Amino Acid Sequence of the Diazooxonorleucine Binding Site of *Acinetobacter* and *Pseudomonas 7A* Glutaminase–Asparaginase Enzymes†

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ABSTRACT: *Acinetobacter* glutaminase-asparaginase was treated with [6-^{14}C]diazo-5-oxonorleucine, reduced with sodium borohydride, and cleaved with cyanogen bromide. Radioactivity was present only in a 96-residue N-terminal peptide which eluted as the second peptide peak on Sephadex G-50. Radioactivity was released with the threonine in position 12 during automatic sequencing of this peptide. The amino acid sequence of a 60-residue N-terminal segment and a 16-residue C-terminal segment of this peptide was determined. *Pseudomonas 7A* glutaminase-asparaginase was treated with [6-^{14}C]diazo-5-oxonorleucine and reduced with sodium borohydride. Radioactivity was released with the threonine in residue 20 during automatic sequencing of the whole enzyme. Analysis of 26 N-terminal residues showed that an 8-residue segment containing the radioactive threonine was identical with that in *Acinetobacter* glutaminase-asparaginase and in *Escherichia coli* asparaginase. Additional identical residues were noted in the N-terminal regions of these enzymes.

L-Asparaginase from *E. coli* has antitumor activity toward transplantable mouse tumors and human acute lymphocytic leukemia (for reviews, see Adamson and Fabro, 1968; Wriston and Yellin, 1973). The partial sequence of this enzyme from *E. coli* B (Gumprecht and Wriston, 1973) and the complete sequence from *E. coli* A 1-3 have been reported (Maita et al., 1974). The segment of the enzyme which covalently binds the alternate substrate 5-diazo-4-oxo-L-norvaline has a sequence of Arg-Pro-Ser-Thr-Ser-Met (Peterson et al., 1977).

A glutaminase-asparaginase from *Acinetobacter glutaminasifacans* (ATCC 27197) has been purified and characterized (Roberts et al., 1972; Holcenberg et al., 1975). It has a different spectrum of antitumor activity toward mouse transplantable tumors and human leukemic cells in vitro than *E. coli* asparaginase (Holcenberg et al., 1973; Schmid and Roberts, 1974; Shrek et al., 1973; Holcenberg and Roberts, 1977). Recently, Roberts has described the purification of a glutaminase-asparaginase from *Pseudomonas 7A*. This enzyme has a higher ratio of glutaminase to asparaginase activity, a much longer plasma half-life and greater antitumor activity than *Acinetobacter* glutaminase-asparaginase (Roberts, 1976). These glutaminase-asparaginase enzymes and *E. coli* asparaginase have similar catalytic activity toward asparagine. Each enzyme is composed of four subunits with a molecular weight of 33 000–36 000 (Holcenberg et al., 1976). On the other hand, the *Acinetobacter* and *Pseudomonas 7A* enzymes have a K_m for glutamine over 600 times lower and a V_{max} for glutamine over 40 times higher than *E. coli* asparaginase (Roberts et al., 1972; Roberts, 1976). These catalytic differences led us to investigate the differences in the catalytic site of these enzymes.

Preliminary experiments showed that the *Acinetobacter* enzyme was irreversibly inhibited by methanesulfonyl fluoride, an inhibitor of serine proteases. This inhibition suggested that serine or threonine hydroxyl groups are involved in the binding of substrates to both the *Acinetobacter* and *E. coli* enzymes. In contrast, cysteine is the binding group for the glutamyl transferase enzymes that have been studied (Hartman, 1973).

5-Diazo-4-oxo-L-norvaline does not readily bind to the glutaminase-asparaginases, but the next larger homologue 6-diazo-5-oxo-L-norleucine (DON)[1] binds irreversibly. Therefore, radioactive DON was prepared and bound to the enzymes. This paper describes the partial sequence of the N-terminal CNBr peptide and the binding of [^{14}C]DON to a threonine at residue 12 of *Acinetobacter* glutaminase-asparaginase. Twenty-six N-terminal residues of *Pseudomonas 7A* glutaminase-asparaginase were sequenced. [^{14}C]DON binds to a threonine at residue 20. An 8-residue segment containing these threonines is identical in both glutaminases and in *E. coli* asparaginase. A preliminary abstract of this work has appeared (Holcenberg and Ericsson, 1976).

