lanes 2 and 3 of Fig. 4) were cut from the same gel and sliced into 2 mm segments which were then assayed for AGA activity. ●, 'Native' enzyme; △, enzyme heated 3 min at 100 °C.

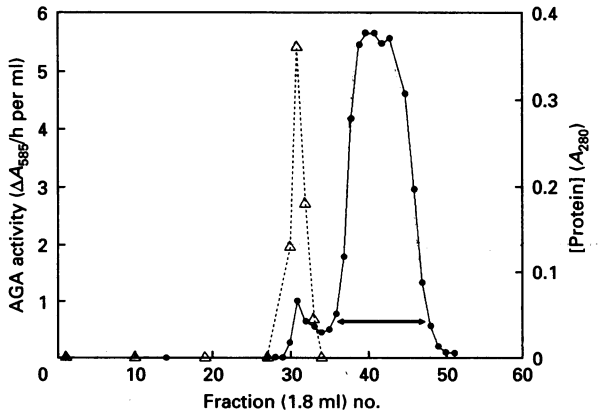

Fig. 6. Sephadex G-100 chromatography of aspartylglucosaminidase purified up to and including the hydroxyapatite step

●, AGA activity; △, A_{280}; ↔, fractions pooled for additional purification (by hydroxyapatite) and enzyme characterization by SDS/PAGE (Fig. 7).

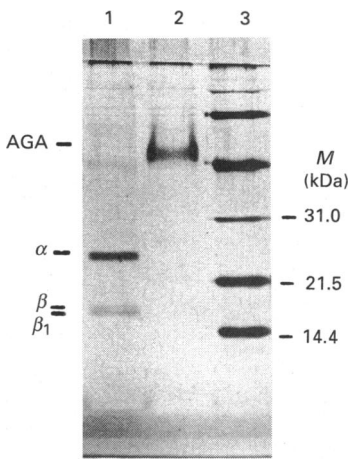

Fig. 7. SDS/PAGE of pooled Sephadex G-100 material

The lanes are: 1, AGA heated 100 °C for 3 min; 2, 'native' AGA; and 3, molecular-mass (*M*) markers.

Furthermore when samples were heated at 100 °C before electrophoresis this pattern is not observed in the subunits (about 25, 17, and 18 kDa) which appear to be generated from the 'native' ≈ 56 K structure (lane 3, Fig. 4).

Lanes identical with 2 (non-heated) and 3 (heated) of Fig. 4 were cut from the same gel and each sliced into 2 mm sections starting from the top of the separating gel. Individual slices were cut into approx. 1 mm cubes which were added to AGA assays as enzyme source and incubated overnight at 56 °C. AGA activity was readily detected at the 56 kDa region of the gel (Fig. 5 below). On heating, AGA activity and silver staining are nearly completely lost from this location of the gel, with the corresponding appearance of smaller polypeptides devoid of AGA activity (Figs. 4 and 5). Thus the band at 56 kDa is AGA and the polypeptides of smaller size generated on heating appear to be its subunits.

Given the relatively small apparent size of the AGA subunits < 25 kDa, it seemed that removal from our 'native' hydroxyapatite material (Fig. 4, lane 2) of those polypeptides smaller than about 50 kDa would enable us to generate (from the hydroxyapatite prep) and recover in pure form the subunits of AGA after denaturation (provided there are no other proteins than AGA at 60 kDa+ which could generate subunits of a similar size).

This separation was attempted using Sephadex G-100 columns (Fig. 6). Protein (A_{280}) was monitored to fraction 35, but not in those fractions with AGA activity (36–47). Fractions with appreciable AGA activity were pooled and concentrated and their protein content and distribution analysed by SDS/PAGE (Fig. 7). This material was 24500-fold purified over the initial homogenate. Lane 2 of this Figure shows clearly that AGA is the smallest 'native' protein remaining in the preparation. When aliquots of the G-100 Sephadex pool are denatured, α, β and β_1 subunits are formed and can be cut from the gel as pure polypeptides or blotted to membranes for *N*-terminal amino acid sequence studies.

To remove small amounts of several polypeptides larger than 56 kDa present in G-100-Sephadex-purified material, aliquots were run on a second hydroxyapatite (0.5 cm × 2 cm) column. Elution was with 2.0 ml aliquots of potassium phosphate buffer, pH 7.4, increasing in concentration (in 1 mM steps) from 1 to 15 mM. A number of fractions could be selected which contained

