Biochem. J. (1989) 260, 101–108 (Printed in Great Britain)

Purification and characterization of rat liver glycosylasparaginase

Ole K. TOLLERSRUD and Nathan N. ARONSON, Jr.
Department of Molecular and Cell Biology, The Pennsylvania State University, University Park, PA 16802, U.S.A.

1. Rat liver glycosylasparaginase [N^4-(β-*N*-acetylglucosaminyl)-L-asparaginase, EC 3.5.1.26] was purified to homogeneity by using salt fractionation, CM-cellulose and DEAE-cellulose chromatography, gel filtration on Ultrogel AcA-54, concanavalin A–Sepharose affinity chromatography, heat treatment at 70 °C and preparative SDS/polyacrylamide-gel electrophoresis. The purified enzyme had a specific activity of 3.8 μmol of *N*-acetylglucosamine/min per mg with N^4-(β-*N*-acetylglucosaminyl)-L-asparagine as substrate. 2. The native enzyme had a molecular mass of 49 kDa and was composed of two non-identical subunits joined by strong non-covalent forces and having molecular masses of 24 and 20 kDa as determined by SDS/polyacrylamide-gel electrophoresis. 3. The 20 kDa subunit contained one high-mannose-type oligosaccharide chain, and the 24 kDa subunit had one high-mannose-type and one complex-type oligosaccharide chain. 4. *N*-Terminal sequence analysis of each subunit revealed a frayed *N*-terminus of the 24 kDa subunit and an apparent *N*-glycosylation of Asn-15 in the same subunit. 5. The enzyme exhibited a broad pH maximum above 7. Two major isoelectric forms were found at pH 6.4 and 6.6. 6. Glycosylasparaginase was stable at 75 °C and in 5 % (w/v) SDS at pH 7.0.

INTRODUCTION

Glycosylasparaginase [N^4-(β-*N*-acetylglucosaminyl)-L-asparaginase, EC 3.5.1.26] is a lysosomal enzyme that hydrolyses the amide bond between asparagine and *N*-acetylglucosamine that forms a major type of oligosaccharide–peptide linkage in glycoproteins (Makino *et al.*, 1966; Conzelman & Sandhoff, 1987). The hydrolysis is initiated only when both the α-amino and α-carboxy groups of the asparagine residue have become free as a result of proteolytic activity (Makino *et al.*, 1968). Thus no native glycoprotein is acted upon by the enzyme. The oligosaccharide chain linked to the 4-position of the inner *N*-acetylglucosamine residue in glycoproteins does not need to be cleaved before this asparaginase reaction (Makino *et al.*, 1968; Tarentino & Maley, 1969; Kohno & Yamashina, 1972). However, fucose, which is sometimes linked to the 6-position of this terminal *N*-acetylglucosamine residue in complex-type oligosaccharide chains, must be removed before the cleavage by glycosylasparaginase (Tarentino *et al.*, 1975; Yamashita *et al.*, 1979). Following breakage of the amide bond by the enzyme, the amino group still linked to the 1-position of the *N*-acetylglucosamine residue is non-enzymically hydrolysed (Makino *et al.*, 1968). The enzyme thus furnishes an oligosaccharide chain with a free reducing-end *N*-acetylglucosamine unit that in rats and humans can be removed by an enzymic activity termed di-*N*-acetylchitobiase (Kuranda & Aronson, 1986; Baussant *et al.*, 1986). In other species the degradation of the oligosaccharide part is apparently conducted entirely by exoglycosidases acting from the non-reducing end (Song *et al.*, 1987).

Effects of chronic deficiency of glycosylasparaginase have been studied in humans suffering from the congenital lysosomal storage disease aspartylglycosaminuria. Mainly N^4-(β-*N*-acetylglucosaminyl)-L-asparagine accumulates in the patient's liver and urine, and this storage results in various physiological defects and mental retardation (Maury, 1982). The exact mutation that causes the disease is not known. Glycosylasparaginase has been partially purified from a variety of species and tissues among mammals (Conzelman & Sandhoff, 1987) and to apparent homogeneity from pig kidney (Kohno & Yamashina, 1972) and human liver (McGovern *et al.*, 1983; Kalkkinen *et al.*, 1987). Apart from studies on substrate specificities, kinetic data and pH–activity curves, the enzyme has been incompletely described. Our investigation was initiated to extend previous characterization of the enzyme, especially the molecular properties, which are not well understood. We chose the rat liver enzyme because it has not yet been purified and the degradative pathway for asparagine-linked glycoproteins in rat liver is well characterized (Kuranda & Aronson, 1987).

EXPERIMENTAL

Enzyme assay

Glycosylasparaginase was assayed in a final volume of 50 μl consisting of 50 mM-sodium phosphate buffer, pH 7.5, 5 mM-N^4-(β-*N*-acetylglucosaminyl)-L-asparagine (Bachem, Torrance, CA, U.S.A.) and enzyme. After incubation for appropriate time intervals at 37 °C, the reaction was stopped by adding 150 μl of 0.25 M-sodium borate buffer, pH 8.8, and heating at 100 °C for 3 min. The *N*-acetylglucosamine released was determined by the Morgan–Elson reaction according to the procedure of Levvy & McAllan (1959). One unit of enzyme activity was defined as the amount that liberates 1 μmol of *N*-acetylglucosamine/min.

Purification of glycosylasparaginase

Previously reported steps. Glycosylasparaginase was purified from 50–80 commercial frozen rat livers up to

Abbreviation used: PAGE, polyacrylamide-gel electrophoresis.

and including the DEAE-cellulose step as previously described (Tollersrud *et al.*, 1988).

The enzyme at this step was concentrated to about 5 ml in an Amicon ultrafiltration cell fitted with a YM-10 membrane (Amicon Corp., Danvers, MA, U.S.A.).