Experimental Section

Materials. Acinetobacter glutaminasificans (ATCC 27197) was cultured and supplied as a frozen paste by Grain Processing Co. The glutaminase-asparaginase was purified to homogeneity as described (Roberts et al., 1972; Holcenberg et al., 1975). Glutaminase-asparaginase from *Pseudomonas 7A* was also prepared in homogeneous form as previously described (Roberts, 1976). *E. coli* asparaginase (lot CA 971) labeled with [5-^{14}C]-5-diazo-4-oxo-L-norvaline was a gift from Dr. Robert Handschumacher, Yale University School of Medicine. All enzyme preparations had a specific activity greater than 120 IU/mg of protein before lyophilization. Enzyme activity was assayed by ammonia formation from asparagine (Roberts et al., 1972).

Trypsin (L-1-tosylamido-2-phenylethyl chloromethyl ketone treated) was obtained from Worthington Biochemical Corp. Cyanogen bromide and glutamate were obtained from Cal-

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[1] Abbreviations used: DON, 6-diazo-5-oxo-L-norleucine; Pth, phenylthiohydantoin.

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biochem, Sephadexes from Pharmacia Fine Chemicals, and Bio-Rex 70 from Bio-Rad. Trifluoroacetic anhydride, dicyclohexylamine, thionyl chloride, and Diazald were from Aldrich, molecular sieve 3A was from Fischer Scientific, Norite A from J. T. Baker, sodium borohydride from Ventron Corp., Celite from Johns Manville, 2-(2-ethoxyethoxy)ethanol from Matheson Coleman and Bell, and cellulose thin-layer strips from Eastman. *N*-Methyl[^{14}C]-*N*-nitroso-*p*-toluenesulfonamide (6.95 mCi/mmol) was purchased from New England Nuclear.

Protein concentration was determined by absorbance at 280 nm or by alkaline hydrolysis and ninhydrin reaction.

Synthesis of [6-^{14}C]-6-Diazo-5-oxo-L-norleucine. *N*-Trifluoroacetyl-L-glutamic anhydride was prepared as described (Weyland and Reiher, 1955) and hydrolyzed with a 15-fold excess of anhydrous methanol. The α-methyl ester was precipitated from a benzene solution by addition of a 1.2-fold excess of dicyclohexylamine. It was washed with benzene and petroleum ether, and dried (mp 188 °C). The acid chloride was formed as described (Weyland and Geiger, 1957) and purified by sublimation at 70–80 °C in a vacuum of about 50 μm.

[^{14}C]Diazomethane was generated by slight modification of the method previously described (Hartman, 1963). Fifty milligrams of Diazald was added to 1.5 mg (50 μCi) of *N*-methyl[^{14}C]-*N*-nitroso-*p*-toluenesulfonamide (total of 240 μmol) in 2 mL of dry ether in a 10-mL pear-shaped flask fitted with a distilling adapter (Kontes K275070), a 10-mL receiver flask and tygon tubing submersed in a test tube containing 2 mL of dry ether. Both the receiver and test tube were cooled in a dry ice–cellsolve bath. Diazomethane was generated by addition of 2 mL of octyl alcohol containing 20 mg of sodium metal and distilled at 65 °C with a slow stream of dry nitrogen. After 10 min, 1 mL of ether was added and distillation was continued for an additional 15 min. Freshly sublimated *N*-trifluoroacetyl-L-glutamic acid-1 methyl ester-5 chloride (20 mg, 81 μmol) was dissolved in the 2 mL of ether in the test tube and added to the diazomethane in the receiver flask. The flask was stoppered and incubated in the dark for 30 min at room temperature. The yellow diazomethane color gradually disappeared during incubation. The ether was evaporated with a stream of dry nitrogen. The blocked [^{14}C]diazooxonorleucine was suspended in 0.5 mL of methanol and 1.7 mL of 0.2 N NaOH at −15 °C and incubated at that temperature for 48 h (Handschumacher et al., 1968). The solution was then diluted with 5 mL of water and adjusted to pH 6 with acetic acid. The material was adsorbed on a 0.6 × 12 cm column of a mixture of acid-washed Norite and Celite (1:1 by weight) and eluted with 1% acetone. The peak of [6-^{14}C]-6-diazo-5-oxo-L-norleucine was pooled and lyophilized. The overall yield was 22.4 μmol with a specific activity of 0.19 μCi/μmol (theoretical sp act. 0.21 μCi/μmol). This material was stable for several months in a desiccator at −20 °C. Only one ultraviolet spot was noted after chromatography in 90% ethanol on cellulose thin-layer sheets or Whatman 3MM paper. About 85% of the radioactivity migrated with this spot. The material eluted from this spot had the same specific activity as the original material, indicating that the loss of radioactivity was caused by reaction of the [^{14}C]DON with the chromatography material.

