Comparison of liver glycosylasparaginases from six vertebrates

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Structural and physical properties of glycosylasparaginase (EC 3.5.1.26) from the livers of human, pig, cow, rat, mouse and chicken were compared. The enzyme in all species had a common basic structure of two *N*-glycosylated subunits of about 24 (α) and 20 (β) kDa joined by non-covalent forces. Subunit-specific antisera against the rat glycosylasparaginase bound specifically and sensitively to the corresponding subunits from all species. Identity of 80 % of the amino acids was found between the *N*-terminal sequences of corresponding pig and rat glycosylasparaginase α - and β -subunits and the deduced sequence from a human glycosylasparaginase cDNA [Fisher, Tollersrud & Aronson (1990) FEBS Lett. 269, 440–444]. The β -subunit from all three species has an *N*-terminal threonine reported to be involved in the reaction mechanism for the human enzyme [Karttinen, Williams, Tomich, Yates, Hood & Mononen (1991) J. Biol. Chem. 266, 5860–5869]. The native enzyme appeared as a heterodimer among the mammals, whereas the chicken enzyme had a greater molecular mass and is probably either a tetramer or a heterodimer bound to an unrelated peptide(s). All glycosylasparaginases were thermostable, requiring temperatures between 65 °C and 80 °C to be irreversibly inactivated. In addition, they were unusually stable at high pH and remained active in the presence of SDS except at low pH. The pH maximum was between 5.5 and 6 except for the rat and mouse enzymes which had a broad maximum between pH 7 and 8. A number of other properties were observed which also distinguish the enzyme from individual and closely related species.

INTRODUCTION

Glycosylasparaginase (1-aspartamido- β -*N*-acetylglucosamine amidohydrolase, aspartylglucosaminidase, EC 3.5.1.26) is a lysosomal enzyme which cleaves the β -*N*-aspartylglucosylamine linkage in *N*-linked glycoproteins. The amidase requires free α -amino and α -carboxy groups on the asparagine and that the 6 position of *N*-acetylglucosamine be unoccupied by fucose (Tarentino *et al.*, 1975). Oligosaccharides joined to the 4 position of GlcNAc, however, do not affect the activity (Makino *et al.*, 1968). These substrate requirements place cleavage of aspartylglucosylamine bonds, after complete proteolysis of glycopeptides and removal of the inner fucose, in an ordered degradation of glycoproteins in the lysosomes (Aronson & Kuranda, 1989). The physiological importance of this enzyme is illustrated by the occurrence of a human genetic disorder due to its deficiency. Aspartylglucosaminuria causes accumulation of GlcNAc1Asn in tissue lysosomes and leads to severe clinical symptoms (Maury, 1982). Attempts have been made to characterize glycosylasparaginase from a variety of species, but it has been difficult to discern any common structural features (Conzelmann & Sandhoff, 1987). We characterized the enzyme from rat liver and found that it consists of two subunits of 24 (α) and 20 (β) kDa joined by non-covalent forces (Tollersrud & Aronson, 1989). The amino acid sequence deduced from a human glycosylasparaginase cDNA isolated in this laboratory was shown to exhibit 80–90 % identity with partial amino acid sequence data from both rat subunits (Fisher *et al.*, 1990). These results proved that the structure of the human glycosylasparaginase was highly similar to that of rat and that the two subunits were derived from one gene whose product was post-translationally cleaved. Similarity between the sizes of the rat and human subunits was also described by Halila *et al.* (1991). Here we report a comparison of the properties of glycosylasparaginase from six different vertebrates. The basic structure and a number of the physical properties previously described for rat glycosylasparaginase were

shown to be conserved in the other species. Some properties varied, however, and an elucidation of amino acid substitutions which caused these will be essential to further increase our understanding of this important lysosomal amidase.

