The apparent active site of human leukocyte glycosaparaginase (N\(^4\)-(\(\beta\)-acetylglucosaminyl)-L-asparaginase (EN-\((\beta\)-acetylglucosaminyl)-L-asparaginase EC 3.5.1.26) has been studied by labeling with an asparagine analogue, 5-diazo-4-oxo-L-norvaline. Glycosaparaginase was purified 4,600-fold from human leukocytes with an overall recovery of 12%. The purified enzyme has a \(K_m\) of 110 \(\mu\)M, a \(V_{max}\) of 34 \(\mu\)mol \(\times 1^{-1} \times \min^{-1}\), and a specific activity of 2.2 units/mg protein with \(N^4-(\beta\)-N-acetylglucosaminyl)-L-asparaginase as substrate. The carbohydrate content of the enzyme is 15%, and it exhibits a broad pH maximum between 7 and 9. The 88-kDa native enzyme is composed of 19-kDa light (L) chains and 25-kDa heavy (H) chains and it has a heterotetrameric structure of L\(_2\)H\(_2\)-type. The glycosaparaginase activity decreases rapidly and irreversibly in the presence of 5-diazo-4-oxo-L-norvaline. At any one concentration of the compound, the inactivation of the enzyme is pseudo-first-order with time. The inhibitory constant, \(K_i\), is 80 \(\mu\)M and the second-order rate constant \(1.25 \times 10^4\ \text{M}^{-1}\ \text{min}^{-1}\) at pH 7.5. The enzyme activity is competitively protected against this inactivation by its natural substrate, \(\beta\)-asparaginylglycopeptides, indicating that this inhibitor binds to the active site or very close to it. The covalent incorporation of \([5-\text{\textsuperscript{14}}\text{C}]\text{diazo-4-oxo-L-norvaline}\) paralleled the loss of the enzymatic activity and one inhibitor binding site was localized to each L-subunit of the heterotetrameric enzyme. Four peptides with the radioactivity label were generated, purified by high performance liquid chromatography, and sequenced by Edman degradation. The sequences were overlapping and all contained the amino-terminal tripeptide of the L-chain. By mass spectrometry, the reacting group of 5-diazo-4-oxo-L-norvaline was characterized as 4-oxo-L-norvaline that was bound through an \(\alpha\)-ketone ether linkage to the hydroxyl group of the amino-terminal amino acid threonine.

The lysosomes of mammalian cells contain a glycosaparaginase (\(N^4-(\beta\)-acetylglucosaminyl)-L-asparaginase, asparaglycosaminase, asparaglycosaminidase, glycosylasparaginase, EC 3.5.1.26) that is involved in the degradation of the N-glycosidic linkage between asparagine and \(N\)-acetylgulosamine in various asparaginylglycopeptides. The substrate \(N^4-(\beta\)-N-acetylglucosaminyl)-L-asparaginase (asparaglycosaminase) is hydrolyzed by the enzymes to aspartate and L-amino-N-acetylgulosamine. The latter product is further cleaved nonenzymatically to ammonia and \(N\)-acetylgulosamine (1).

Human liver has been reported to contain a monomeric \(\beta\)-asparaglycosaminidase of 80 kDa (2) and a trimeric enzyme of 60 kDa consisting of three nonidentical polypeptides (3), both with a pH maximum at 6.1, in addition to an undetailed asparaglycosaminidase with a pH maximum at 7.5 (4). The rat liver asparaglycosaminidase is a heterodimeric protein of 43 kDa that contains two noncovalently bound subunits and has a broad maximum between pH 6.5 and 10 (5). The amino-terminal sequences of its subunits have been determined (5).

Lack of the glycosaparaginase activity in humans results in a lysosomal storage disease called asparaglycosaminuria (McKusick 20840) characterized with severe psychomotor retardation and accumulation of glycosaparaginases in tissues and body fluids (1).

Bacterial (6) and yeast (7) asparaginases can hydrolyze D- or L-asparagine with free \(\alpha\)-amino and \(\alpha\)-carboxyl groups to yield aspartate and ammonium ion. In addition to asparaginase, bacterial glutaminase-asparaginases are able to hydrolyze glutamine to yield glutamic acid and ammonium ion (8). Inhibitors of these related amidohydrolases have proved useful in understanding the characteristics of the enzymes. An L-asparaginase analogue 5-diazo-4-oxo-L-norvaline (DONV)\(^1\) has been used to label the active site of Escherichia coli L-asparaginase (9). Glutaminase-asparaginases are similarly inhibited and labeled by the next larger homologue of DONV, 6-diazo-4-oxo-L-norleucine (DON), which binds to threonine hydroxyls in an 8-residue segment identical in both Acinetobacter and Pseudomonas 7A glutaminase-asparaginase enzymes (10). DONV has been shown to irreversibly inhibit hen oviduct (11) and rat liver glycosaparaginase (12), but no further characterization of the adduct was done. The effect of the amidohydrolase inhibitors on human glycosaparaginases has not been studied. In this paper, we describe purification of heterotetrameric glycosaparaginase from human leukocytes and report our findings that 5-diazo-4-oxo-L-norvaline is an irreversible inhibitor of the enzyme. We also show that the compound is an active site-directed inhibitor or affinity label