Ultrogel AcA-54 chromatography. The concentrated enzyme was applied to an Ultrogel AcA-54 (I.B.F. Biotechnics, Villeneuve-la-Garenne, France) column (1.5 cm × 85 cm) equilibrated with 50 mM-sodium phosphate buffer, pH 7.0, containing 0.15 M-NaCl. The flow rate was 0.44 ml/min, and 2 ml fractions were collected.

Concanavalin A–Sepharose chromatography. The combined active fractions (22 ml) from Ultrogel AcA-54 chromatography were loaded at a flow rate of 0.8 ml/min on to a concanavalin A–Sepharose (10 mg of concanavalin A/ml; Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) column (1.5 cm × 8 cm) equilibrated with 50 mM-sodium phosphate buffer, pH 7.0, containing 0.15 M-NaCl. Glycosylasparaginase was bound to the column, and was then eluted by 50 mM-sodium phosphate buffer, pH 7.0, containing 0.15 M-NaCl and 0.1 M-methyl α-mannoside.

Heat treatment. Methyl α-mannoside in the eluate from concanavalin A–Sepharose chromatography was removed by concentration of the enzyme three times with the above phosphate buffer in an Amicon ultrafiltration cell fitted with a YM-10 membrane. The enzyme sample was finally concentrated to about 2 ml and heated at 70 °C for 20 min. Precipitated protein was removed by centrifugation at 12000 *g* for 5 min in a Beckman Microfuge.

Preparative SDS/PAGE. Preparative SDS/PAGE was performed at room temperature with 3.3% polyacrylamide in the stacking gel (3.8 mm thickness × 20 mm length × 130 mm) and 12.5% polyacrylamide in the separating gel (3.8 mm × 86 mm × 130 mm) according to the Laemmli (1970) system. Glycerol was added to the enzyme to 3% (v/v), SDS to 0.5% and a few grains of Bromophenol Blue. The current was maintained at 15 mA during migration through the stacking gel (about 2 h) and at 40 mA during separation through the separating gel (about 4 h). When the dye marker reached the lower-reservoir buffer, the gel was removed, and four slices from the top of the separating gel, each about 4 mm, were cut out with a razor blade. Each of the slices was cut into small pieces, which were transferred to a 50 ml tube containing 25 ml of 0.2 M-sodium phosphate buffer, pH 7.5. The tubes were gently shaken overnight at 4 °C. Tubes containing enzymic activity were pooled and concentrated to about 5 ml in an Amicon ultrafiltration unit fitted with a YM-10 membrane. The enzyme was finally run on an Ultrogel AcA-54 column as described above under 'Ultrogel AcA-54 chromatography' to remove acrylamide and SDS.

Purification of rat liver superoxide dismutase

During the gel-filtration step on Ultrogel AcA-54 of the purification of glycosylasparaginase as described above under 'Ultrogel AcA-54 chromatography' a blue–green fraction was eluted at an apparent molecular mass of 34 kDa. The fractions containing the most intense colour were pooled (6 ml) and heated at 70 °C for 20 min. Aggregated protein was removed by centrifugation at 12000 *g* for 5 min in a Beckman Microfuge. The bluish protein left in the supernatant migrated as a single 18 kDa band on SDS/PAGE. The molecular mass, amino acid composition, metal content (determined by a Perkin–Elmer 5100 Atomic absorption spectrophotometer) and u.v.–visible-absorption spectrum of this protein (results not shown) showed it to be superoxide dismutase (Crapo & McCord, 1976; Fridovich, 1982). Many of the physical properties of this enzyme during SDS/PAGE turned out to be similar to those of glycosylasparaginase, and these results were useful for comparison.

N-Terminal sequence analysis

About 10 μg of the purified glycosylasparaginase in 30 μl of 50 mM-sodium phosphate buffer, pH 7.0, containing 0.15 M-NaCl, 1 M-2-mercaptoethanol, 0.5% SDS, 3% (v/v) glycerol and some Bromophenol Blue was denatured and dissociated into subunits by heating at 100 °C for 3 min. The dissociated enzyme was applied to a SDS/polyacrylamide gel and electrophoresed in accordance with Laemmli (1970). After electrophoresis the gel was soaked in transfer buffer [10 mM-3-cyclohexylaminopropane-1-sulphonic acid buffer, pH 11.0, containing 10% (v/v) methanol] for 10 min with one change. During this time a poly(vinylidene difluoride) membrane (Immobilon; Millipore Corp., Bedford, MA, U.S.A.) was rinsed with 100% methanol and soaked in transfer buffer. The gel was overlayed with the Immobilon membrane and electroblotted (Trans-Blot Cell; Bio-Rad Laboratories, Richmond, CA, U.S.A.) at 0.3 A for 6 h in transfer buffer and with cooling. The membrane was then soaked in distilled water for 5 min, stained with 0.1% Coomassie Brillant Blue R-250 in 10% (v/v) acetic acid/40% (v/v) methanol for 10 min and destained with 10% (v/v) acetic acid/50% (v/v) methanol for 10 min at room temperature. The membrane was finally soaked in distilled water for 10 min and air-dried. The bands corresponding to the 24 kDa and 20 kDa subunits were cut out and the *N*-terminal of each peptide was sequenced by automated Edman degradation as described by Matsudaira (1987).

Amino acid analysis

Amino acid analysis was carried out on purified rat liver glycosylasparaginase and superoxide dismutase. Freeze-dried samples (10–20 μg) were hydrolysed for 24 h at 110 °C in 6 M-HCl. Amino acid composition was then determined with an amino acid analyser.