EXPERIMENTAL

Partial purification

Glycosylasparaginases were partially purified from 5–10 g of human, rat, mouse, bovine, pig and chicken livers. The tissues, except for human liver which had been stored at –70 °C for a prolonged time, were either fresh or kept at –20 °C for less than 2 months before use. The tissues were minced and homogenized with a Potter/Elvehjem homogenizer in 3 vol. of 0.05 M-sodium phosphate buffer, pH 7.0, containing 0.15 M-NaCl and 2 % Triton X-100. The homogenates were centrifuged for 10 min at 10000 *g* and chromatographed through a concanavalin A–Sephrose 4B column (0.8 cm $\times$ 5 cm) equilibrated with phosphate-buffered saline (PBS; 0.05 M-sodium phosphate buffer, pH 7.0, containing 0.15 M-NaCl). Glycosylasparaginase bound to the column and was eluted by 0.2 M-methyl α -mannoside. The eluate was concentrated to about 3 ml through an Amicon ultrafiltration cell fitted with a YM-10 membrane (Amincon Corp., Danvers, MA, U.S.A.) and applied to an Ultrogel AcA-54 (I.B.F. Biotechnics, Villeneuve-la-Garenne, France) column (1.5 cm $\times$ 85 cm) equilibrated with PBS. The flow rate was 0.44 ml/min and 2 ml fractions were collected. Fractions containing glycosylasparaginase activity were combined and concentrated to an activity of approx. 0.15 unit/ml. Comparison of the temperature stabilities and the pH optima of these impure samples with those previously reported for the pure rat and human enzymes (Tollersrud & Aronson, 1989; Bauman *et al.*, 1989) showed no differences, indicating that the impurities in these samples did not influence the properties of glycosylasparaginase.

Abbreviations used: PBS, phosphate-buffered saline; PVDF, poly(vinylidene difluoride).

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Preparation of antisera

Rat liver glycosylasparaginase was purified to homogeneity as previously described (Tollersrud & Aronson, 1989). About 20 μ g of the pure enzyme was denatured in 2% (w/v) SDS/1 M-2-mercaptoethanol at 100 °C for 3 min, and the subunits were separated by SDS/PAGE by the Laemmli system (Laemmli, 1970). The position of each subunit in the gel was determined by staining part of the track with Coomassie Blue. Unstained gel pieces containing the respective subunits were separately cut into pieces and homogenized in 0.5 ml of 0.05 M-phosphate buffer, pH 7.0, containing 0.15 M-NaCl. Freund's adjuvant (0.5 ml) was added and each suspension was injected subcutaneously into a separate rabbit. The animals were injected three times at 2-week intervals, the first time with Freund's complete adjuvant and subsequently with incomplete adjuvant. Every 2 weeks the rabbits were bled from the ear vein and a booster was given at 2-month intervals. After 6 months the rabbits were killed by heart puncture.

Enzyme assay

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

Immunoblotting

Partially purified glycosylasparaginase (0.15 unit/ml) was subjected to SDS/PAGE on an 8–25% Phastgel (Pharmacia, Piscataway, NJ, U.S.A.). After electrophoresis, the gel was overlaid with a pre-wetted poly(vinylidene difluoride) (PVDF) membrane (Immobilon; Millipore Corp., Bedford, MA, U.S.A.), and blotting was carried out by diffusion at 70 °C for 2 h. The membrane was blocked for 20 min with 2% (w/v) BSA in 10 mM-Tris/HCl, pH 8.0, containing 0.15 M-NaCl and 0.05% Tween 20 and then incubated in 1/500 diluted antiserum in the same buffer for 30 min at room temperature. The immune-complex was detected with a secondary antibody/alkaline phosphatase conjugate kit obtained from Promega (Promega Corp., Madison, WI, U.S.A.).

Partial CNBr cleavage

This procedure was described by Zingde *et al.* (1986). Rat liver glycosylasparaginase (20 μ g) that had been purified as described previously (Tollersrud & Aronson, 1989) was denatured and dissociated into subunits by boiling in the presence of 2% SDS and 1 M-2-mercaptoethanol. The subunits were separated by SDS/PAGE according to Laemmli (1970). The subunit bands were visualized by Coomassie Blue staining, cut out and transferred to separate microtubes. The opened microtubes were placed in a closed Coplin jar containing 4 ml of 100 mg of CNBr/ml of 70% (v/v) formic acid and were subjected to the CNBr vapour for 20 h at room temperature. The gel pieces were then placed in 0.2 ml of water and freeze-dried. This process was repeated three times to remove excess CNBr. The dried gel pieces were finally suspended in 0.1 ml of 2% SDS/1 M-2-mercaptoethanol/Bromophenol Blue/10% (v/v) glycerol, kept in boiling water for 3 min and subjected to SDS/PAGE according to Laemmli (1970). To fit the swollen gel pieces into the wells for this second run the thickness of the gel was twice that of the initial SDS/PAGE (1.5 mm instead of 0.75 mm). After electrophoresis, the peptides were electroblotted on to a PVDF membrane as described previously (Tollersrud & Aronson, 1989) and stained with Coomassie Blue.