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\(^1\) The abbreviations used are: DONV, 5-diazo-4-oxo-L-norvaline; HPLC, high performance liquid chromatography; ONV, 4-oxo-L-norvaline; DON, 6-diazo-4-oxo-L-norleucine; PTC, phenylthiocarbamyl; TEA, triethylamine; PFTC, phenylthioformic acid; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; PAGS, polyaspartamid gel electrophoresis; SDS, sodium dodecyl sulfate.
and that a threonine hydroxyl is alkylated to form an ether linkage with the reacting group of the inhibitor.

MATERIALS AND METHODS

RESULTS

5-Diazo-4-oxo-L-norvaline at each concentration caused progressive, irreversible inhibition of glycoasparaginase activity in a pseudo-first-order reaction under conditions that maintained the activity of the enzyme in the absence of the reagent (Fig. 1). The activity of the enzyme could not be restored by extensive dilution or dialysis of the inhibited enzyme. The hyperbolic relationship between the pseudo-first-order constant and concentration of DONV (data not shown) indicates that the inhibitor forms a reversible, prealkylation complex with the enzyme (16, 17). Thus, the inhibition corresponds to the mechanism

$$E + I \underset{k_i}{\overset{k_i}{\rightleftharpoons}} E-I \underset{k_{ii}}{\overset{k_{ii}}{\rightleftharpoons}} E - X, \ K_i = k_{ii}/k_i,$$

where $E - X$ is the irreversibly inactivated enzyme and follows the form

$$k_{obs} = k_i[I]/(K_i + [I]).$$

The second-order rate constant corresponding to $[I] \ll K_i$ is given by $k_i/K_i$.

A plot of $1/k_{obs}$ against $1/(DONV)$ showed a linear relationship typical of hyperbolic saturation (Fig. 1, inset). The steady-state constant ($K_i$) and the rate constant ($k_i$) in 50 mM phosphate buffer, pH 7.5, calculated from the slope were $K_i = 80 \mu M$, $k_i = 0.10 \text{ min}^{-1}$ and the second-order rate constant $= 1.25 \times 10^4 \text{ M}^{-1} \text{ min}^{-1}$. In order to determine if DONV reacts with some group at the active site of the enzyme, we studied the effect of the substrate, aspartylglucosamine, on the inactivation rate. The presence of substrate in the same medium with the inactivator competitively inhibited the inactivation of glycoasparaginase with an inhibition constant $K_c = 98 \mu M$ (Fig. 2).

To determine the region of glycoasparaginase modified by DONV, the enzyme was radioactively labeled in the presence of $[5^{-14}C]DONV$ for 24 h. The completely inhibited enzyme was further incubated for another 2 h in the presence of 1,000-fold excess of unlabeled DONV and injected onto the reverse-phase HPLC column. Monitoring of the eluent by the absorbance at 225 nm and radioactivity showed that the light chain of the enzyme eluting at 34% of solvent B was effectively radiolabeled (Fig. 3). Background levels of radioactivity were found with the heavy subunit eluting at 43% of solvent B and any of the other components in the chromatograms. Taking into consideration that the glycoasparaginase-DONV adduct lost about 20% of its label during repeated chromatographic runs in 0.1% trifluoroacetic acid, 1.3 mol of $[5^{-14}C]DONV$ was bound/mol of enzyme or 0.7 mol/mol of L-subunit.

After total proteolysis of the $[5^{-14}C]DONV$-labeled L-chain with chymotrypsin, the peptides were separated by reverse-
DONV-labeled L-chain was stable in 70% formic acid and parallel experiments where the modified protein was subjected to treatment with Edman degradation. The absence of significant secondary amino acid or unknown peaks indicated high purity of the peptides. The chymotryptic peptide CH1 was a tripeptide of Thr-Ile-Gly. The peptide CH2 consisted of seven amino acids with the same Thr-Ile-Gly sequence at its amino-terminal end (Table I). The radioactive chymotryptic fragments CH1 and CH2 showed identical tripeptide sequences of Thr-Ile-Gly in Edman degradation (Table I). All the sequences obtained from the peptides were identical to the amino-terminal end of the intact L-subunit (Table I). Further localization of the inhibitor-binding site by this method was unsuccessful, since the radioactivity could not be localized to any particular cleavage cycle presumably because it was either flushed away by 100% trifluoroacetic acid before the actual sequencing cycles or found to be retained on the sample filters after the sequencing procedure. The combined evidence demonstrates that the reacting site of 5-diazo-oxo-norvaline to human leukocyte glycoasparaginase is within a few amino-terminal amino acids of its L-subunit.

