Human leucocyte glycosylasparaginase is an α/β -heterodimer of 19 kDa α -subunit and 17 and 18 kDa β -subunit

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Human lysosomal glycosylasparaginase (AGA; EC 3.5.1.26) consists of two glycosylated subunits, α and β . Treatment with 3 % SDS at 45 °C as part of a new purification scheme did not affect enzyme activity, but the α -subunit migrated as an apparent 19 kDa peptide on SDS/PAGE instead of as a 24 kDa peptide, as observed without this SDS treatment. The N-terminal sequence was similar to that of the 24 kDa form, and, after reversed-phase h.p.l.c., the 19 kDa form was transformed to an apparent 24 kDa peptide on SDS/PAGE, indicating that their primary structures were identical. As the molecular mass of the α -subunit deduced from its cDNA was 19.5 kDa, the variation might be due to

incomplete SDS coating of the 24 kDa form. This was confirmed by the tendency of the 24 kDa variant to polymerize even in the presence of SDS. The molecular mass of the β -subunit was 17 and 18 kDa in accordance with previous reports. Chemical cross-linking with 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide resulted in the appearance of a 38 kDa peptide on SDS/PAGE which reacted with both the subunit-specific antisera on Western-blot analysis. On SDS/PAGE at pH 10.2 the active enzyme migrated as an apparent 43 kDa peptide. These results indicate that native human glycosylasparaginase is a heterodimer.

INTRODUCTION

Glycosylasparaginase (aspartylglucosaminidase; AGA; EC 3.5.1.26) is a lysosomal amidase that cleaves the amide bond in the β -position of oligosaccharide-linked asparagines (Makino et al., 1968). The enzyme requires a free α -amino and α -carboxy group of asparagine for hydrolysis and therefore catalyses one of the final steps during the ordered degradation of N-linked glycoproteins in the lysosomes (Aronson and Kuranda, 1989). The size and type of the oligosaccharide chain do not affect the cleavage catalysed by the enzyme, except that fucose on the 6 position of the inner GlcNAc must be released before hydrolysis, probably because of steric hindrance (Tarentino et al., 1975).

AGA is synthesized as a monomer and post-translationally cleaved into two non-covalently linked subunits of 24 kDa (α) and 17 and 18 kDa (β) (Fisher et al., 1990; Ikonen et al., 1991) via two proteolytic events, one early, most probably in the endoplasmic reticulum and one late in the lysosomes (Ikonen et al., 1993). The first cleavage is necessary to generate an active enzyme (Ikonen et al., 1993). There has been some controversy with respect to the quaternary structure of human AGA (Conzelman and Sandhoff, 1987; Baumann et al., 1989; Kaartinen et al., 1991; Tollersrud and Aronson, 1992). Furthermore we realized that the reported molecular mass of the α -subunit did not correspond to the estimated molecular mass based on its cDNA sequence. We therefore characterized the native molecular mass of the native enzyme by two independent and previously unreported methods and investigated a 19 kDa variant of the α -chain as judged by SDS/PAGE that corresponded to the molecular mass deduced from its cDNA sequence.

EXPERIMENTAL

Enzyme assay

AGA activity was determined by assaying the release of *N*-acetylglucosamine from the substrate *N*⁴-(β -*N*-acetylglucosaminyl)-*L*-asparagine as described previously (Tollersrud and Aronson, 1989). One unit of enzyme activity is defined as the amount that liberates 1 μ mol of *N*-acetylglucosamine/min at 37 °C.

Purification of AGA

Crude extract

Human leucocytes obtained from the Finnish Red Cross Blood service were isolated and homogenized as previously described (Halila et al., 1991). The resulting crude extract was salted out at 70 % (NH₄)₂SO₄ and the precipitate dissolved in a minimum volume of PBS and dialysed against PBS.

SDS treatment

If not otherwise stated, all further steps were carried out at room temperature. The dialysate was adjusted to about 20 mg of protein/ml by adding PBS, and 20 % SDS was added stepwise during stirring to a final concentration of 3 %. The solution was heated at 46 °C for 20 min. (NH₄)₂SO₄ was then slowly added at room temperature to 35 % saturation while mixing. To remove the brown glue-like precipitate, the suspension was first centrifuged at 16000 *g* (10000 rev./min) for 10 min, and the supernatant with traces of debris was finally filtered through a Millipore 45 μ m membrane. The clear yellowish solution was dialysed against about 8 litres of PBS in two sessions.

Abbreviations used: EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide; AGA, glycosylasparaginase (aspartylglucosaminidase); Con A, concanavalin A.