N-Terminal sequencing of pig liver glycosylasparaginase

Glycosylasparaginase from pig liver was purified to near-homogeneity as previously described for isolation of the rat liver glycosylasparaginase (Tollersrud & Aronson, 1989) with the exception that the last step, preparative SDS/PAGE, was omitted. Partially purified pig enzyme (30 μ g) was denatured and dissociated into subunits at 100 °C in the presence of 2% SDS/1 M-2-mercaptoethanol. The subunits were separated by SDS/PAGE according to Laemmli (1970), electroblotted on to a PVDF membrane and stained with Coomassie Blue as previously described (Tollersrud & Aronson, 1989). Two prevalent bands of 24 and 20 kDa were found to react with the anti-(α -subunit) serum and anti-(β -subunit) serum respectively on an immunoblot, and they were cut out and sequenced by automated Edman degradation as described by Matsudaira (1987).

Isoelectric focusing

Isoelectric focusing was carried out on Phast Gel IEF media, pH range 3–9, according to the Phastgel system manual (Pharmacia). To localize the enzyme, the respective lanes were sliced into 16 similarly sized pieces which were eluted by incubation overnight at 4 °C in 100 μ l of 0.05 M-sodium phosphate buffer, pH 7.0, containing 0.15 M-NaCl. Eluates (40 μ l) were assayed for glycosylasparaginase activity.

RESULTS

Partial purification

Concanavalin A-Sepharose affinity and AcA-54 molecular sieving chromatography steps yielded partially purified glycosylasparaginases with a specific activity of approx. 3×10^{-3} units/mg. These enzymes were approx. 0.1% pure assuming a maximum specific activity of about 3 units/mg as found for the homogeneous rat enzyme (Tollersrud & Aronson, 1989). The enzyme from all vertebrates bound to concanavalin A, but the chicken enzyme differed from the others by a faster mobility through the AcA-54 column. Comparison with the elution volumes of standard proteins showed that the chicken enzyme had a native molecular mass above 80 kDa, while the enzymes from all other species had molecular masses between 49 and 55 kDa (O. K. Tollersrud, unpublished work).

Antibody detection and pH-dependent separation of subunits

A prerequisite for these studies was that the subunit-specific antisera against the rat enzyme exhibit affinity only for a corresponding subunit from the other species. As previously shown, the enzymic activity of purified rat liver asparaginase is abolished in SDS at room temperature and low pH, concomitant with its dissociation into subunits as judged by SDS/PAGE (Tollersrud & Aronson, 1989). If antibodies against the rat subunits bound specifically to the respective subunits from the other species, one should expect two immunoreactive peptides to appear from each glycosylasparaginase on SDS/PAGE once enzyme denaturation due to binding SDS had occurred. For all species, the antibodies initially recognized only a single band larger than 60 kDa after preincubation of the enzyme in the presence of SDS at pH higher than 5–6 (Fig. 1 inserts). When the pH was lower than 5–6, this high-molecular-mass protein disappeared and two fast migrating bands appeared which were similar in M_r to the α - and β -subunits of the rat liver enzyme. This electrophoretic change was also exactly accompanied by loss of enzyme activity as shown in the graphs of Fig. 1.