To identify the residues involved in the covalent modification of the glycoasparaginase by 5-diazo-oxo-norvaline, the enzyme was labeled with nonradioactive DONV. The L-subunit was purified under conditions identical to those used for [5-14C]DONV labeling, digested with trypsin, and the peptides were isolated by HPLC as described earlier. The mass spectrum of the HPLC fraction collected at 30% of solvent B (data not shown) consisted of two strong (M+H)+ signals at m/z 997 and 1,125. The ion at m/z 997 matches the value expected for the N-terminal tryptic peptide from the α-subunit. Formation of the DONV-peptide adduct by nucleophilic substitution would increase the mass of this peptide by 128 daltons to m/z 1,125. Analysis of the ion at m/z 1,125 by collision-activated dissociation (22-24) produced the mass spectrum in Fig. 5. The predicted fragment ions, type b and y+ (22), are displayed with the structure in the figure, and those ions observed in the mass spectrum are underlined. To provide additional support for the localization of the label to

| Cycle | Amino acid | Amino acid | Amino acid | Amino acid |
|-------|------------|------------|------------|------------|
|       | CH1        | CH2        | CB1        | CB2        |
| 1     | Thr        | Thr        | Thr        | Thr        |
| 2     | Ile        | Ile        | Ile        | Ile        |
| 3     | Gly        | Gly        | Gly        | Gly        |
| 4     | Met        | Met        | Met        | Met        |
| 5     | Val        | Val        | Val        | Val        |
| 6     | Val        | Val        | Val        | Val        |
| 7     | Ile        | Ile        | Ile        | Ile        |
| 8     | His        | His        | His        | His        |
| 9     | Lys        | Lys        | Lys        | Lys        |
| 10    | Thr        | Thr        | Thr        | Thr        |
| 11    | Gly        | Gly        | Gly        | Gly        |
| 12    | His        | His        | His        | His        |
| 13    | Ile        | Ile        | Ile        | Ile        |
| 14    | Ala        | Ala        | Ala        | Ala        |
| 15    |             |             |             |             |

1 nmol of each subunit was used for sequencing and picomoles of phenylthiohydantoin-amino acids analyzed are shown in parenthesis. X, no amino acid signal above the background was detected.


DISCUSSION

In this work, glycoasparaginase was purified 4,600-fold from human leukocytes. Throughout the purification of the enzyme, we monitored its activity with its natural substrate using a new, specific assay for glycoasparaginase activity (15). The purified enzyme was obtained with a 12% overall yield. Gel electrophoresis under reducing conditions revealed that the enzyme protein runs as two separate bands nominated as light (L) and heavy (H) subunits with apparent Mr of 19,000 and 25,000, respectively. Since the amounts of L- and H-subunits according to SDS-PAGE and reverse-phase HPLC are very similar, and on native PAGE the enzyme runs as a single band at 83,000, a quaternary structure of L2H2-type with an apparent Mr of 88,000 for the native glycoasparaginase is suggested. The pH optimum of glycoasparaginase of human leukocytes and chorionic villous cells is 7-9 (15), which is extraordinarily high for lysosomal enzymes that usually have an acidic optimum. At pH 4-5, the leukocyte glycoasparaginase had only 6-10% of its activity at 7.5, which may reflect unknown catabolic characteristics of the enzyme different from most lysosomal exoglycosidases. The enzymatic activity of the dissociation intermediates of human leukocyte glycoasparaginase suggests that its action requires association of at least one of each chain, which by themselves are enzymatically inactive. A recent report indicates the presence of a heterodimeric glycoasparaginase of Mr, 43,000 with a broad pH maximum between 6.5 and 10 in rat liver (6). The amino-terminal sequences of the rat enzyme have high similarity to those of the human leukocyte enzyme.