Miscellaneous methods

SDS/PAGE in denaturing conditions was performed with 3.3% polyacrylamide in the stacking gel (0.75 mm × 2.5 cm × 13 cm) and 12.5% polyacrylamide in the separating gel (0.75 mm × 8.6 cm × 13 cm) according to the Laemmli (1970) system. To the enzyme samples were added glycerol to a final concentration of 3% (v/v), SDS to 0.5%, 2-mercaptoethanol to 1 M and a few grains of Bromophenol Blue. The treated enzyme was heated at 100 °C for 3 min before application.

The molecular mass of the native enzyme was determined by comparative chromatography on Ultrogel AcA-54, with bovine serum albumin, ovalbumin, carbonic anhydrase and cytochrome *c* (Sigma Chemical Co., St. Louis, MO, U.S.A.) as standards.

Isoelectric focusing was carried out on Phast Gel IEF media (pH range 3–9) in a Phast System instrument from Pharmacia. Plates were pre-run at 2 kV for 75 V·h and the enzyme was applied to both the anodal and the cathodal position. Focusing was at 2 kV for 410 V·h. The plates were stained with Coomassie Brilliant Blue R-350.

Protein was determined by the Folin phenol method by using a microassay described by Peterson (1983).

Endoglycosidase H and *N*-Glycanase (Genzyme Corp., Boston, MA, U.S.A.) digestions were used to determine the presence of oligosaccharide on rat glycosylasparaginase. A mixture of 5 μl of enzyme (1.0 mg/ml), 5.4 μl of 0.55 M-sodium phosphate buffer, pH 8.6, 0.5 μl of 5% (w/v) SDS and 0.5 μl of 1 M-2-mercaptoethanol was boiled for 3 min to denature the glycosylasparaginase. Then 2.5 μl of 7.5% (v/v) Nonidet P-40 and 2 μl of *N*-Glycanase (250 units/ml) were added and the mixture was incubated overnight at 37 °C. During Endoglycosidase H treatment, 5 μl of glycosylasparaginase (1.0 mg/ml) was incubated at 37 °C with 5 μl of 0.10 M-sodium citrate buffer, pH 5.5, and 2 μl of Endoglycosidase H (1.0 unit/ml). After 10 h, an additional 2 μl of Endoglycosidase H was added and the reaction mixture was kept overnight at 37 °C.

RESULTS

Purification of glycosylasparaginase

Initial stages of purification have previously been reported (Tollersrud *et al.*, 1988). A new, highly specific, step now included is preparative SDS/PAGE without inclusion of 2-mercaptoethanol after having heated the enzyme at 70 °C. Upon such preparative SDS/PAGE (Fig. 1*a*) the enzyme ran abnormally slowly (apparent molecular mass greater than 70 kDa), without loss of enzymic activity and with the same approximate mobility as when the electrophoresis was run without SDS (Fig 1*b*). However, the main contaminant, having a molecular mass of about 45 kDa, migrated much faster in the presence of SDS (Fig. 1*a*) than in its absence (Fig. 1*b*), thereby allowing its separation. The sharp band on top of the preparative gel in Figs. 1(*a*) and 1(*b*) appeared only when the enzyme sample was heat-treated, and is thus probably heat-denatured proteins that had aggregated. The different stages of complete purification are summarized in Table 1. From the crude homogenate the degree of purification was 3800-fold with an 18% recovery.

Molecular mass and quaternary structure

SDS/PAGE of glycosylasparaginase in denaturating conditions (heating at 100 °C and 2-mercaptoethanol included) gave two polypeptide species of molecular masses 20 kDa and 24 kDa respectively (Fig. 2, lane 3). Since the native molecular mass as determined by molecular sieving on an Ultrogel AcA-54 column was 49 kDa (result not shown), the enzyme was probably a dimer of these unequal subunits. Further evidence for this was provided by an experiment in which the enzyme was heat-denatured at 100 °C, but not treated with 2-mercaptoethanol, before SDS/PAGE. As shown in Fig. 2 (lane 2), a minor band of molecular mass 48 kDa appeared in addition to the major bands corresponding to the two subunits. This indicated that the enzyme indeed was a dimer. Thus the subunits were linked by non-covalent forces, since the enzyme partially dissociated in the absence of reducing agent. Note also that in the absence of 2-mercaptoethanol the mobility of each subunit increased to that of proteins of 1–2 kDa smaller molecular mass (Fig. 2, compare lane 2 with lane 3). This

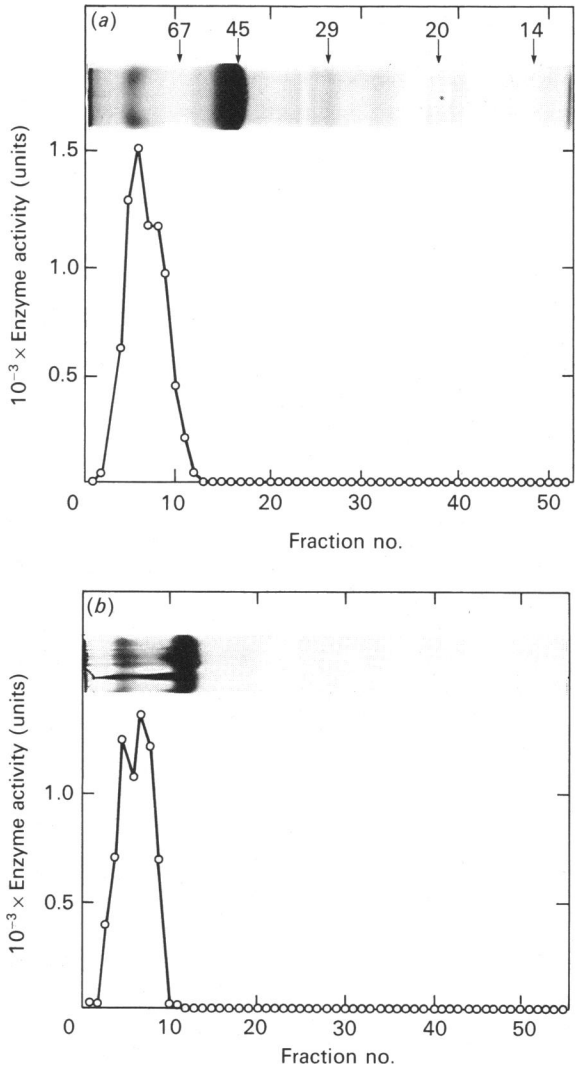

Fig. 1. PAGE of partially purified rat liver glycosylasparaginase in the presence (*a*) and in the absence (*b*) of SDS