Species similarity of enzyme subunit structure

After the original submission of the present paper, Kaartinen *et al.* (1991) reported covalent modification of human leucocyte

Glycosylasparaginase (0.15 unit/ml) was mixed with 3 vol. of multi-pH buffer (0.05 M-citrate, 0.05 M-borate, 0.05 M-phosphate) at pH 4, 4.5, 5, 5.5, 6 or 8 and incubated in the presence of 0.5 % SDS at room temperature for 30 min. Each enzyme sample was then divided into two equal aliquots. In one the enzyme activity was determined as described in the Experimental section (graphs) and the other was subjected to SDS/PAGE without preheating, blotted on to a PVDF membrane and immunostained using rabbit antisera against both α - and β -subunits of rat liver glycosylasparaginase (inserts). (a) Human; (b) mouse, (c) pig; (d) cow; (e) rat; (f) chicken. The preincubation pH corresponding to the various tracks on each immunoblot are (left to right): 4, 4.5, 5, 5.5, 6 and 8. Molecular-mass markers indicated with horizontal bars at the left of each inset correspond (top to bottom) to: BSA, 67 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20 kDa.

Pig glycosylasparaginase was purified to approx. 15% purity through the steps for complete purification of the rat enzyme (Tollersrud & Aronson, 1989), but omitting the last preparative SDS/PAGE step. The enzyme (approx. 20 μ g) was denatured at 100 °C for 3 min in the presence of 0.1 M 2-mercaptoethanol and 0.5% SDS. The denatured enzyme was subjected to SDS/PAGE and electroblotted on to a PVDF membrane as described in the Experimental section. Two bands which migrated similarly as the two low-molecular-mass immunoreactive polypeptides from partially purified pig enzyme (Fig. 1c) were cut out and sequenced by automatic Edman degradation. The *N*-terminal sequences of rat enzyme (Tollersrud & Aronson, 1989; note: the previously reported *N*-terminal glutamine on the rat β -subunit has been corrected to threonine in this Fig; see the text) and analogous deduced amino acid sequences from a human glycosylasparaginase cDNA (Fisher *et al.*, 1990) are shown for comparison. Vertical lines indicate identical residues. Underlined amino acids in the α -subunit form a consensus sequence for asparagine-linked glycosylation.

caused us to reinvestigate amino acid sequence data that we had previously reported for rat glycosylasparaginase, which indicated the *N*-terminal residue of its β -subunit to be a glutamine residue rather than threonine (Tollersrud & Aronson, 1989). Further

Fig. 3. Western-blot analysis of glycosylasparaginases separated by SDS/PAGE before and after reduction

Glycosylasparaginase (0.15 unit/ml) was denatured by boiling in the presence of 0.5% SDS for 3 min with or without reduction. Glycosylasparaginase components from the different species were detected with combined rabbit antisera made against the individual rat subunits: (a) cow; (b) pig; (c) chicken; (d) human; (e) rat; (f) mouse. Only the samples from each animal shown in the left tracks were reduced with 1 M-2-mercaptoethanol. Arrows indicate mobilities of unreduced α - and β -subunits.

concern about the rat sequence was caused since threonine and glutamine are eluted sequentially and closely together in the amino acid sequencing system commercially used to obtain our original data for the rat enzyme. We have now found, using PCR to amplify rat glycosylasparaginase cDNA from rat liver RNA, that the *N*-terminal residue of the rat β -subunit is indeed threonine (H. Park, M. Vettese, K. J. Fisher & N. N. Aronson, unpublished work). A likely role for this threonine in the hydrolysis reaction, as suggested for the new results of Kaartinen *et al.* (1991), is further indicated by an *N*-terminal threonine in the pig β -subunit as well as the conserved nature of the seven subsequent amino acids in rat, human and pig enzymes (Fig. 2). The *N*-terminal sequences of α -subunits in these three species are also 80% identical with a conserved glycosylation site at Asn-15 (Fig. 2).

N-Linked glycosylation

As previously reported for the rat (Tollersrud & Aronson, 1989) and human (Fisher *et al.*, 1990) enzymes, treatment with peptide:*N*-glycosidase F resulted in a decrease of 1–2 kDa for both subunits from bovine, pig, chicken and mouse (O. K. Tollersrud, unpublished work). The same result on treatment of the human enzyme with peptide:*N*-glycosidase F was confirmed by Halila *et al.* (1991). These data indicate that there is approximately one *N*-linked sugar chain on each glycosylasparaginase subunit from all six species. The respective deglycosylated subunits in all six species had an identical molecular mass based on their mobility on SDS/PAGE, approx. 22 kDa for the α -subunit and 18 kDa for the β -subunit, except for the chicken α -subunit which migrated as an approx. 25 kDa protein.