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Concanavalin A (Con A)–Sephacrose

Con A–Sephacrose (20 ml) was added to the dialysate during stirring and kept overnight at 4 °C. Con A was precipitated in a Sorvall centrifuge at 350 g (1500 rev./min) for 5 min, and the pellet was washed three times with 200 ml of PBS. Elution from Con A was accomplished by adding 100 ml of 0.2 M methyl α -mannopyranoside in PBS. After being stirred for 30 min, the suspension was centrifuged as described above and the supernatant collected, and concentrated using an Amicon ultrafiltration cell fitted with a PM-30 membrane; to obtain even smaller volumes a Centricon-30 miniconcentrator from Amicon was used.

Preparative SDS/PAGE

The last step, preparative SDS/PAGE, was carried out as previously described by Tollersrud and Aronson (1989) with the modification that the pH of the separating gel was adjusted to 8.65 instead of 8.8. The final preparation had a specific activity similar to previous reports (Baumann et al., 1989; Tollersrud and Aronson, 1989) and was judged pure by SDS/PAGE.

Western-blot analysis

Proteins were separated on the Phast system from Pharmacia using 8–25 % gradient SDS/PAGE. Transfer to a poly(vinylidene fluoride) membrane (Immobilon; Millipore) was carried out at 70 °C for 2 h by passive diffusion. The Protoblot AP system from Promega was used for antigen detection according to the manufacturer's instructions. The subunit-specific antisera have been described previously (Halila et al., 1991).

Reversed-phase h.p.l.c.

Reversed-phase h.p.l.c. was carried out using a TSK TMS 250 column (0.21 cm $\times$ 4 cm) equilibrated with 0.1 % trifluoroacetate, with a linear gradient of acetonitrile (3–100 %) (flow rate 1 ml/min) as described by Baumann et al. (1989).

Cross-linking with 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (EDC)

Cross-linking was performed as described by Parson and Pierce (1979). Enzyme (1 μ l), 0.1 M phosphate, pH 4.0, (1 μ l) and 0.2 M EDC (1 μ l) were mixed and incubated for 20 min at 4 °C and then 40 min at room temperature. Then 1 μ l of 0.1 M borate, pH 9.0, and 1 μ l of 0.1 M 2-aminoisobutyric acid, 0.5 μ l of 1 % SDS and 0.5 μ l of 2-mercaptoethanol were added and the sample was boiled for 3 min and analysed by Western blot.

Amino acid sequencing

N-Terminal sequencing was performed by automated Edman degradation using a modified Applied Biosystems 477 A on-line sequencer.

RESULTS AND DISCUSSION

Molecular mass of the α -subunits

The recent elucidation of the C-terminal processing of the α -subunit has made it possible to deduce the molecular mass of the mature α -subunit from the cDNA sequence (Ikonen et al., 1991, 1993). Assuming that the single N-linked oligosaccharide chain

constitutes about 1.5 kDa (Fisher et al., 1990; Halila et al., 1991; Rip et al., 1993), the total molecular mass was calculated to be 19.5 kDa. This is far from the previously reported values of 23–25 kDa (Fisher et al., 1990; Halila et al., 1991; Kaartinen et al., 1991; Enomaa et al., 1992; Rip et al., 1993) based on the mobility on SDS/PAGE.

As shown in Figure 1, SDS denaturation of the α -subunit resulting in an apparent 19 kDa peptide on SDS/PAGE occurred after a new purification step which included incubation of the enzyme in 3 % SDS at 45 °C, and it required reduction by 2-mercaptoethanol. A small amount of the 19 kDa conformer was nevertheless generated in the absence of reduction by 2-mercaptoethanol (Figure 1, lanes 2–4; Figure 2c). This may reflect a heterogeneity in the disulphide pattern of the α -subunit or indicate that, in the absence of reduction, a small portion of the subunit behaves as a 19 kDa conformer after heat denaturation, whereas the majority requires reduction to produce this conformer. As it was impossible to distinguish between the physical properties of the native enzyme before and after step 2 in Figure 1, it was possible that either proteolytic cleavage at a disulphide bridge or a small conformational change caused the shift on SDS/PAGE.

To address the first possibility we carried out N-terminal analysis of the subunits after separation by reversed-phase h.p.l.c. Only one N-terminal sequence was found for each subunit (Figure 2a), which corresponded to previous reports (Mononen et al., 1991; Rip et al., 1993), indicating that no internal cleavage of the modified α -chain had occurred. During the Edman degradation of the α -subunit, two amino acids appeared in each cycle. These could be aligned to give two sequences that were one amino acid out of phase with each other (Figure 2a). Such a partial lack of the N-terminal serine has previously been reported for rat liver α -subunit (Tollersrud and Aronson, 1989). Rip et al. (1993), however, found that the human liver α -subunit had completely lost its N-terminal serine, whereas Mononen et al. (1991) reported a single sequence of the human leucocyte α -subunit with the N-terminal serine intact. Thus it seems that the N-terminal serine of the human α -subunit has a tendency to be removed, but that the degree of this cleavage varies as a consequence of the purification protocol or the source of enzyme.