PAGE was carried out as described in the Experimental section. A 100 μg portion of partially purified enzyme (0.3 unit/mg) in 30 μl of 50 mM-sodium phosphate buffer, pH 7.0, containing 0.15 M-NaCl was heated (no SDS present) at 70 °C for 20 min. Glycerol was added to 3% (v/v) as well as some Bromophenol Biue, and, for (*a*) only, SDS was included to 0.5% before electrophoresis. One lane was stained with Coomassie Blue, and another lane was cut into approx. 50 pieces with a razor blade for determination of enzyme activity. Each gel piece was added to a mixture of 60 μl of water, 20 μl of 0.2 M-sodium phosphate buffer, pH 7.5, and 10 μl of 17 mM-N^4-(β-*N*-acetylglucosaminyl)-L-asparagine, and this solution was then incubated at 37 °C for 4 h. Released *N*-acetylglucosamine was determined in 50 μl of the buffer by the Morgan–Elson reaction as described in the Experimental section. The migration of standard proteins on SDS/PAGE for completely denatured proteins is indicated in (*a*).

Table 1. Purification of rat liver glycosylasparaginase

For experimental details see the text.

Step	Total protein (mg)	Total activity (units)	$10^{-4}\times$ Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude extract	23750	24	10	1	100
$(NH_4)_2SO_4$ fractionation (35–70% satn.)	16250	22.5	14	1.4	94
CM-cellulose chromatography	1540	14	91	9.1	58
DEAE-cellulose chromatography	315	15	450	45	62
Ultrogel AcA-54 gel filtration	55	9.5	1700	170	40
Concanavalin A–Sepharose chromatography	5	7.0	15000	1500	29
Heat treatment (70 °C)	4.5	9.0	20000	2000	37
Preparative SDS/PAGE	1.2	4.5	38000	3800	18

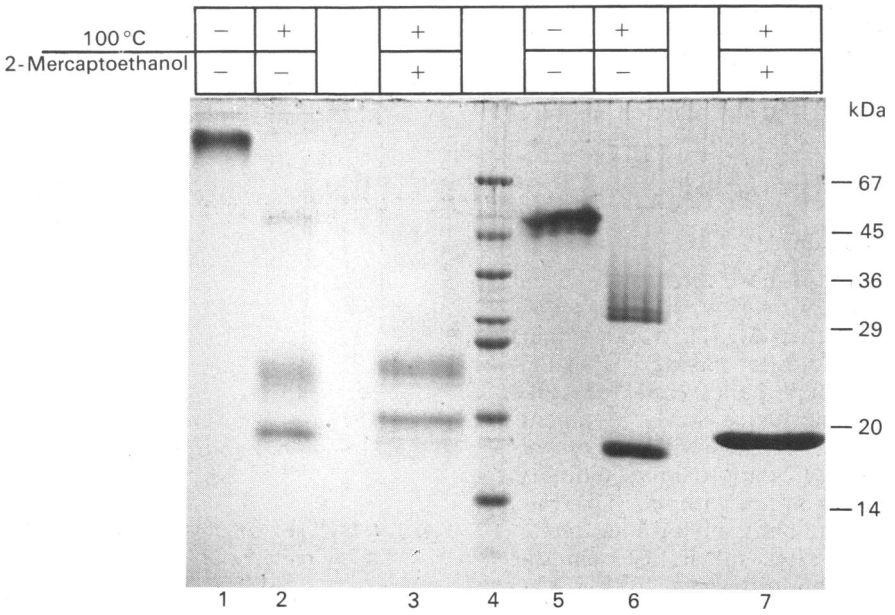

Fig. 2. SDS/PAGE of purified glycosylasparaginase and superoxide dismutase

Portions (2 μg) of glycosylasparaginase (lanes 1–3) or portions (10 μg) of superoxide dismutase (lanes 5–7) were subjected to SDS/PAGE after pretreatment of the enzymes in different ways. In lanes 1 and 5 the enzymes were added to 3% (v/v) glycerol and 0.5% SDS. In lanes 2 and 6 the enzymes were additionally heated in boiling water for 3 min. In lanes 3 and 7 the enzymes were treated with 2-mercaptoethanol to 1 M and 0.5% SDS before the heating in boiling water. Molecular-mass standards are in lane 4. The gel was stained with Coomassie Blue.

behaviour indicated that each subunit contained intrachain disulphide bridges. These bonds probably stabilized the quaternary structure, since the enzyme dissociated completely when heated at 100 °C in the presence of 2-mercaptoethanol and SDS (Fig. 2, lane 3).