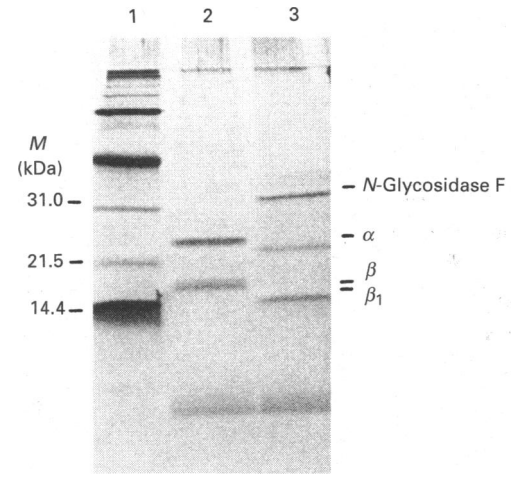

Fig. 8. SDS/PAGE of AGA after denaturation and treatment with *N*-glycosidase-F

The lanes are: 1, molecular-mass (*M*) markers; 2, AGA after heating at 100 °C for 3 min; and 3, AGA heated at 100 °C for 3 min and treated with *N*-glycosidase-F as indicated in the text.

Table 2. Terminal amino acid sequence of subunits of AGA

	Sequence				
Subunit Residue no....	5	10	15	20	25
α	_SPLP	LVVNT	WPFK_*	ATEAA	W
β	TIGMV	VIHKT	GHIAA	GTSTN	GIKFK I
β_1	TIGMV	VIHKT	GHIAA	GTSTN	GI

* *N*-Glycosylation site.

only the 56 kDa polypeptide. These were enriched 26000-fold over the starting homogenate.

The results of *N*-glycosidase-F studies indicate that α, β and β_1 are *N*-glycosylated, since a size reduction is noted in SDS-containing gels for each of them (Fig. 8). In addition, only a single band is present instead of the $\beta\beta_1$doublet, suggesting these two polypeptides may differ only in the size or number of the *N*-glycan(s) carried (lanes 2 and 3; Fig. 8). The additional band in lane 3 is *N*-glycosidase-F (molecular mass 35 kDa).

On the basis of a number of determinations the 'native' molecular mass of human liver AGA in SDS-containing gels appeared to be 56 kDa. On denaturation the following structures and sizes are obtained (α = 24.6 kDa; β = 18.4 kDa and β_1 = 17.4 kDa). When denatured samples were treated with *N*-glycosidase-F, the following changes in molecular mass were obtained (24.6→23.0 kDa, 18.4→15.8 kDa and 17.4→15.8 kDa).

For the preparation of subunits for amino-acid-sequencing studies, the extent of transfer from gel to membrane was monitored by using pre-stained protein standards (Bio-Rad). The conditions used for blotting gave quantitative transfer of polypeptides under 50 kDa and a progressively lower efficiency with increasing molecular mass. The transfer of AGA subunits was verified by silver staining (Bio-Rad) the SDS-containing gel (after blotting) for protein.

The lightly stained AGA subunits were excised from Pro Blott membrane and their *N*-terminal peptide sequence determined (Table 2). In each case the *N*-terminus was accessible and sequence corresponding to the first 21, 26, and 22 positions was obtained for α, β and β_1 respectively.

DISCUSSION

We have purified human liver AGA to homogeneity and determined its subunit composition. The overall purification was 26000-fold, and recovery of enzyme activity over 10%. Although several groups have purified AGA from human liver to apparent homogeneity there is considerable discrepancy in the results of these studies as they relate to size and structure (particularly so with the earlier descriptions) (Savolainen, 1976; Dugal & Strömme, 1977; McGovern *et al.*, 1983; Baumann *et al.*, 1989). Working with AGA purified 155–228-fold from AGU and normal patient liver respectively, Savolainen (1976) determined a native molecular mass of 150 kDa for both the control and the 'pathological' AGA. Dugal & Strömme (1977) were unable to localize AGA activity to any one or more of eight protein bands seen on PAGE of liver enzyme purified 17500-fold. Other studies resulted in native molecular-mass estimates of 63 and 80 kDa based on gel filtration and 84 kDa by PAGE (Dugal, 1978; McGovern *et al.*, 1983). The native 84 kDa structure ran in gels under denaturating conditions at 76 kDa suggesting that AGA was a monomer (McGovern *et al.*, 1983).

Our results much more closely resemble those obtained by Baumann *et al.* (1989), who were able to purify small amounts of liver AGA by h.p.l.c. procedures and found a molecular mass of 60 kDa based on Bio-Gel P-100 analysis. This enzyme consisted of three non-identical subunits of 24, 18 and 17 kDa (referred to as α, β, and β_1 by analogy to the rat enzyme, even though the exact molecular sizes may differ among different species) under denaturing conditions (Tollersrud & Aronson, 1989; Fisher *et al.*, 1990). We have obtained molecular masses of 56 kDa for 'native' AGA in SDS/PAGE gels and values of 24.6, 18.4 and 17.4 for α, β and β_1 by SDS/PAGE after boiling. The subunits do not have enzyme activity and cannot be re-activated by combining the subunits under neutral conditions (Baumann *et al.*, 1989).