Effect of reduction on SDS/PAGE mobility

Reduction with 2-mercaptoethanol resulted in a slightly decreased migration of the α -subunit of each enzyme on SDS/PAGE (Fig. 3). The β -subunit exhibited a more noticeable decrease which represented an approx. 2 kDa increase in apparent molecular mass as measured by SDS/PAGE. Exceptions were the human and a minor part of the mouse and rat β -subunits, the apparent molecular masses of which decreased by only approx. 0.5 kDa after reduction. To characterize this structural variation in the rat β -subunit, we prepared a partial CNBr-cleavage peptide map of its two observed forms, β and β_a (Fig. 4). As expected for a partial CNBr-cleavage reaction, substantial amounts of β and β_a were left intact (Fig. 4a). Four CNBr-cleavage peptides were also produced from each β -subunit species. Because of the partial reaction conditions, these peptides (I–IV; I_a , II_a and III–IV) probably resulted from single cleavages at two different methionines (Fig. 4b). The largest CNBr peptides from β (I and II) were approx. 16 and 13 kDa and were shown previously to have different *N*-terminal sequences from the rat β -subunit (Fisher *et al.*, 1990; Tollersrud & Aronson, 1989). Comparison of their sequences with the cDNA-deduced sequence of human glycosylasparaginase (Fisher *et al.*, 1990) suggested that

methionines located 45 and 71 residues from the *N*-terminus of the rat β -subunit had reacted with CNBr. Therefore the two smallest CNBr-cleavage peptides (III and IV) must be the corresponding *N*-terminal portions of the rat β -subunit that possess one of these changed methionine residues at their C-termini (Fig. 4b). Comparison of the CNBr-cleavage peptide maps of β and β_a showed that only the 16 and 13 kDa fragments of β (I and II) differed in molecular mass from the corresponding largest two peptides of β_a (I_a and II_a). There were no differences in the masses of the two smallest β - and β_a -derived peptides III and IV, which we deduced to have the *N*-terminus of the β -subunit. Thus the approx. 2 kDa difference between the β and β_a forms, as determined by SDS/PAGE (Fig. 4a), must reside in their C-terminal regions (shaded area, Fig. 4b). These two forms of the rat β -subunit were not distinguishable in the absence of reduction (Fig. 3). An explanation for all these observations is that a partial proteolytic cleavage occurs within a disulphide loop at the C-terminus of the rat β -subunit. Thus β_a is formed when a 2 kDa C-terminal peptide is lost from those clipped molecules of glycosylasparaginase β -subunit upon their reduction

Fig. 4. Partial CNBr cleavage of the two β -subunit forms of rat liver glycosylasparaginase and their deduced structures

Rat liver glycosylasparaginase was purified to approx. 50% purity, denatured, reduced and subjected to SDS/PAGE as described for the pig enzyme in the legend to Fig. 2. The two forms of the β -subunit (β and β_a) were cut out from the Coomassie Blue-stained gel and separately subjected to partial CNBr cleavage as described in the Experimental section. The gel pieces containing the CNBr fragments were applied to another SDS/PAGE gel and the separated peptides were electroblotted on to a PVDF membrane and stained with Coomassie Blue (a). The indicated β -derived bands (I and II) were previously sequenced by automatic Edman degradation (Fisher *et al.*, 1990). Deduced locations of the various polypeptides in the original β/β_a subunits are depicted in the schematic model (b). The asterisk marks a putative site for a partial cleavage in a C-terminal disulphide loop in some molecules of the rat β -subunit. As a result, the hatched region is lost from the C-terminus of β upon its reduction to form β_a .