The SDS/PAGE pattern of rat liver superoxide dismutase was included as a control (Fig. 2, lanes 5–7). This well-characterized heat-stable enzyme is composed of two (identical) subunits joined by strong non-covalent interactions, is stabilized by intrachain disulphide bridges and migrates abnormally upon SDS/PAGE when not denatured by boiling in the presence of SDS and 2-mercaptoethanol before application (Abemethy *et al.*, 1974; Fridovich, 1982). These properties coincided with those found for glycosylasparaginase. A comparison between lanes 1–3 and 5–7 in Fig. 2 shows that the migration patterns for the two proteins on SDS/PAGE after various pretreatments were similar, thus confirming an apparent structural similarity between these two otherwise unrelated enzymes.

Amino acid composition and *N*-terminal sequence

The amino acid composition obtained from about 15 μg of enzyme is shown in Table 2 (part *a*). The published amino acid composition of the pig enzyme (Kohno & Yamashina, 1972) is included for comparison. The *N*-terminal sequence of each rat subunit is given in Table 2 (part *b*). There is no similarity between the sequences obtained for these two subunits. During each cycle of Edman degradation for the 24 kDa subunit two

Table 2. (*a*) Amino acid composition of rat liver glycosylasparaginase and (*b*) *N*-terminal sequences of its subunits

The amino acid composition of the pig kidney enzyme (for comparison) is taken from Kohno & Yamashina (1972). Tryptophan was determined spectrophotometrically (Edelhoch, 1967). Abbreviation: N.D., not determined. In (*b*) the predicted site of oligosaccharide attachment is underlined.

(*a*) Amino acid composition

Amino acid	Composition (residues/100 residues) Rat liver enzyme	Pig kidney enzyme	Amino acid	Composition (residues/100 residues) Rat liver enzyme	Pig kidney enzyme
Glycine	11.8	10.4	Tryptophan	1.2	N.D.
Alanine	10.6	10.8	Cysteine	N.D.	2.0
Valine	6.8	5.5	Methionine	3.3	2.3
Isoleucine	3.8	5.0	Proline	6.3	3.7
Leucine	6.3	6.0	Aspartic acid	6.5	9.8
Serine	6.3	7.5	Glutamic acid	7.0	6.8
Threonine	7.8	8.0	Lysine	4.5	4.5
Phenylalanine	3.8	3.9	Arginine	4.0	3.9
Tyrosine	3.3	3.1	Histidine	1.8	1.7

(*b*) *N*-Terminal sequences

20 kDa subunit:

```
          5                   10                  15                  20
Gln-Ile-Gly-Met-Val-Val-Ile-His-Lys-Thr-Gly-His-Thr-Ala-Ala-Gly-Thr-Ser-Thr-Asn-Gly-Leu
```

24 kDa subunit:

```
          5                   10                  15
I   Ser-Asn-Pro-Leu-Pro-Leu-Val-Val-Asn-Thr-Trp-Pro-Phe-Lys-Asn-Ala-Thr-Glu
              5                   10                  15
II      Asn-Pro-Leu-Pro-Leu-Val-Val-Asn-Thr-Trp-Pro-Phe-Lys-Asn-Ala-Thr-Glu
```

amino acid peaks occurred. These amino acids could be aligned to give two sequences that were one amino acid out of phase from each other. Thus about half of the 24 kDa polypeptide lacked an *N*-terminal serine residue. The 24 kDa subunit also was apparently *N*-glycosylated in position 15, since no amino acid was detected by the Sequenator at this position, and position 17 is threonine. These results fitted the Asn-Xaa-Thr/Ser signal requirement for *N*-glycosylation (Hart *et al.*, 1979).

N-Linked oligosaccharide chains

The distribution of *N*-linked oligosaccharide chains on each subunit was determined by Endoglycosidase H and *N*-Glycanase treatments. As shown in Fig. 3 the 20 kDa subunit was transformed into an 18 kDa polypeptide both by Endoglycosidase H and by *N*-Glycanase. A partial cleavage of the glycosylasparaginase by Endoglycosidase H did not result in any intermediate molecular-mass bands (Fig. 3, lane 2), indicating that the 20 kDa subunit contained a single Endoglycosidase H-sensitive (high-mannose-type) oligosaccharide chain. The 24 kDa subunit was transformed into a 23 kDa polypeptide by Endoglycosidase H and into a 21.5 kDa polypeptide by *N*-Glycanase (Fig. 3). These changes indicated that the 24 kDa subunit contained at least two oligosaccharide chains, one Endoglycosidase H-sensitive (high-mannose type) and one Endoglycosidase H-resistant (complex type). The fact that glycosylasparaginase cleaved by Endoglycosidase H still adsorbed on concanavalin A–Sepharose gave further evidence that the 24 kDa subunit contained an Endoglycosidase H-insensitive oligosaccharide chain. The enzyme activity of this form bound to the lectin column and was released by 10 mM-methyl α-glucoside (results not shown), a behaviour that

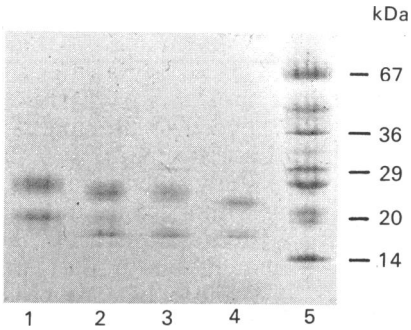

Fig. 3. Treatment of glycosylasparaginase with Endoglycosidase H and *N*-Glycanase

Portions (5 μg) of enzyme were either untreated (lane 1), or incubated in the presence of Endoglycosidase H for 10 h (lane 2) or 24 h (lane 3) or treated with *N*-Glycanase overnight (lane 4) as described in the Experimental section. The enzyme was then heated in boiling water for 3 min in the presence of 3% (v/v) glycerol, 0.5% SDS, 1 M-2-mercaptoethanol and Bromophenol Blue. Approx. 0.5 μg was subjected to SDS/PAGE with use of a Phast Gel system (Pharmacia) with a 8–25% gradient of polyacrylamide. Molecular-mass standards were applied to lane 5. The gel was stained with Coomassie Blue R-350.