Since the free α-amino and α-carboxyl groups of asparagine were essential for the hydrolysis of the substrate, we synthesized an asparagine analogue 5-diazo-oxo-α-norvaline and studied its action on human leukocyte glycoasparaginase. Incubation of the compound with glycoasparaginase resulted in irreversible loss of its enzymatic activity. The specificity of the interaction between the asparagine analogue and the enzyme is demonstrated in several ways: the glycoasparaginase activity is inhibited by DONV under conditions that maintain the activity of the enzyme without inhibitor, the inhibition follows pseudo-first-order kinetics at any one concentration of the inhibitor, the enzyme activity is partially

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**Fig. 5.** Collision-activated dissociation mass spectrum of the ion at m/z 1,125 of the peptide ONV-Thr-Ile-Gly-Met-Val-Val-Ile-His-Lys, residues 1-9 of DONV-labeled L-chain of human leukocyte glycoasparaginase. The collision energy was 27 eV, and the argon pressure in the collision cell was 3 × 10⁻⁵ Torr. The structure is labeled with fragment ions of type b, above, and type y”, below. Those fragment ions observed in the mass spectrum are underlined.

**TABLE II**

Fragment ions and ion intensities observed in the collision activated dissociation mass spectra of 4-oxo-α-norvaline-Thr-Ile-Gly-Met-Val-Val-His-Ile-Lys and 4-oxo-α-norvaline-Thr-Ile-Gly-Met-Val-Val-His-Ile-Lys methyl ester

| Sequence ions | m/z | Intensity | m/z | Intensity |
|---------------|-----|-----------|-----|-----------|
| y*            | 147 | 161       | 1,153| 0.64      |
| y*2           | 294 | 298       | 298 | 5.77      |
| y*3           | 397 | 411       | 411 | 2.42      |
| y*4           | 496 | 510       | 510 | 2.37      |
| y*5           | 595 | 609       | 609 | 1.46      |
| y*6           | 726 | 740       | 740 | 0.61      |
| y*7           | 783 | 797       | 797 | 1.61      |
| y*8           | 896 | 910       | 910 | 0.45      |
| (M+H)*        | 1,125| 100.0   | 1,153| 100.0    |

* Free acid collision-activated dissociation mass spectrum.

* Relative intensities.

* Methyl ester collision-activated dissociation mass spectrum.
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Table III

Comparison of the N-terminal sequence of human leukocyte glycoasparaginase L-chain (I), rat liver glycosylasparaginase light chain (II) (5), Acinetobacter glutaminase-asparaginase (III) (10, 28), Pseudomonas T/A glutaminase-asparaginase (IV) (10), and E. coli L-asparaginase (V) (30).

A known reaction site for DONV (I) and DON (III, IV) are indicated in boldface.

| Peptide | Sequence |
|---------|----------|
| I       | Thr-Ile-Gly-Met-Val-Val-Ile-His-Lys-Gly-His-Ile-Ala-Ala- |
| II      | Gln-Ile-Gly-Met-Val-Val-Ile-His-Lys-Thr-Gly-His-Ile-Ala-Ala- |
| III     | Lys-Asn-Asp-Val-Val-Val-Val-Ala-Thr-Gly-Thr-Ile-Ala- |
| IV      | Leu-Asp-Arg-Val-Ile-Leu-Thr-Gly-Thr-Ile-Ala- |
| V       | Leu-Pro-Asp-Ile-Thr-Ile-Leu-Thr-Gly-Thr-Ile-Ala- |

protected by its natural substrate, and a single amino acid residue is covalently labeled by the inhibitor.

The stoichiometry of [5-14C]DONV binding to glycoasparaginase was 0.7 mol of DONV/mol of L-subunit indicating the existence of two inhibitor-binding sites/one enzyme molecule or one each L-chain. The labeling efficiency is in accordance with the sequencing finding that some of the inhibitor-binding sequence was found to be without the label. The inhibitor-binding site to glycoasparaginase could be localized within the three most amino-terminal amino acid residues of the L-chain by automated Edman degradation sequencing of [5-14C]DONV-labeled chymotryptic and cyanogen bromide peptides. The cyanogen bromide cleavage produced two peptides, CB1 and CB2, that had different retention times in reverse-phase HPLC, but generated identical sequences upon Edman degradation. The small amounts of the peptides limited mass spectrometric analysis, and no structure-related mass spectra could be recorded for them. The structural difference between CB1 and CB2 is most likely ascribed to a mixture of DONV-containing tetrapeptides with homoserine and homoserine lactone at their carboxy-terminal ends. Cyanogen bromide cleavage is known to produce both compounds, and it has been demonstrated that even octapeptides with identical sequences are separated from one another by reverse-phase HPLC due to the presence of either homoserine or homoserine lactone at the carboxyl terminus (23). The final identification of the amino acid modified by the inhibitor was accomplished by mass spectrometry of tryptic peptides. The reacting group of DONV was shown to be 4-0xo-L-isoleucine bound through an α-ketone ether linkage to the hydroxyl group of the amino-terminal amino acid, threonine. More generally, our data show the applicability of mass spectrometry in characterization and localization of modifications in proteins. Active-site-directed irreversible inhibitors of enzymes produce labile adducts often difficult to characterize by chemical means (9, 10) and especially in such cases, mass spectrometry should be considered as the method of choice.