Labeling of Acinetobacter Glutaminase-Asparaginase. In a typical experiment, *Acinetobacter* glutaminase-asparaginase (54 mg, 7300 IU of glutaminase, 1.5 μmol of subunits) was dissolved in 1.5 mL distilled water. [^{14}C]DON (2.3 μmol) in 0.5 mL of water was added and the solution incubated in the dark for 30 min at room temperature. After incubation, only 5% of the enzyme activity remained. The solution was chromatographed on a 0.9 × 60 cm column of Sephadex G-25 (fine) equilibrated and developed in 0.01 M sodium phosphate buffer, pH 7.2. Radioactive enzyme eluted in the void volume and separated well from the radioactive salt peak. The labeled enzyme peak was pooled and contained 35 mg of protein, and 0.15 μCi (sp act. 0.15 μCi per mol of subunit or 0.8 mol of [^{14}C]DON per mol of subunit). The ultraviolet absorbance of the material in the salt peak indicated that two-thirds of the DON not bound to the enzyme had been degraded during the incubation.

The product of enzyme and [^{14}C]DON was unstable in acid. Therefore, within several hours, the labeled enzyme was diluted to 6 mL, adjusted to pH 6.8 with formic acid, and reduced by addition of 5 mg of solid sodium borohydride. pH was maintained at 6–7 with formic acid. This reduced radioactive enzyme was usually lyophilized and treated with cyanogen bromide. About half of the radioactivity appeared in the salt peak after this treatment. After reduction of one preparation, a small aliquot of the radioactive enzyme was chromatographed on a 1 × 40 cm Sephadex G-25 (fine) column in 9% formic acid. Fifty-seven percent of the radioactivity appeared in the salt peak. In another experiment, the entire sample of 39 mg of reduced enzyme labeled with [^{14}C]DON was lyophilized, dissolved in 3 mL of 60% formic acid and chromatographed on a 0.9 × 58 cm column of Sephadex G-25 (fine) previously equilibrated with 9% formic acid. The peak in the void volume contained 61% of the radioactivity and 82% of the protein. No further loss of radioactivity occurred on subsequent reaction with cyanogen bromide. Thus, a small radioactive peptide was not produced by reaction with cyanogen bromide.

Cyanogen Bromide Cleavage. After reaction with [^{14}C]-DON and reduction with sodium borohydride, the lyophilized enzyme was dissolved in 70% formic acid at a final concentration of 5 mg of protein per mL. Approximately 10 mg/mL of cyanogen bromide was added, and the mixture was incubated in the dark for 24 h at room temperature. The peptide mixture was diluted approximately tenfold, shell frozen, and lyophilized.

Column Chromatography. The peptides from cyanogen bromide cleavage were chromatographed on a 2.5 × 100 cm column of Sephadex G-75 or G-50 (fine) in 9% formic acid at a flow rate of about 10 mL/h. Column eluates were monitored for radioactivity, ultraviolet absorbance, and protein by hydrolysis in base and reaction with ninhydrin. Fractions were pooled, shell frozen, and lyophilized. Some fractions were further purified by rechromatography in 9% formic acid on the same Sephadex G-50 column or on a 1.5 × 100 cm column of Sephadex G-25 (superfine). Bio-Rex 70 (greater than 400 mesh) was equilibrated with 9% formic acid. Tryptic peptides were chromatographed on a 1 × 30 cm column of this resin by elution with a 200-mL gradient of 9% and 90% formic acid. The radioactive peptide eluted before the other peptides.

Trypsin Digestion. Three-tenths of a milligram of radioactive peptide and 10 mg of unlabeled CNBr peptide CB2 were dissolved in 5 mL of water in a 10-mL jacketed beaker and adjusted to pH 8 with 0.1 N NaOH at 37 °C. The solution became cloudy. Solid trypsin (0.5 mg, 91 units) was added and the pH maintained at 8 by automatic titration with 4.4 μmol of 0.1 N NaOH. This titration represents approximately six cleavages per mol of peptide. The solution partially cleared during 3 h of incubation at 37 °C. The material was shell frozen and lyophilized. Eighty percent of the radioactivity in the lyophilized powder dissolved in 9% formic acid while residual material dissolved in 30–50% formic acid. These peptides were purified by gel filtration on Sephadex G-25 (superfine).