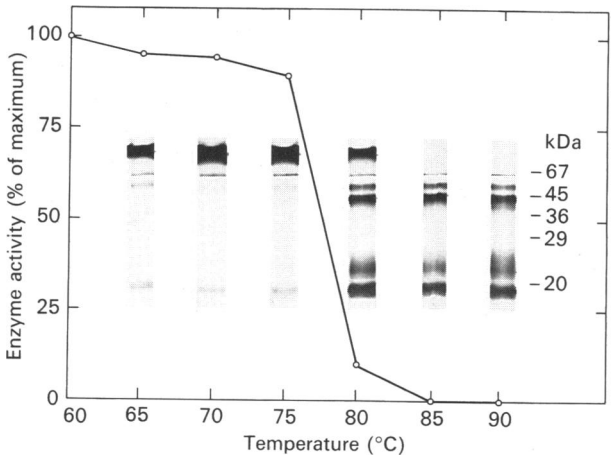

Fig. 4. Glycosylasparaginase stability as a function of temperature

Purified glycosylasparaginase (0.5 unit/ml) was incubated in 50 mM-sodium phosphate buffer, pH 7.0, containing 0.15 M-NaCl for 15 min at the temperatures indicated. The enzyme was chilled on ice, and one sample was assayed for enzyme activity and a second was mixed with 3% (v/v) glycerol, 0.5% SDS and some Bromophenol Blue and analysed by SDS/PAGE according to the Laemmli system. The gel was silver-stained.

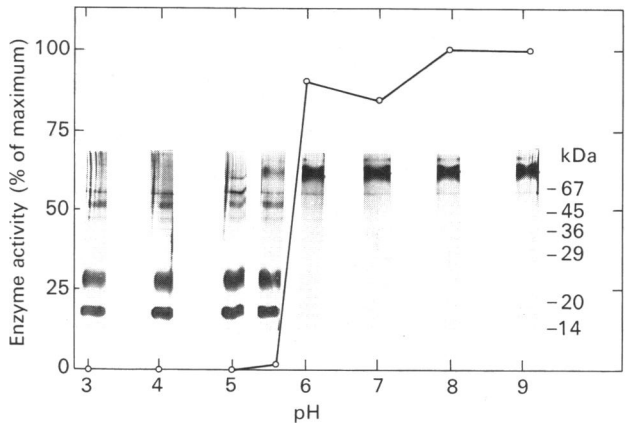

Fig. 5. Glycosylasparaginase stability as a function of pH in the presence of SDS

Purified glycosylasparaginase (0.5 unit/ml) was incubated at room temperature in 50 mM-sodium acetate buffer, pH 3–6, containing 0.15 M-NaCl and 0.5% SDS or 50 mM-sodium phosphate buffer, pH 6.5–9, containing 0.15 M-NaCl and 0.5% SDS. After 1 h the pH was adjusted to 7.5 by addition of 0.2 M-sodium phosphate buffer, pH 7.5. The enzyme activity was determined on a portion of each treated sample, and another portion was mixed with 3% (v/v) glycerol and Bromophenol Blue and analysed by SDS/PAGE according to the Laemmli system. The gel was silver-stained. Glycosylasparaginase was not heated before electrophoresis.

is typical of glycoproteins containing complex-type oligosaccharide chains (Kornfeld, 1983).

Stability towards heat, SDS and pH variations

In agreement with previous reports for glycosylasparaginase from human liver (Dugal & Strømme, 1977; McGovern *et al.*, 1983) and pig kidney (Kohno & Yamashina, 1972), we found that the rat liver enzyme was very heat-stable, as shown in Fig. 4. Below 75 °C the enzyme remained active and migrated as a single major band above 70 kDa on SDS/PAGE (Fig. 4, left-hand insets), which corresponded to the migration of native enzyme depicted in Figs. 1(*a*) and Fig. 2 (lane 1). Heat denaturation occurred only at temperatures above 75 °C and was concomitant with a change in the migration of the protein on SDS/PAGE, as shown in the right-hand insets of Fig. 4. In addition to the three bands of molecular masses 48, 24 and 20 kDa, which corresponded to a partially dissociated enzyme (Fig. 2, lane 2), the heat-denatured glycosylasparaginase exhibited a major band migrating as 43 kDa (Fig. 4 insets). Since this band did not appear when the enzyme was heated in the presence of SDS (Fig. 2, lane 2), its occurrence is probably due to the formation of a new and more compact structure of the heat-denatured enzyme. From an Arrhenius plot we found that the heat of activation was 25.9 kJ/mol (6.2 kcal/mol) (results not shown).

Glycosylasparaginase activity decreased by about 40% in the presence of 0.1% SDS at pH 7.0 (results not shown). Increasing the SDS concentration to 5% (w/v) did not lower the activity further. The partial loss of activity in the presence of SDS was restored by dialysis. As shown in Fig. 5, enzyme activity in the presence of 0.5% SDS was irreversibly abolished at pH values below 5.5. As judged from SDS/PAGE (Fig. 5 insets), this irreversible decrease of activity again occurred together with partial dissociation of the enzyme into 48 kDa, 24 kDa and 20 kDa bands, as shown in Fig. 2 (lane 2). Between pH 6 and 9, however, the enzymic activity was unaffected by SDS, and the enzyme ran as one major band above 70 kDa on SDS/PAGE (Fig. 5 insets), corresponding to the migration of native enzyme as seen in Fig. 1(*a*) and Fig. 2 (lane 1). The faint sharp band migrating as 55 kDa (Figs. 4 and 5 insets) was an artifact that appeared during silver-staining and is probably due to 2-mercaptoethanol (Marshall & Williams, 1984).