We have extended the studies of Baumann *et al.* (1989) by demonstrating that each of the human liver subunits is *N*-glycosylated. Glycosylation of all α- and β-subunits has also been described for human leucocyte AGA, which appears to contain two α-subunits, i.e. α and α_1, in addition to the two β-structures, and for rat liver AGA (Halila *et al.*, 1991).

Ikonen *et al.* (1991) found the *N*-terminus of human liver AGA and its α-subunit to be blocked and this precluded direct sequencing. Similarly the *N*-termini of α and α_1 from human leucocytes was also blocked (Halila *et al.*, 1991). However the 24.6 kDa α-subunit that we obtained from human liver and that obtained from rat liver were not blocked. We obtained the sequence _SPLP/LVVNT/WPFK-/ATEAA/W for the *N*-terminus of the α-polypeptide. Rat liver α-subunit yielded two amino acid peaks during each cycle of the Edman degradation procedure (Tollersrud & Aronson, 1989). This resulted from the presence of two sequences that were one amino acid out of phase from each other i.e. SNPLP/LVVNT/WPFKN/ATE and _NPLP/LVVNT/WPFKN/ATE, indicating that about half the 24 kDa(α) polypeptide had lost the terminal serine. Our material from human liver α-subunit appears to have completely lost the first serine residue [on the basis of the cDNA and deduced amino acid sequence presented by Ikonen *et al.* (1991) for human leucocytes], but otherwise conforms to the sequence they present. *N*-Terminal sequencing of rat α-subunit gave a sequence of 17 amino acids; of these, one differs in position from what is seen in the human material (Tollersrud & Aronson, 1989).

Blockage of the *N*-terminus of the β-subunits has not been reported. We have shown that β and β_1 are the same in polypeptide structure on the basis of SDS/PAGE and *N*-terminal sequencing of the two forms. The same observation was made in

human leucocyte β and β_1 (Halila *et al.*, 1991) and indirectly in human liver (i.e. β and β_1 were not separated, but sequenced as a single preparation) (Baumann *et al.*, 1989; Ikonen *et al.*, 1991). In liver from the rat, only a single 20 kDa β-band was obtained for the β-subunit(s) (Tollersrud & Aronson, 1989).

There have been relatively few attempts to purify AGA from human sources. Detailed analysis of the AGA protein has been hampered by the apparent low abundance of this activity in human tissues (Baumann *et al.*, 1989). Several groups have carried out partial purification by conventional means followed with final purification of relatively small amounts by h.p.l.c. (Baumann *et al.*, 1989; Halila *et al.*, 1991). In other cases unusual and labour-intensive sources (suggested to be somewhat enriched in AGA) such as Sendai-virus-induced human leucocytes have been used (Halila *et al.*, 1991). This procedure required 140 litres of human blood as starting material and required a 13000-fold purification over the initial leucocyte homogenates. We have obtained pure AGA from human liver. This required a purification of about 26000-fold over the liver homogenate specific activity. The enzyme is a heterodimer containing two non-identical subunits, both of which are glycosylated.

Glycoproteins occur widely and abundantly in all cell types, including those of the nervous system. Normal cell growth thus requires the capacity for the continuous degradation of substantial amounts of glycosylated protein. The accumulation in nervous tissue of products resulting from the incomplete breakdown of glycoproteins appears to be the basis for neurological involvement of the glycoprotein storage diseases, although little is known of the actual mechanisms involved.

Glycoprotein degradation is a lysosomal process which begins with the removal of the protein backbone by a series of lysosomal peptidases, followed by a sequential removal of sugars from the non-reducing end of the oligosaccharide (Aronson & Kuranda, 1989). For *N*-linked glycans the hydrolysis of chitobiosyl and asparagine-to-*N*-acetylglucosamine linkages is also required for proper disposal.

The present study has provided new information concerning the size, subunit composition, glycosylation and *N*-terminal sequence of subunits of human liver AGA. The ability to isolate milligram quantities of AGA should enable us to generate antibodies to human AGA and its α- and β-subunits, to investigate both normal and abnormal synthesis assembly and transport to lysosomes of this acid hydrolase.

The sequence of the AGA gene has recently become available (Ikonen *et al.*, 1991; Fisher *et al.*, 1990) and will enable one to identify the mutation(s) in our patients. Recently a $Cys^{163} \rightarrow Ser$ alteration was identified as the mutation responsible for AGU in most, if not all, Finnish cases (Ikonen *et al.*, 1991; Fisher & Aronson, 1991). However, non-Finnish patients with AGU did not have this mutation (Fisher & Aronson, 1991), suggesting that these individuals (including our four Canadian patients) carry unique mutation(s).

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