Labeling of Pseudomonas 7A Glutaminase-Asparaginase. Enzyme (15 mg of protein, 1150 IU of glutaminase, 0.4 μmol

of subunits) was dissolved in 1 mL of 0.01 M sodium phosphate buffer, pH 7.4, and incubated for 1 h in the dark at room temperature with 1 μmol of [^{14}C]DON. The enzyme solution still had 68% of the original enzyme activity. An additional 0.9 μmol of [^{14}C]DON was added. After 30 min, only 24% of the enzyme activity remained. The enzyme was chromatographed on a Sephadex G-25 column as described above. The protein peak had 9 mg of protein, 200 IU, and 0.17 μCi of [^{14}C]DON per μmol of subunit (0.9 mol of DON bound/mol of subunit). The radioactive peak that eluted with the salt peak had no UV absorbance, indicating that the unbound DON was hydrolyzed. The radioactive enzyme was reduced with 5 mg of sodium borohydride, lyophilized, and chromatographed on a 1.5 × 100 cm column of Sephadex G-25 (superfine) in 9% formic acid. The single protein peak was lyophilized and analyzed for amino acid sequence. Native *Pseudomonas 7A* glutaminase-asparaginase was dialyzed in 5% acetic acid, lyophilized, and analyzed for sequence.

Amino Acid Analysis. Proteins and peptides were hydrolyzed 24–96 h in constant-boiling HCl in sealed tubes at 110 °C. No tryptophan was detected in the radioactive CNBr fragment by hydrolysis with methanesulfonic acid (Penke et al., 1974) or by alkaline hydrolysis (Hugli and Moore, 1972). Analyses were performed by AAA Laboratories, Mercer Island, on a Durrum D500 analyzer.

Sequenator Analyses. Automated Edman degradations were performed by L. Ericsson with a Beckman sequencer Model 890B according to the method of Edman and Begg (1967) as modified by Hermodson et al. (1972). A peptide program according to Crewther and Inglis (1975), program 2, was used where indicated except that dimethylbenzylamine was substituted for quadrol and no solvents containing ethyl acetate were used for extraction. Solvents used in the sequenator were Sequenol grade from Pierce and Burdick-Jackson solvents. Pth-amino acids were analyzed as trimethylsilyl derivatives by gas chromatography by comparison with known standards. With radioactive peptides, aliquots of the aqueous layer were removed for counting in a liquid scintillation counter. Histidine and arginine were identified in the aqueous extracts by diazotized *p*-anisidine and phenanthrenequinone spot tests, respectively. The identities of some amino acid derivatives were verified by high pressure liquid chromatography. Samples containing the phenylthiohydantoin-amino acids were dissolved in methanol and an aliquot injected onto a Waters Associates μ-Bondapak C-18 column. Chromatography involved using a linear gradient from 14 to 56% methanol in aqueous buffer (Bridgen et al., 1976) pumped at 2.2 mL/min and a Waters Associates high pressure liquid chromatograph (Model 6000A).

Results

Inhibitor Studies. Acinetobacter glutaminase-asparaginase was incubated with inhibitors of glutamyl transferase enzymes, serine proteases, sulfhydryl enzymes, and metal chelators to obtain clues about the nature of the catalytic site. Previously, *p*-mercuribenzoate was shown to neither inhibit nor react with the enzyme (Roberts et al., 1972). The absence of sulfhydryl groups was confirmed when incubation of 1.0–2.5 mg/mL enzyme with 0.1 μmol/mL of 5,5′-dithiobis(2-nitrobenzoic acid) in the presence or absence of 7 M urea produced no significant change in absorbance at 412 nM (Ellman, 1959). Enzyme activity was unchanged after incubation of 1 mg/mL protein at 0 °C for 2 h in a solution containing 0.05 M sodium phosphate buffer, pH 7.5, and 1 mM ethylenediaminetetraacetic acid or 1 and 10 mM 1,10-phenanthroline.

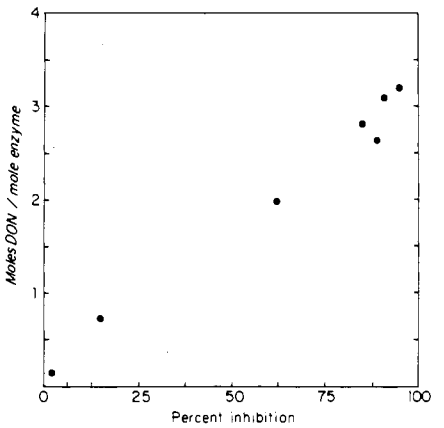

FIGURE 1: DON binding to *Acinetobacter* glutaminase-asparaginase. Enzyme was incubated with 0.2 to 3.7 mol of [^{14}C]DON/mol of enzyme as described in the text. After gel filtration, residual enzyme activity and radioactivity were measured.