The binding of the substrate analogues to the hydroxyl group of threonine or serine residues is a common feature between glycoasparaginase and other amidohydrolases. The diazo compounds, like the actual substrates of the enzymes (26), are obviously attached to the active center of amidohydrolases through their α-amino- and α-carboxyl groups. This is followed by a nucleophilic attack in which the diazo nitrogens are cleaved off and the positively charged carbonium ion reacts with the negatively charged oxygen and a covalent oxygen-carbon bond is formed (10). Increased acidity of the hydroxyl group is suggested by this reaction in absence of catalyst. The acidity of amines is not great enough to react with the diazo group without a catalyst (27). Since both glycoasparaginase and asparaginases recognize the asparagine moiety with free α-amino and α-carboxyl groups and are inhibited with the same substrate analogue, it is reasonable to assume that this general reaction scheme applies to glycoasparaginase as well.

The amino-terminal sequence of the human leukocyte glycoasparaginase L-subunit shows considerable homology to that of bacterial amidohydrolases, and it is highly similar to the light subunit of the rat liver glycosylasparaginase (Table III). The primary structure of the L-subunit of the human glycoasparaginase has two significant differences compared to the other sequences: it is the only enzyme to contain an amino-terminal threonine as well as to lack threonine at the position 13 that corresponds to the DON-binding threonine at position 12 in Acinetobacter glutaminase-asparaginase (10, 28). Located within the conserved 8-residue fragment found also in Pseudomonas T/A glutaminase-asparaginase (10), E. coli asparaginase (28), and E. coli asparaginase (30). The presence of the amino-terminal threonine in human leukocyte glycoasparaginase is apparently of functional importance, since this particular residue forms the covalent adduct with [5-14C]DONV and is replaced in the rat liver enzyme by glutamine (5). DONV is known to inhibit rat liver glycoasparaginase, but whether it is bound to the threonine at position 13 or elsewhere remains to be shown.

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[Panel Table]
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SUPPLEMENTAL MATERIAL TO GLYCOSASPARAGINASE FROM HUMAN LEUKOCYTES: INACTIVATION AND COVALENT MODIFICATION WITH DIACETONASOYGLYCEMIC

BY VESA KAARTINEN, JULIAN C. WILLIAMS, JOHN TOMICH, JOHN R. YATES III, LEROY E. ROYD, AND BIRKA MONonen

MATERIALS AND METHODS

Materials Leukocytes were isolated from the buffy coat of human blood obtained from the Finnish Red Cross Transfusion Service, Kuopio, Finland. The donors were healthy Finnish blood donors with no history of current or previous use of medications known to influence the activity of the tested enzymes.

Methods

1. Glycosasparaginase activity was determined by measuring the release of aspartate from N-AcetylglucosaminyI-N-acetylglucosamine using the method of Kashiwagi et al. (1987) Methods Enzymol. 150, 175-185. The enzyme was purified from human leukocytes by ammonium sulfate precipitation, followed by gel filtration on Sephacryl S-200, ion exchange on DEAE-Sepharose CL-6B, and electrophoresis on a 10% polyacrylamide gel. The purified enzyme was dialyzed against 50 mM sodium phosphate buffer, pH 7.5, and concentrated to 1 mg/ml.

2. The reaction was carried out in a 100 mM sodium phosphate buffer, pH 7.5, and the reaction was monitored by measuring the release of aspartate using the method of Kashiwagi et al. (1987) Methods Enzymol. 150, 175-185.

3. The enzyme was assayed by measuring the release of aspartate from N-AcetylglucosaminyI-N-acetylglucosamine using the method of Kashiwagi et al. (1987) Methods Enzymol. 150, 175-185.

4. The enzyme was purified from human leukocytes by ammonium sulfate precipitation, followed by gel filtration on Sephacryl S-200, ion exchange on DEAE-Sepharose CL-6B, and electrophoresis on a 10% polyacrylamide gel. The purified enzyme was dialyzed against 50 mM sodium phosphate buffer, pH 7.5, and concentrated to 1 mg/ml.

5. The enzyme was assayed by measuring the release of aspartate from N-AcetylglucosaminyI-N-acetylglucosamine using the method of Kashiwagi et al. (1987) Methods Enzymol. 150, 175-185.

6. The enzyme was purified from human leukocytes by ammonium sulfate precipitation, followed by gel filtration on Sephacryl S-200, ion exchange on DEAE-Sepharose CL-6B, and electrophoresis on a 10% polyacrylamide gel. The purified enzyme was dialyzed against 50 mM sodium phosphate buffer, pH 7.5, and concentrated to 1 mg/ml.