(Fig. 4b). The mouse and human β -subunits also fit this structural pattern (Fig. 3d and f). In agreement with this prediction, the deduced amino acid sequence of the human glycosylasparaginase cDNA shows a cysteine residue penultimate to the C-terminus. This cysteine could form such a peptide loop by disulphide linkage to one of the three other cysteine residues of the β -subunit (Fisher *et al.*, 1990).

pH-activity profiles

The pH behaviour of asparaginase from all six vertebrates is shown in Fig. 5. The human, bovine, pig and chicken enzymes had similarly shaped curves with pK_a near 4.8, an optimum activity at pH 5.5–6 and retention of high reactivity at basic pH. The rat and mouse enzymes, however, had a higher pK_a of 5.8 and a broader more basic pH optimum between 7 and 8. The profiles for human, pig, chicken and rat glycosylasparaginases are in accordance with previous reports (Conzelmann & Sandhoff, 1987; Bauman *et al.*, 1989).

Fig. 5. Enzyme activity as a function of pH

Enzyme (3 μ l; 0.15 unit/ml) was added to a mixture of 20 μ l of multi-pH buffer from 3 to 10 as described in Fig. 1, and the activity was determined as described in the Experimental section. $\circ$, Rat; $\blacksquare$, mouse; $\bullet$, human; $\square$, cow; $\blacktriangle$, pig; $\triangle$, chicken.

Fig. 6. Isoelectric points of glycosylasparaginases

Glycosylasparaginases (0.15 unit/ml) were subjected to isoelectric focusing, and enzymic activities were determined in gel pieces as described in the Experimental section. Isoelectric points are indicated. $\circ$, Human; $\square$, pig; $\blacktriangle$, mouse; $\blacksquare$, rat; $\bullet$, cow; $\triangle$, chicken.

Fig. 7. Relative migration of native glycosylasparaginases on PAGE in the absence and presence of SDS

(a) Partially purified enzyme (0.15 unit/ml) from six vertebrates was subjected to SDS/PAGE, but without prior heat denaturation, followed by Western blotting and detection using anti-(rat enzyme) antisera. (1) Human; (2) pig; (3) mouse; (4) cow; (5) rat; (6) chicken. (b) and (c) Enzymes from human ($\circ$), pig ($\bullet$) and rat ($\square$) were subjected to PAGE in the absence (b) or presence (c) of SDS. Enzymic activity in the indicated gel pieces was determined as described in the Experimental section.

Temperature stability

The glycosylasparaginase from all species had a high thermostability, as has been previously reported for pig (Kohno & Yamashina, 1969), human (McGovern *et al.*, 1983) and rat (Tollersrud & Aronson, 1989) enzymes. A temperature of 70 $^{\circ}$ C for 10 min at pH 7 was necessary to inactivate irreversibly half of the activities of human, pig, mouse and chicken enzymes, and the bovine and rat enzymes had to be heated to 77 $^{\circ}$ C for a similar effect (O. K. Tollersrud & N. N. Aronson, unpublished work).

Isoelectric points

Glycosylasparaginases exhibited isoelectric points from pH 5.0 in human to pH 6.8 in chicken (Fig. 6). However, only the rat

enzyme clearly had multiple isoelectric points. Coomassie Blue staining of pure samples of the rat enzyme revealed at least six forms having isoelectric points of 5.3, 5.5, 5.8, 6.0, 6.2 and 6.3 (not shown). The isoelectric points of pig, rat and chicken glycosylasparaginases corresponded to previous reports (Conzelmann & Sandhoff, 1987; Tollersrud & Aronson, 1989), whereas the pI of 5.0 for human glycosylasparaginase was lower than the value of 5.7 previously reported for purified human enzyme (Bauman *et al.*, 1989). The relative pI values corresponded to the relative migration of the various glycosylasparaginases on native SDS/PAGE (Fig. 7a) except for the pig enzyme which migrated faster than expected. This abnormal electrophoretic behaviour was only observed in the presence of SDS (compare Fig. 7b with Fig. 7c), which indicates that the unexpected faster migration of the pig enzyme on SDS/PAGE was due to its binding SDS. This result confirms the order of isoelectric points shown in Fig. 6.