Incubation of 1 mg/mL of the *Acinetobacter* enzyme for 2–4 h at room temperature with 1 M methanesulfonyl fluoride in 0.08 M sodium phosphate buffer, pH 7.5, and 8% dimethylformamide resulted in 75% inhibition of enzyme activity. In contrast, enzyme activity was unchanged after incubation of the same enzyme and buffer concentrations at room temperature for up to 23 h in 10 mM diisopropyl phosphorofluoridate and 8% isopropyl alcohol or 1 mM phenylmethanesulfonyl fluoride and 2% isopropyl alcohol. The solvents alone did not inhibit the enzyme.

A 1 mg/mL solution of *Acinetobacter* glutaminase-asparaginase containing 0.005% methylene blue or 0.01% rose bengal was exposed at 25 °C to light from a 100-W tungsten lamp at a distance of 15 cm. After 15 min, enzyme activity decreased to 13 and 35%, respectively, with methylene blue and rose bengal. No appreciable activity was lost without light or dye. Activity was not restored by 15-min incubations in 10 mM 2-mercaptoethanol or 1 mM dithiothreitol. Incubations for 15 min in light and methylene blue inactivated *Pseudomonas 7A* glutaminase-asparaginase to 12% of control but did not appreciably inactivate *E. coli* asparaginase.

The *Acinetobacter* enzyme was rapidly and irreversibly inactivated by 0.1–1 mM DON. After removal of unreacted DON by gel filtration or ammonium sulfate precipitation, the enzyme had the same ultraviolet absorbance as the native enzyme.

The stoichiometry of DON binding to *Acinetobacter* glutaminase-asparaginase is shown in Figure 1. Enzyme was incubated at room temperature for 30 min with 0.2 to 3.7 mol of [^{14}C]DON/mol of enzyme. Enzyme and excess DON were separated on a 0.9 × 40 cm column of Sephadex G-25 (fine) and the enzyme was tested for residual specific enzyme activity and radioactivity. Inhibition of enzyme specific activity increased with increasing amounts of [^{14}C]DON bound. Nearly complete inhibition was seen when 3.2 mol of DON was bound per mol of enzyme or 0.8 mol of DON per mol of 35 000-dalton subunit (Holcenberg et al., 1975). Most of the radioactivity bound to the enzyme was removed by incubation in 50% formic acid for 1 h at room temperature.

In a separate experiment 1 mg of enzyme was incubated at room temperature for 30 min with 88 nmol of [^{14}C]DON with and without 2 μmol of L-asparagine and then chromatographed on a Sephadex G-25 column. The enzyme incubated without asparagine had only 6% of the original specific enzyme activity and had 2.7 mol of DON bound per mol of enzyme. The enzyme incubated with asparagine had not lost enzyme activity

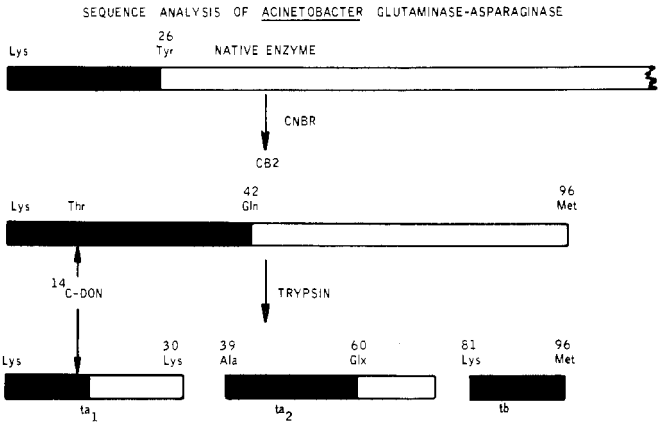

FIGURE 2: Flow diagram of the sequence analysis of *Acinetobacter* glutaminase-asparaginase. Shaded areas indicate the sequenced segments.