7. The protection of glycosasparaginase by its substrate, aspartylglucosaminlyl-N-acetylglucosamine (L), against the inactivation caused by DONV (5) was studied in various concentrations of the compounds in 50 mM phosphate buffer, pH 7.5, and the inhibition constant (K_{i}) was derived from (16, 17).
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Glycoasparaginase Chromatography. After methylation of glycoasparaginase in 0.5 M methanolic hydrobromic acid, RPCP, 18 h, with mannitol and myo-inositol as internal standards, the quantitative monosaccharide analysis was performed by capillary liquid chromatography using a Perkin Elmer gas chromatograph model 8500 equipped with a flame-ionization detector (Perkin Elmer, Norwalk, CT, U.S.A.). The trimethylsilylated monosaccharide derivatives were separated on a 25-cm capillary column (25 m × 0.1 mm, i.d. coated with 5% SE-54 (Nicolet Inc., Loveland, Finland)).

Amino Acid Analysis. The purified protein was hydrolyzed in 6 M HCl at 110 °C for 16 h and the liberated amino acids were analyzed as their methylthiolate derivatives, with an automatic LKB 4151 Alpha Plus amino acid analyzer (LKB-Products AB, Bromma, Sweden), using scintillation as an internal standard. Protein was determined by BCA using perosulfate-Fe(III) reagent.

Polyacrylamide gel electrophoresis. The native PAGE electrophoresis was performed with the PhastSystem™ according to the manufacturer's instructions using precast 8-25% gradient gels (PhastSystem™, Separation Technique Gel No. 120). Protein bands and molecular weight standards were localized by silver staining. SDS-PAGE was performed under non-reducing conditions (21). The enzyme preparation was further purified by releasing by 0.5 M NaCl and dialyzed against 0.1 M phosphate buffer, pH 7.0, containing 0.5 M NaCl and eluted with 6.8 M urea. The effect of pH on the enzyme activity was determined with Tris-HCl and phosphate buffers, pH 7.0, and NaCl of 0.5 M concentration. The purified protein was hydrolyzed at 110 °C for 20 h to determine the amount of amino acids. The sample was evaporated to dryness with a Savant SpeedVac™. The synthetic peptide was prepared using automated solid phase synthesis techniques on a Beckman System 240. The amino acids of the peptide were analyzed using the Bio-Rad protein sequencer equipped with online 120A PTH analyzer. The amino acids in the peptide were sequenced with a Ceres Instruments 120A PTH analyzer and used for peptide sequencing. Details of the chromatography are given in the legends of Figs. 3 and 4.

Peptide Purification and Analysis. The [14C]DONV-labeled L-chain (7 nmol, 135 mCi) was digested with chymotrypsin (2.7 µg) in 2 M urea, 0.1 M ammonium bicarbonate, pH 7.0, at 37 °C for 4 h and the amino acids obtained from the tryptic digests were performed under similar conditions for 6 h. For cyanogen bromide cleavage, the enzyme preparation was frozen by HPLC. The purified and sequenced peptide was used for peptide sequencing. Details of the chromatography are given in the legends of Figs. 3 and 4.

Results

Glycoasparaginase. The purification scheme is summarized in Table IV. The capillary- and polycarbonate- and back techniques on DEAE-Sephadex A-50 yielded an activity of 280 mU/mg. The capillary- and polycarbonate- and back techniques on DEAE-Sephadex A-50 yielded an activity of 280 mU/mg. The capillary- and polycarbonate- and back techniques on DEAE-Sephadex A-50 yielded an activity of 280 mU/mg. The capillary- and polycarbonate- and back techniques on DEAE-Sephadex A-50 yielded an activity of 280 mU/mg. The capillary- and polycarbonate- and back techniques on DEAE-Sephadex A-50 yielded an activity of 280 mU/mg.
The yield from 1 L of packed leukocytes was 340 µg of pure and active enzyme with a specific activity of 2.2 U/mg. The yield of purification was 12 % and the specific activity increased 4600-fold as compared with that of the crude extract (Table IV).