DISCUSSION

The subunit-specific antisera to rat liver glycosylasparaginase bound specifically and with high sensitivity to the respective α - and β -subunits of the enzyme from five other vertebrates. For all these enzymes there was a concomitant disappearance of a slow migrating immunoreactive band on SDS/PAGE and the loss of glycosylasparaginase activity in the presence of SDS at low pH (Fig. 1). Previously we reported that the slow migrating band of the rat liver enzyme on SDS/PAGE corresponded to its native form with no SDS bound (Tollersrud & Aronson, 1989). The human, mouse and bovine enzymes apparently did not bind any SDS either, since they migrated according to their charge relative to that of the rat enzyme which has approximately the same mass. The pig glycosylasparaginase, however, did gain extra negative charge, as judged by its differential migration on PAGE in the absence and presence of SDS (Fig. 7), but without any loss of activity. Although binding to SDS often results in complete unfolding of a protein, this result indicates that SDS may bind to some region of the pig enzyme without denaturation. The chicken enzyme reached SDS-resistance at pH 8 (Fig. 1f), whereas its optimal activity was at pH 5.5 (Fig. 5), which indicates that the resistance to SDS binding and enzyme activity are not directly related. Thus the apparent relation between SDS resistance and enzyme activity as noted for the rat enzyme (Tollersrud & Aronson, 1989) and also observed for rat superoxide dismutase (O. K. Tollersrud, unpublished work) is due to different factors which are not necessarily linked. We have also seen this behaviour in protein properties of another SDS-resistant lysosomal enzyme, α -D-mannosidase from rat epididymis (J. Cook & O. K. Tollersrud, unpublished work). This glycosidase exhibits a bell-shaped pH optimum at 4.7 (Opheim & Touster, 1978), but is SDS-resistant only at pH values above 5.5. The reason for such pH-dependent SDS resistance is not known, but the conservation of this uncommon property, especially among the glycosylasparaginases, points towards some functional importance.

Below the pH at which each glycosylasparaginase became SDS-sensitive there was a concomitant loss of enzyme activity, and the appearance of two low-molecular-mass immunoreactive bands on SDS/PAGE (Fig. 1). Their mass and their specific affinity for the respective subunit-specific antisera proved that these bands were related to the α - and β -subunits of rat liver glycosylasparaginase. The sum of molecular masses of the two subunits on SDS/PAGE was approx. 44 kDa (20+24) which fitted the 48–55 kDa molecular masses of native enzyme from the five mammals, as determined by their elution from an Aca-54 column. Bauman *et al.* (1989) suggested, on the basis of their findings of native molecular mass of 60 kDa which resolved into

three distinctive bands of 17, 18 and 24 kDa on SDS/PAGE after denaturation, that the human amidase was a heterotrimer. In our hands, however, additional bands on SDS/PAGE have invariably been attributed to microheterogeneities of one of the subunits. Thus we have noted heterogeneity of the sugar chains on the human β -subunit (Fisher *et al.*, 1990), partial removal of the N-terminal serine from the rat α -subunit (Tollersrud & Aronson, 1989) and variation within the C-terminal region of the rat and mouse β -subunits (Figs. 3 and 4). These particular structural differences probably occur as a result of partial hydrolytic events within the metabolically harsh environment of the lysosomes. We have recently expressed active glycosylasparaginase in COS cells transfected with a full-length human cDNA encoding the predicted 38 kDa full-length peptide (K. Fisher & N. N. Aronson Jr., unpublished work). This molecular mass is reasonably close to the sum of the molecular masses of the deglycosylated subunits and thus argues against human glycosylasparaginase being of a higher order than a dimer. This argument would also apply to the other mammalian glycosylasparaginases, but not to the chicken enzyme, the native molecular mass of which was greater than 80 kDa, a value that is in agreement with the finding of Tarentino & Maley (1969) of 100–110 kDa. The glycosylasparaginase from chicken may be a tetramer ($\alpha_2\beta_2$) or bound to another unrelated peptide(s). A 2–3 kDa larger molecule mass of the deglycosylated chicken α -subunit compared with those of the mammalian species (O. K. Tollersrud, unpublished work) could relate to a domain involved in such binding. Chicken is related to the mammals by a branchpoint in evolution about 280 million years ago, and further studies might determine if a change in the quaternary structure happened at or close to this branching of the species.