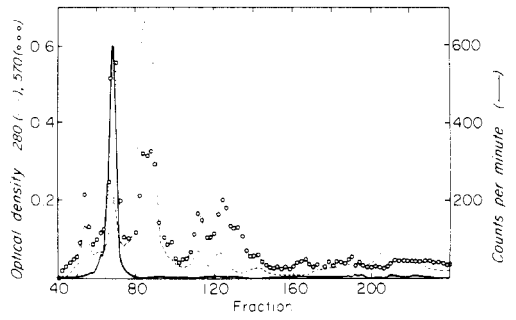

FIGURE 3: Fractionation of CNBr fragments of ^{14}C-labeled *Acinetobacter* glutaminase-asparaginase on a column of Sephadex G-50 (fine) (2.5 × 100 cm) equilibrated with 9% formic acid: rate, 8 mL/h; 3-mL fractions collected; 90% of the radioactivity added to the column was recovered in tubes 61–75; (○) optical density at 570 nm of fractions after base hydrolysis and reaction with ninhydrin; (dashed line) optical density at 280 nm; (solid line) radioactivity in 50 μL.

and had less than 0.3 mol of DON bound per mol of enzyme.

Sequence Analysis of Acinetobacter Glutaminase-Asparaginase. Figure 2 shows the scheme used to analyze the amino acid sequence. The N-terminal sequence of the whole protein was determined for 18 and 26 amino acids in two experiments. Assuming a 95% yield at each turn, the content of valine at turn 4 indicated that 88% of the N-terminal amino groups are free. A similar analysis of a succinylated preparation of enzyme revealed only 6% free N-terminal amino acids. The enzyme was incubated with [^{14}C]DON, reduced with sodium borohydride, chromatographed on Sephadex G-25, reacted with cyanogen bromide, lyophilized, and then applied to a Sephadex G-50 column. Figure 3 illustrates the elution pattern on Sephadex G-50 from a typical cyanogen bromide reaction. Five protein peaks were noted (circles and dashed line). Only the second peak had any appreciable radioactivity (solid line). The material in this peak was rechromatographed on the same column. The amino acid analysis of this peptide, CB2, from two separate experiments is presented in Table I. The analysis indicates that the peptide has approximately 96 amino acids with 6 lysine and 1 arginine residues. CB2 was identified as the N terminus by sequence analysis of 42 residues (Figure 1).

Figure 4 shows the chromatographic pattern on Sephadex G-25 (superfine) of a typical tryptic digest of CB2. The radioactivity was present in the first pool (tubes 19–30). Sequential analysis showed that this fraction contained two peptides. The radioactive peptide, ta_1, was soluble in 9% formic acid and eluted from a Bio-Rex 70 column at low formic acid

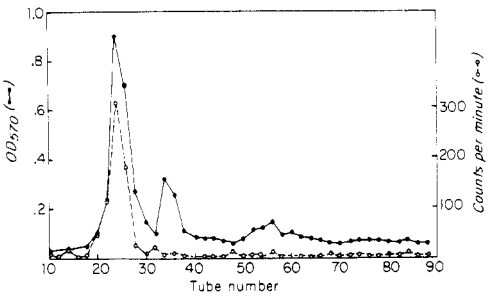

FIGURE 4: Fractionation of tryptic digest of ^{14}C-labeled CB2 recovered and purified from Sephadex G-50 (Figure 3) on a column of Sephadex G-25 (superfine) (1.5 × 100 cm) equilibrated with 9% formic acid: rate, 8.6 mL/h; 1-mL fractions collected; 88% of the radioactivity added to the column was recovered in tubes 19–30; (●) optical density at 570 nm of 50-μL aliquots of fractions after base hydrolysis and reaction with ninhydrin; (○) radioactivity of 20-μL aliquots of the fractions.

TABLE I: Amino Acid Composition of CNBr Peptide II.[a]

Amino Acid	Residues/subunit
Ala	13.00 (13)
Arg	1.38 (1)
Asp	12.38 (12)
Glu	6.46 (6)
Gly	7.80 (8)
His	1.50 (1)
Ile	6.69 (7)
Leu	7.36 (7)
Lys	5.95 (6)
Phe	0.81 (1)
Pro	2.89 (3)
Ser	8.25 (8)
Thr	8.11 (8)
Tyr	1.50 (1)
Val	12.67 (13)
Hse	0.77 (1)
	96

[a] Amino acid analyses were performed on 6 N HCl hydrolyses of one preparation for 24, 48, 72, and 96 h. Composition was calculated from the average of these time points for all amino acids except Ile, Leu, Val, Hse, Ser, and Thr. Standard deviations were less than 3.3%. Maximum values (96 h) were used for Ile, Leu, and Val. Ser and Thr values were calculated by extrapolation to zero time by linear least-square equations. No tryptophan was found after 48 h of alkaline hydrolysis at 135 °C (Hugli and Moore, 1972). Values are best fit to integers by the method of DeLaage (1968) using no weighting factor.

concentration. This peptide was sequenced through 14 residues and identified as the N terminus (Figure 1).