Isoelectric point and pH optimum

Upon isoelectric focusing of 0.5 μg of enzyme two major bands were observed at pH 6.4 and 6.6 (results not shown). In agreement with studies on partially purified rat liver glycosylasparaginase (Mahadevan & Tappel, 1967), we found that the pure enzyme had a broad pH optimum beginning at 7.0 and extending to at least pH 9, and that the enzyme was stable upon incubation at pH down to 3.0 for 2 h at room temperature (results not shown).

DISCUSSION

Glycosylasparaginase migrated as a protein of molecular mass above 70 kDa on SDS/PAGE provided that the enzyme was not denatured by boiling before its application to the electrophoresis gel [Figs. 1(*a*) and 2 (lane 1)]. When PAGE was carried out in the absence of SDS (Fig. 1*b*), the enzyme migrated with a similar rate. These two results indicated that untreated glycosylasparaginase, which had an actual molecular mass of about 44 kDa (sum of two subunits depicted in Fig. 2, lane 3), did not attain additional negative charge in the presence of SDS at the pH range used, 6.8–8.8. This is not a unique feature, since we found that rat liver

superoxide dismutase behaved similarly, i.e. the untreated 32 kDa enzyme migrated as a 55 kDa protein on SDS/PAGE (Fig. 2, lane 5), and this migration was similar when the electrophoresis was carried out in the absence of SDS (results not shown). Some proteins do not appear to bind SDS under certain conditions (Nelson, 1971), and rat liver glycosylasparaginase and superoxide dismutase probably belong to that group. Since most proteins readily bind SDS, glycosylasparaginase could be separated from contaminating proteins by preparative SDS/PAGE. The enzyme sample first had to be heat-treated, since two contaminating proteins were otherwise found to co-migrate with the enzyme on this preparative SDS/PAGE. The heat treatment at 70 °C probably denatured those proteins, thus making them susceptible to SDS binding.

Rat liver glycosylasparaginase is a glycoprotein of apparent native molecular mass 49 kDa that consists of two different subunits that are joined by strong non-covalent forces and have molecular masses of 20 and 24 kDa as judged by SDS/PAGE. Both the molecular mass and quaternary structure differ from data reported in other studies that obtained the enzyme from different species and tissues. The molecular mass reported for the native enzyme varies from 31 kDa in rat kidney to 150 kDa once claimed for the human liver enzyme (Conzelman & Sandhoff, 1987). Most of these studies, however, were conducted on partially purified samples, and therefore no further information about molecular properties could be obtained. Only the pig kidney and the human liver enzymes have been purified to apparent homogeneity. The pig kidney enzyme was found to be a monomer of molecular mass about 70 kDa (Kohno & Yamashina, 1972), and its amino acid composition closely resembles that of the rat liver enzyme (Table 2, part *a*). This compositional similarity might indicate that the rat liver enzyme originated from a one-chain precursor that was post-translationally cleaved in rat liver, but not in pig kidney. Such a variation in chain number for purified lysosomal cathepsin D from different species has been reported as well (Diment *et al.*, 1988). It is common to find that lysosomal enzymes have been post-translationally cleaved to multisubunit structures in the lysosomes. One still cannot, however, exclude the possibility that pig kidney glycosylasparaginase might have a quaternary structure and that the treatment before SDS/PAGE was not sufficient to dissociate the extremely stable enzyme. We found that the rat liver enzyme had to be boiled in the presence of SDS and 2-mercaptoethanol to be completely dissociated; mere incubation at 37 °C was not sufficient. The human liver enzyme was reported to be an 80 kDa monomer by McGovern *et al.* (1983), but more recently Kalkkinen *et al.* (1987) found it to consist of three subunits of molecular masses 24, 18 and 17 kDa joined by non-covalent forces. The discrepancy again might be due to insufficient conditions for dissociation before SDS/PAGE by McGovern *et al.* (1983) or to limited proteolysis occurring during the purification procedure used by Kalkkinen *et al.* (1987). Since the exact sample treatment before SDS/PAGE was not reported for either the pig kidney or the human liver enzyme, any conclusions with regard to their quaternary structure remain ambiguous.

Glycosylasparaginase was heterogeneous as judged by the migration of the subunits on SDS/PAGE (Fig. 3) and by isoelectric focusing of the native enzyme. One of the isoforms was caused by a partial removal of the *N*-terminal serine residue from the 24 kDa subunit (Table 2, part *b*). A similar *N*-terminal cleavage has been reported to occur in the α-chain of β-hexosaminidase from human fibroblasts (Little *et al.*, 1988), but it is not known whether this cleavage serves any purpose. Besides proteolysis, enzyme heterogeneity might also be caused by limited hydrolysis of oligosaccharide chains. Variations of isoelectric points have often been attributed to partial cleavage of sialic acid in the lysosomes (Goldstone & Koenig, 1974).

It may be difficult to relate the high pH optimum of rat liver glycosylasparaginase with a physiological function in the acidic lysosomal compartments. However, rat liver enzyme activity with similar pH optimum and K_m to those of our pure enzyme has been exclusively localized to the lysosomal fraction of the cell (Mahadevan & Tappel, 1967; Ogushi & Yamashina, 1968). At pH 5.0 the enzyme did retain 40% of maximum activity. It is conceivable that the enzyme could function with this activity level *in vivo*. Alternatively the microenvironments of the lysosomes might allow for a higher activity as compared with conditions *in vitro*.