**TABLE IV**

| Homogenate | Total units | Total protein | Yield | Specific activity | Fold |
|------------|-------------|---------------|-------|------------------|------|
| 6400       | 12 800      | 100           | 0.5   | 1                |      |
| Caprylic acid | 9400       | 4300          | >100  | 2.2              | 4.5  |
| DEAE II    | 6330        | 1.376         | >100  | 4.6              | 9.5  |
| DEAE I     | 6624        | 2.88          | >100  | 23.0             | 46   |
| Cos A      | 5060        | 18            | 79    | 281              | 585  |
| S-200 HR   | 1180        | 3.2           | 30    | 587              | 1015 |
| Alkyl S    | 1105        | 0.88          | 17    | 1255             | 2615 |
| Mono Q     | 751         | 0.34          | 12    | 2210             | 4600 |

**Molecular weight and quaternary structure.** On native PAGE, glycoasparaginase ran as a single protein band with an apparent Mr of 83,000 (Fig. 7A). By gel filtration on a Sephacryl S-200 HR column calibrated with marker proteins, the Mr was estimated to 76,000 (data not shown). When the enzyme preparation was incubated in 2.5 % SDS and 3 % mercaptoethanol for 10 minutes at 60 °C, the protein completely dissociated to give two protein bands with the relative molecular weight of 25,000 and 19,000 (± 12 % SDS-PAGE (Fig. 7B)). In a pH gradient, the native enzyme distributed between pH 4.6-5.2 on isoelectric focusing (Fig. 7C) and at least five separate bands indicating charge heterogeneity.

**Fig. 7.** Molecular weight, subunit composition and isoelectric point of purified human leukocyte glycoasparaginase.

A. Polyacrylamide gel electrophoresis of the purified enzyme (lane 1) and the molecular weight markers (lane 2). Electrophoresis was carried out on a 8-25 % gradient gel, which was stained with silver.

B. SDS-polyacrylamide gel electrophoresis of the purified enzyme (lane 1) and the molecular weight markers (lane 2) under reducing conditions. Electrophoresis was carried out on a 12 % gel, which was stained with silver.

C. Isoelectric focusing of the purified enzyme (lane 1) and the marker proteins (lane 2). IEF was performed on a pH 4.5-9.0 gradient gel, which was stained with silver.

Glycoasparaginase activity was lost simultaneously with the disappearance of the native enzyme during the incubation before SDS-PAGE electrophoresis (Fig. 8). The intensity of the new bands at 19,000 and 25,000 increased rapidly with the progress of dissociation. At the same time, protein bands with glycoasparaginase activity were consistently observed at 67,000 and 55,000 regions until the native enzyme band had completely disappeared. The proteins at 19,000 and 25,000 were enzymatically inactive (Fig. 8). On these basis we conclude that glycoasparaginase of human leukocytes is a polymeric and most obviously a heterotrimetric protein of L2H12-type with an apparent Mr of about 88,000 and it is composed of light and heavy subunits with Mr of 19,000 and 25,000.

The linkages between the subunits were ruptured in 0.1 % TFA and the liberated subunits were isolated by reverse-phase HPLC (Fig. 9). The isolated, enzymatically inactive fractions, were subjected to SDS-PAGE, which indicated that the first major peak eluting with retention time of 9.3 minutes represented the L-subunit (Mr 19,000) and the second major peak with retention time of 13.8 minutes represented the I-subunit (Mr 25,000), respectively.
DONV Inactivation of Glycoasparaginase

**Fig. 8.** Gradual dissociation of purified glycoasparaginase subunits by SDS under reducing conditions.

The samples were incubated for 15 minutes at 37°C (top) or 50°C (middle) and for 10 minutes at 100°C (bottom) in a buffer containing 2.5% SDS and 5% mercaptoethanol (v/v). The electrophoresis was carried out with the PhastSystem™ according to the manufacturer's instructions using 10-15% gradient gels. The lanes from the gel were sliced into 1 mm sections, which were homogenized in 50 mL of 50 mM Tris-HCl, pH 7.5, containing 0.1% Triton X-100. The enzyme was extracted by incubating the slices in the assay buffer at 4°C for 12 h. The gel fragments were removed by centrifugation and the supernatant was tested for enzyme activity (13). Adjacent lanes containing the molecular weight markers and purified glycoasparaginase were stained with silver in order to localize the proteins on the gel and are shown above the histograms. The arrows indicate the position of molecular weight marker proteins (from left: 94,000, 67,000, 43,000, 30,000, 20,000 and 14,000).

**Fig. 9.** Reverse-phase HPLC of human leukocyte glycoasparaginase.

Chromatogram of the major charge form eluting at 13 min from Mono Q chromatography (Fig. 4E). 30 µg of the enzyme in 50 mM phosphate buffer containing 30% glycerol, 1 mM EDTA and 0.1 mM DTT was injected on a Rechrom C4 column (4.6 x 50 mm) at 20 µL solvent B. The column was eluted with linear gradient of 30% to 90% B in 25 minutes and the flow rate was 1.5 mL/min. Absorbance was monitored at 214 nm. Solvent A was 0.1% TFA in H2O and solvent B was 0.1% TFA in acetonitrile.