Curiously, reversed-phase h.p.l.c. resulted in the conversion of the apparent 19 kDa form to a 24 kDa variant on SDS/PAGE (Figure 2b, lane 3). Thus the primary structures of the two forms were probably identical. Furthermore there was substantial polymerization of the 24 kDa subunit (Figure 2b, lanes 1 and 2), the polymers becoming disaggregated only after reduction (Figure 2b, lane 3). Dimerization of the α -subunit was also observed after SDS denaturation of the native enzyme in the absence of

Figure 1 Migration of the α -subunit of AGA on SDS/PAGE after various purification steps

AGA (2 μ l; 0.1 unit/ml) from various purification steps, as described in the Experimental section, was boiled for 3 min after the addition of either 1 μ l of 2 % SDS alone (lanes 1–4) or SDS plus 0.5 μ l of 2-mercaptoethanol (lanes 5–8). The samples were subjected to SDS/PAGE and Western-blot analysis using the antiserum against the α -subunit as described in the Experimental section. Lanes 1 and 5, crude extract; lanes 2 and 6, SDS treatment; lanes 3 and 7, Con A–Sephacrose; lanes 4 and 8, preparative SDS/PAGE.

Figure 2 Analysis of the subunits of AGA obtained by reversed-phase h.p.l.c.

About 30 μ g of purified AGA was subjected to reversed-phase h.p.l.c. (a) and each peak was subjected to N-terminal sequence analysis as described in the Experimental section. The peak corresponding to the α -subunit was subjected to Western-blot analysis using antisera against both the α - and β -subunits (b) after pretreatment with 1% SDS (lane 1), and additional 3 min boiling (lane 2) or both boiling and 1 M 2-mercaptoethanol (lane 3). Lane 4, Western blot of the peak corresponding to the β -subunit that was not pretreated before SDS/PAGE. (c) Western blot of AGA before reversed-phase h.p.l.c. after pretreatment consisting of boiling in the presence of 1% SDS. In this case only the antiserum against the α -subunit was used.

Figure 3 Chemical cross-linking of AGA with EDC

About 0.3 unit/ml AGA was chemically cross-linked with EDC as described in the Experimental section, and subjected to Western-blot analysis using the antiserum against either α -subunit (lanes 1 and 2) or β -subunit (lane 3). Lane 1 is enzyme in the absence of EDC and lanes 2 and 3 show enzyme in the presence of EDC.

reducing agents, as seen from the appearance of a 48 kDa peptide in Figure 2(c), as also suggested by Enomaa et al. (1992).

In addition to the $\alpha\alpha$ -homodimers and the monomers, there is a band of about 38 kDa (Figure 2c). This band probably corresponds to the 43 kDa band from heat-denatured rat liver AGA (Tollersrud and Aronson, 1989) and the 36 kDa band from human leucocyte AGA (Enomaa et al., 1992). The 24 kDa monomer and the 48 kDa $\alpha\alpha$ -homodimer were also observed in both these reports. As the 38 kDa band cross-reacted with the antiserum against the α -subunit, it is probably the $\alpha\beta$ -heterodimer

and not a $\beta\beta$ -homodimer as suggested by Enomaa et al. (1992). The 43 kDa band from rat liver enzyme was shown to be an $\alpha\beta$ -heterodimer, as it was immunostained with antisera against both the α - and β -subunits (O.K. Tollersrud, unpublished work). Its molecular mass is higher than that of the human enzyme, because in rat the β -subunit is 20 kDa instead of the 17, 18 kDa observed for the human β -subunit (Tollersrud and Aronson, 1992). The slightly lower-molecular-mass satellite of the 38 kDa band in Figure 2(c) may be due to the heterogeneity of the β -subunit or heterogeneous conformations of the α -subunit within the $\alpha\beta$ -heterodimer. As seen from the very broad band of the 24 kDa α -subunit monomer (Figure 2c), the subunit probably exists in several different conformations in this system.