The other peptide, ta_2, eluted from Bio-Rex 70 at higher formic acid concentration and was soluble in 30–40% formic acid. These properties were used to separate the peptides which were then rechromatographed on the Sephadex G-25 column. Two preparations of ta_2 were sequenced for 21 and 22 residues. ta_2 was identified as beginning at residue 39 by a 4-residue overlap with the sequence of CB2 (Figure 1). These preparations of ta_2 were not completely free of contaminating ta_1. Quantitative comparison of the major and minor sequences indicated that the preparations contained about 20% ta_1 and 80% ta_2.

The second peak, tb, from the tryptic digest (tubes 32–38, Figure 4) was lyophilized and rechromatographed on the same column. This peptide had 16 residues by amino acid analysis and was identified as the C terminus of CB2 by the presence of homoserine. Thirteen of the 16 residues were identified by sequence analysis.

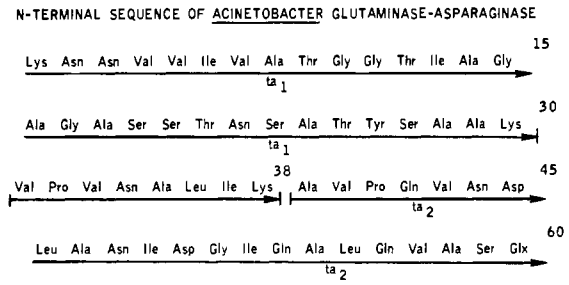

FIGURE 5: Sequence analysis of N-terminal sequence of *Acinetobacter* glutaminase-asparaginase.

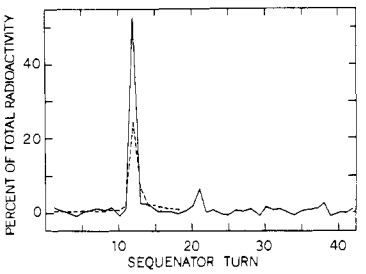

FIGURE 6: Radioactivity released at each turn of automatic Edman degradation of CNBr fragment CB2 (solid line) and tryptic peptide ta_1 (dashed line). Radioactivity is presented as the percent of the total radioactivity of the peptide applied to the sequenator.

The third peak, tc (tubes 49–68), represented a small peptide or peptides and was not purified further.

Figure 5 represents the sequence analysis of the first 60 amino acid residues of CB2.

Identification of Threonine Bound to [^{14}C]*DON.* During sequence analysis of the cyanogen bromide peptide CB2 and tryptic peptide, ta_1, a portion of the extract from each turn was counted for radioactivity. The radioactivity released at each turn is presented in Figure 6. In both sequence analyses, most of the radioactivity was released with the threonine in residue 12. A small portion was released with residues 13 and 14. Assuming a 95% yield at each turn, in the two experiments residues 12 and 13 contained 97% and 60% of the radioactivity in the peptides. The gas chromatogram of the extract from turn 12 showed some unidentified peaks which probably represent the threonine-DON reaction product.

Sequence Analysis of Pseudomonas 7A Glutaminase-Asparaginase. The whole enzyme after reaction with [^{14}C]-DON and reduction with $NaBH_4$ was analyzed for radioactivity and amino acid sequence. This preparation had 7.3 mg of protein and 57 000 dpm. No appreciable radioactivity was noted in extracts from residues 2–14 and only 240 dpm was released with turn 1. Table II presents the radioactivity released with residues 15–23. Residue 20 had approximately 10% of the counts in the whole enzyme. Assuming a 95% yield at each turn, approximately 47% of the radioactivity was released in steps 20 and 21.

The sequence of the N-terminal 26 amino acids of untreated enzyme was as follows: Lys-Glu-Val-Glu-Asn-Gln-His-Lys-Leu-Ala-Asn-Arg-Val-Ile-Leu-Ala-Thr-Gly-Gly-Thr-Ile-Ala-Gly-Ala-Gly-Ala.

Assuming a 95% yield at each turn, the content of valine at turn 3 indicated that 73% of the N-terminal amino groups are free.