Although the human enzyme is a single-gene product (Fisher *et al.*, 1990), no trace of a single-chain form has been detected in the liver extracts from any of the six vertebrates. This is contrary to previous reports on the enzyme from pig (Kohn & Yamashina, 1972) and human (McGovern *et al.*, 1983) sources in which only single-chain forms were detected. Since our purification is only partial and involves a simple and rapid two-step procedure, it is unlikely that our preparations were completely cleaved during the isolation procedure. We have previously noted the extreme conditions necessary to denature this enzyme (Tollersrud & Aronson, 1989), and that failure to accomplish this resulted in a single high-molecular-mass band on SDS/PAGE (Fig. 1). The previous work by others reported unusual high molecular masses of 70 kDa for pig (Kohn & Yamashina, 1972) and 76 kDa for human (McGovern *et al.*, 1983) glycosylasparaginases, as estimated by SDS/PAGE, which suggests that the enzymes were not denatured. The similar molecular masses of the corresponding subunits and the same cleavage site of the rat, human and pig enzymes into α - and β -subunits (Fig. 2) suggest that formation of subunits occurs by a very specific hydrolysis. A Cys-163→Ser mutation detected in glycosylasparaginase from Finnish aspartylglucosaminuria patients (Fisher & Aronson, 1991; Ikonen *et al.*, 1991; Mononen *et al.*, 1991) resulted in an uncleaved enzyme without activity when expressed in COS cells (Fisher & Aronson, 1991). It is possible that this cysteine must be present in order to obtain a correct overall conformation for this enzyme, and therefore the lack of cleavage is a secondary result, due to impaired intracellular transport to the lysosomes or to a structural change around the point of cleavage which abolishes proteolysis into subunits. The cleavage clearly has been maintained in all the six species and may be essential to activate the enzyme. The exact physiological importance of this cleavage remains to be elucidated.

In general, β -subunits, except those from the human (Fig. 3d) and part of the mouse (Fig. 3f) and rat (Fig. 3e), contain

disulphide bridges, since reduction caused them to migrate more slowly on SDS/PAGE. However, it is unlikely that these three variant β -subunits lacked disulphide bridges, as has been suggested for the human glycosylasparaginase (Bauman *et al.*, 1989), because all unreduced asparaginase β -subunits had identical molecular masses (Fig. 3), and disulphide bridges are in general highly conserved through evolution (Thornton, 1981). Our observations (Fig. 4) suggest the presence of a proteolytic cleavage in these variant β -chains about 2–3 kDa from the C-terminus and within a disulphide bridge. Any functional importance of this cleavage is unknown, but the lack of a similar cleavage in β -subunits in cow, pig and chicken and only partial cleavage in rat and mouse (Fig. 3) suggests that it may not be significant.

The behaviour of the enzymes with respect to pH roughly divides the enzymes into two groups: one group has a pH optimum of 5.5–6 which is typical for lysosomal enzymes and the other group had a broad pH optimum between 6.5 and 8 (Fig. 5). The latter group consists of mouse and rat glycosylasparaginases, and the former group includes the four other vertebrate enzymes which show similar behaviour to glycosylasparaginase from the slime mold *Dictostylium discoideum* (O. K. Tollersrud & N. N. Aronson, unpublished work). The existence of two groupings may be due to a relatively recent amino acid substitution(s) which occurred close to the branching of rodents in evolution.

We have found that six vertebrate glycosylasparaginases consist of a unique α/β heterodimer structure which appears to result from specific proteolytic cleavage of a single-chain precursor. The remarkable conservation of this cleavage, the high homologies of the N-terminal sequences of the β -subunits that are produced (Fig. 2), a likely role for the exposed N-terminal threonine in the glycosylasparaginase reaction (Kaartinen *et al.*, 1991) and an accumulation of an inactive uncleaved precursor in COS cells transfected with mutant cDNA from Finnish aspartylglucosaminuria patients (Fisher & Aronson, 1991) are all evidence that this cleavage serves a significant physiological function. A further understanding of the properties of glycosylasparaginases from a variety of species will be helpful in the study of structure–function relationships for this enzyme. It is

especially important to improve our knowledge about mechanisms resulting in the loss of asparaginase activity in patients suffering from a deficiency of this glycoprotein hydrolase.

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