As we observed no aggregation of the 19 kDa form, there seems to be a difference in the exposure of hydrophobic areas, which is probably due to incomplete SDS coating of the 24 kDa form. This would also explain the difference in migration on SDS/PAGE. The β -subunit, however, exhibited only the doublet band of 17 and 18 kDa (Figure 2b, lane 4) which directly corresponds to its cDNA sequence, the heterogeneity being due to heterogeneous glycosylation (Fisher et al., 1990; Halila et al., 1991; Rip et al., 1993). The two bands of the β -subunit cannot easily be discerned in Figure 2b, as the immunoblot was overexposed to detect any oligomers.

The significance of our observation is shown by the otherwise unexplainable increase of 3 kDa in the molecular mass of the α -chain precursor on SDS/PAGE compared with the mature α -chain (Fisher and Aronson, 1991; Ikonen et al., 1993). The difference in molecular mass based on the deduced cDNA sequence of the processed peptide, however, was calculated to be only 1 kDa (Ikonen et al., 1993). Assuming incomplete SDS coating, the increase in apparent molecular mass of the α -chain precursor on SDS/PAGE was not only due to the size of the extra peptide fragment, but also to its charge.

Removal of the single N-linked oligosaccharide chain on the α -subunit by peptide N-glycosidase F only changes its molecular mass from about 23 to 22 kDa on SDS/PAGE after reduction by 2-mercaptoethanol (Halila et al., 1991) and does not result in

Table 1 Apparent molecular mass of native AGA as a function of pH during SDS/PAGE

SDS/PAGE was carried out in a 'Mighty small' system from Hoefer. The concentration of SDS was 0.1%. About 0.03 unit of AGA was applied to one lane and the sample was run at 20 mA until the Bromophenol Blue front reached the edge of the gel. The Pharmacia Low Molecular Weight kit was used as molecular-mass markers. At end of the run the lanes containing enzyme were cut into about 2 mm-thick slices and each slice was added to 100 μ l of PBS. After diffusion overnight at 4 °C, the enzyme activity was determined as described in the Experimental section. The lane with molecular-mass markers was stained with Coomassie Blue and the relative migration of the active enzyme compared with that of the markers was determined.

Buffer system	pH	Molecular mass (kDa)
Tris/Tricine (Pharmacia Phast gel)	8.0	95
Tris/glycine (Laemmli, 1970)	8.8	70
Ammonia/Caps (McLellan, 1982)	10.2	43

formation of any 19 kDa-sized conformed. Thus the problem of incomplete coating by SDS was probably entirely within the polypeptide chain. Intrachain disulphide bridges probably contribute to this phenomenon, as reduction prevents aggregation and is necessary to obtain the 19 kDa form (Figures 1 and 2b). Reduction is not sufficient to generate the 19 kDa form from unmodified enzyme (Figure 1, lane 5), however, and other factors determining the tertiary structure of the α -subunit must therefore also contribute.

Quaternary structure

The quaternary structure of AGA has been disputed: a tetrameric structure has been suggested on the basis of measurement by SDS/PAGE and gel filtration (Kaartinen et al., 1991) and a heterodimeric structure as determined by gel filtration (Tollersrud and Aronson, 1992). We therefore decided to investigate the quaternary structure by an independent method (chemical cross-linking) and to study the molecular mass by SDS/PAGE at various pH values, as the SDS-resistance of AGA might previously have caused misinterpretation of the molecular mass by this method. Cross-linking of the native enzyme by EDC is depicted in Figure 3. A new band of 38 kDa appears which cross-reacts with both subunit-specific antisera on Western-blot analysis, indicating a heterodimeric structure. This is not due to the formation of the $\alpha\alpha$ - and $\beta\beta$ -homodimers because, in the absence of reduction by 2-mercaptoethanol, the new band appeared at

40 kDa on a Western blot using antiserum against the α -subunit (O. K. Tollersrud, unpublished work). An $\alpha\alpha$ -homodimer would migrate with an apparent molecular mass of 48 kDa as seen in Figures 2(b) and 2(c).

SDS/PAGE was carried out at pH 8.0, 8.8 and 10.2. As shown in Table 1, the enzyme migrated faster at increasing pH and attained an apparent molecular mass of 43 kDa at pH 10.2. This indicates that the enzyme was more negatively charged at increasing pH and that the previous molecular-mass determination by SDS/PAGE at pH 8.0 (Kaartinen et al., 1991) was misleading. This molecular-mass determination does not necessarily reflect the molecular mass of the native enzyme within the lysosomes, where the pH is around 5. The cross-linking experiment with EDC, however, was carried out at pH 4. Thus our results, along with previous molecular-mass determinations by gel filtration (e.g. Tollersrud and Aronson, 1992), demonstrate that, *in vitro* at pH between 4 and 10, human AGA is an $\alpha\beta$ -heterodimer.

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