The stability properties of glycosylasparaginase at neutral pH were comparable with those of superoxide dismutase (Fridovich, 1982). Apparently intrachain disulphide bridges contributed to the stability, since reduction by 2-mercaptoethanol was necessary to dissociate the enzyme completely in the presence of SDS at 100 °C (Fig. 2, lanes 2 and 3). A similar effect of intrachain disulphide bridges has previously been shown for superoxide dismutase (Abemethy *et al.*, 1974). At room temperature, however, glycosylasparaginase was still resistant to binding of SDS in the presence of reducing agent, which shows that other factors also contribute to the remarkable stability. In superoxide dismutase Zn^{2+} stabilizes the structure by forming salt bridges to various parts of the enzyme (Fridovich, 1982). Among proteins from thermophilic bacteria Ca^{2+} stabilizes thermolysin (Amelunxen & Murdock, 1978), and lysine residues add to the stability of α-amylase from *Bacillus licheniformis* (Tomazic & Klibanov, 1988), probably through salt bridges. One still does not know, however, what general structural factors distinguish a thermostable enzyme from a thermolabile one. Presumably a combination between hydrophobic, ionic and other interactions is important (Amelunxen & Murdock, 1978). Since the catalytic activity of glycosylasparaginase and its resistance towards SDS binding coincided both when varying the pH (Fig. 5) and temperature (Fig. 4), the factors responsible for the extraordinary stability of this lysosomal hydrolase might also involve its active site.

This work was supported by U.S. Public Health Service Grant DK-33314 from the National Institute of Diabetes and Kidney Diseases and by a grant from the Pennsylvania State University Agricultural Station. We are grateful to Sigrun Hofmann and Ron Bayline for their technical assistance.

REFERENCES

Abemethy, J. L., Steinman, H. M. & Hill, R. L. (1974) J. Biol. Chem. **249**, 7339–7343

Amelunxen, R. E & Murdock, A. L. (1978) CRC Crit. Rev. Microbiol. **6**, 343–393
Baussant, T., Strecker, G., Wieruszeski, J.-M., Montreuil, J. & Michalski, J.-C. (1986) Eur. J. Biochem. **159**, 381–385
Conzelman, E. & Sandhoff, K. (1987) Adv. Enzymol. Relat. Areas Mol. Biol. **60**, 90–186
Crapo, J. D. & McCord, J. M. (1976) Am. J. Phys. **231**, 1196–1203
Diment, S., Leech, M. S. & Stahl, P. D. (1988) J. Biol. Chem. **263**, 6901–6907
Dugal, G. & Strømme, J. (1977) Biochem. J. **165**, 497–502
Edelhoch, H. (1967) Biochemistry **6**, 1948–1954
Fridovich, I. (1982) in Superoxide Dismutase (Oberley, L. W., ed.), vol. 1, pp. 1–68, CRC Press, Boca Raton
Goldstone, A. & Koenig, H. (1974) FEBS Lett. **39**, 176–181
Hart, G. W., Brew, K., Grant, G. A., Bradshaw, R. A. & Lennarz, W. J. (1979) J. Biol. Chem. **254**, 9747–9753
Kalkkinen, N., Peltonen, L. & Bauman, M. (1987) J. Cell. Biochem. **11C** (Suppl.), 180
Kohno, M. & Yamashina, I. (1972) Biochim. Biophys. Acta **258**, 600–617
Kornfeld, S. (1983) Falk Symp. **34**, 13–22
Kuranda, M. J. & Aronson, N. N., Jr. (1986) J. Biol. Chem. **261**, 5803–5809
Kuranda, M. J. & Aronson, N. N. Jr. (1987) in Lysosomes: Their Role in Protein Breakdown (Glauman, H. & Ballard, F. J., eds.), pp. 241–282, Academic Press, London
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Levvy, G. A. & McAllan, A. (1959) Biochem. J. **73**, 127–132
Little, L. E., Lau, M. H. M., Quon, D. V. K., Fowler, A. V. & Neufeld, E. F. (1988) J. Biol. Chem. **263**, 4288–4292
Mahadevan, S. & Tappel, A. L. (1967) J. Biol. Chem. **242**, 4568–4576
Makino, M., Kojima, T. & Yamashina, I. (1966) Biochem. Biophys. Res. Commun. **24**, 961–966
Makino, M., Kojima, T., Ohgushi, T. & Yamashina, I. (1968) J. Biochem. (Tokyo) **63**, 186–192
Marshall, T. & Williams, K. M. (1984) Anal. Biochem. **139**, 502–505
Matsudaira, P. (1987) J. Biol. Chem. **262**, 10035–10038
Maury, C. P. (1982) J. Inher. Metab. Dis. **5**, 192–196
McGovern, M. M., Aula, P. & Desnick, R. J. (1983) J. Biol. Chem. **258**, 10743–10747
Nelson, C. A. (1971) J. Biol. Chem. **246**, 3895–3901
Ogushi, T. & Yamashina, I. (1968) Biochim. Biophys. Acta **156**, 417–419
Peterson, G. (1983) Methods Enzymol. **91**, 69–105
Song, Z., Li, S.-C. & Li, Y.-T. (1987) Biochem. J. **248**, 145–149
Tarentino, A. L. & Maley, F. (1969) Arch. Biochem. Biophys. **130**, 295–303
Tarentino, A. L., Plummer, T. H. & Maley, F. (1975) Biochemistry **14**, 5516–5523
Tollersrud, O. K., Hofmann, S. & Aronson, N. N., Jr. (1988) Biochim. Biophys. Acta. **953**, 353–356
Tomazic, S. J. & Klibanov, A. M. (1988) J. Biol. Chem. **263**, 3092–3096
Yamashita, K., Tachibana, Y., Takada, S., Matsuda, I., Arashima, S. & Kobata, A. (1979) J. Biol. Chem. **254**, 4820–4827

Received 21 September 1988/23 November 1988; accepted 29 November 1988