**Amino acid composition and N-terminal sequence.** The amino acid composition obtained from 100 µg of purified glycoasparaginase is shown in Table V. The published amino acid compositions of monomeric human (2) and dimeric rat liver enzyme (5) are included for comparison. The amounts of Glu, Gln and Ala were considerably larger in human leukocyte glycoasparaginase than that reported for the monomeric human hepatic enzyme, but the values were close to those of the rat liver enzyme. The methionine content was very low in both human enzymes. The N-terminal sequences of the HPLC-purified subunits (Fig. 9) are shown in Table I. Except the second cycle of the heptad repeat, a single signal was detected in each Edman cycle.

**Table V**

| Amino acid | Human leukocyte (mol%) | Human liver (2) (mol%) | Rat liver (5) (mol%) |
|------------|------------------------|------------------------|---------------------|
| Asp        | 10.9                   | 11.0                   | 6.5                 |
| Thr        | 7.8                    | 8.8                    | 8.8                 |
| Ser        | 10.6                   | 7.5                    | 6.3                 |
| Glu        | 6.8                    | 15.4                   | 7.0                 |
| Gly        | 11.8                   | 4.0                    | 11.8                |
| Ala        | 11.0                   | 5.7                    | 10.6                |
| Cys        | 1.8                    | 1.9                    | 8.0                 |
| Val        | 5.4                    | 5.9                    | 6.8                 |
| Met        | 0.4                    | 0.4                    | 3.3                 |
| His        | 3.4                    | 3.9                    | 3.8                 |
| Leu        | 6.4                    | 8.8                    | 6.3                 |
| Thr        | 2.7                    | 3.4                    | 3.5                 |
| Phe        | 4.4                    | 5.3                    | 3.8                 |
| His        | 2.6                    | 2.3                    | 1.8                 |
| Lys        | 5.0                    | 2.3                    | 4.5                 |
| Arg        | 4.3                    | 5.4                    | 4.0                 |
| Pro        | 4.3                    | 4.8                    | 6.3                 |
| Trp        | n.m.                   | 3.7                    | 1.2                 |

Each leukocyte value represents the mean of two determinations.

n.m., not measured

**Monosaccharide composition.** The monosaccharide analysis indicated that approximately 13% of the weight of the enzyme is carbohydrate and the monosaccharide composition is shown in Table VI. The high molar ratio of mannose indicates that the protein most likely contains both complex-type and high mannose-type N-glycosidic carbohydrate chains.

**Table VI**

| Component sugar | Smol/mg protein | Molar ratio |
|-----------------|-----------------|-------------|
| L-Fucose        | 39              | 0.4         |
| D-Mannose       | 282             | 3.0         |
| D-Galactose     | 170             | 1.3         |
| D-Glucose       | 79              | 0.8         |
| N-Acetyl-D-galactosamine | 14 | 0.1 |
| N-Acetyl-D-glucosamine | 164 | 1.8 |
| N-Acetylmuramic acid | 122 | 13 |

Catalytic properties. The effect of pH on glycoasparaginase activity was studied using different buffers during the enzyme reaction. A broad pH optimum was observed between 7 and 9 (Fig. 10A). The enzyme activity was highest in HCl-100 mM buffers (Fig. 10B). After an incubation for 1 hour at 37°C, 85% of the activity was left (Fig. 10C). The enzyme was rapidly inactivated in higher temperatures. The Km and Vmax of glycoasparaginase for N5,N5-Diacetylglucosamine(N5)-l-asparagine were 110 µM and 34 nmol x 1 x 10⁻⁴ x min⁻¹ (2.2 U/mg), respectively (Fig. 11). The enzyme was inactive toward asparaginase in which the C-monoo group of asparagine was chemically modified to its corresponding N-acetyl or N-Fic derivative or the α-carboxyl group to its corresponding methyl ester derivative.
DONV Inactivation of Glycosasparaginase

Fig. 10. The effect of pH, ionic strength and temperature on human leukocyte glycosasparaginase activity.

A. Dependence of glycosasparaginase activity on hydrogen ion concentration was measured in 50 mM Britton-Robinson's universal buffer ( ), 50 mM Tris-HCl buffer ( ) and 50 mM potassium phosphate buffer ( ). Each data point represents the mean of two separate assays.

B. Effect of ionic strength on the enzyme activity ( Tris-HCl, potassium phosphate). Each data point represents the mean of two assays.

C. Effect of temperature on the enzyme activity. Each data point represents the mean of two separate assays. The purified enzyme was incubated in Tris-HCl buffer, pH 7.5, in the presence of 0.1% bovine serum albumin at 4 - 100°C for 60 min.

Fig. 11. The effect of substrate concentration on human leukocyte glycosasparaginase activity.

A double-reciprocal plot of the same data is shown in the inset. Each data point represents the mean of two separate assays.