Comparison of the N-Terminal Sequences of the Glutaminase–Asparaginase Enzymes and E. coli Asparaginase. Figure 7 presents the sequence analysis of the *Acinetobacter* and *Pseudomonas 7A* enzymes and compares it with the

TABLE II: Radioactivity Released during Sequencing of *Pseudomonas 7A* Glutaminase-Asparaginase.[a]

Residue	Amino acid	dpm
15	Leu	65
16	Ala	123
17	Thr	33
18	Gly	56
19	Gly	224
20	Thr	7787
21	Ile	1791
22	Ala	452
23	Gly	90

[a] [^{14}C]DON was bound to enzyme and reduced with $NaBH_4$. An aliquot of the extract from each turn during automatic sequencing of 7.3 mg of protein (57 000 dpm) was measured in a liquid scintillation counter. dpm is the calculated radioactivity over background (52 dpm) for the entire extract. Efficiency of counting was 79%.

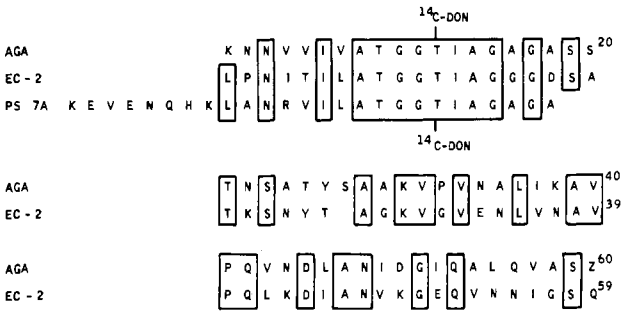

FIGURE 7: Comparison of the N-terminal sequence of *Acinetobacter* and *Pseudomonas 7A* glutaminase-asparaginase and *E. coli* asparaginase. One-letter notations for amino acids are used to conserve space. Identical residues are in boxes. The binding site of [^{14}C]DON to a threonine residue is shown. AGA, EC2, and Ps 7A represent *Acinetobacter* glutaminase-asparaginase, *E. coli* asparaginase, and *Pseudomonas 7A* glutaminase-asparaginase.

published sequence of *E. coli* asparaginase (Maita et al., 1974). Identical residues are in boxes. An 8 amino acid segment is identical in the three enzymes. This contains the threonine residue that binds to [^{14}C]DON in the *Pseudomonas 7A* and *Acinetobacter* enzymes. *E. coli* asparaginase has an identical sequence. Nevertheless, Peterson et al. (1977) showed that [5-^{14}C]-5-diazo-4-oxonorvaline was bound to a serine or threonine residues at positions 117–119 of the *E. coli* enzyme. We treated a portion of Peterson's preparation of *E. coli* asparaginase previously bound to [^{14}C]diazooxonorvaline with $NaBH_4$ and determined the N-terminal amino acid sequence and the radioactivity released with each Edman degradation. The sequence corresponded to that previously published, but no radioactivity was released with residues 8 through 15.

Pseudomonas 7A glutaminase-asparaginase appears to have an 8 amino acid segment at the N terminus not presented in the other enzymes.

Half of the 60 residues shown are identical in the sequences of *Acinetobacter* glutaminase-asparaginase and *E. coli* asparaginase. In addition, 10 of the 16 residues in tb are identical with a segment between residues 77 and 92 of *E. coli* asparaginase. The sequence of tb is as follows

Lys-Pro-Ser-Val-Asn-Gly-Val-
Val-Ile-Thr-His-Gly-(Thr)-Asp-(Thr,Met)

The identical residues in both enzymes are in boxes.

Discussion

This paper reports the covalent binding of [6-^{14}C]DON to a threonine residue in the N-terminal region of glutaminase–asparaginase enzymes from *Acinetobacter glutaminasificans* and *Pseudomonas 7A*. Furthermore, the threonine is part of an identical 8 amino acid segment in both enzymes. The [6-^{14}C]DON–threonine adduct has not been identified. Nevertheless, the acid lability and absence of UV absorbance of the enzyme–DON product suggest that the diazo moiety has been removed with the formation of a keto ether between the hydroxyl group of threonine and the [^{14}C]methylene group of 5-oxonorleucine. Reduction of the enzyme with $NaBH_4$ was required to stabilize the group to the acid conditions used in CNBr cleavage.

A similar adduct has been suggested as the product of a hydroxyl group of threonine or serine in *E. coli* asparaginase and 5-diazo-4-oxonorvaline (Peterson et al., 1977). This reaction requires a nucleophilic attack by the enzyme hydroxyl group on the methylene group of the inhibitor. This type of attack is supported by the identification of 5-hydroxy-4-oxonorvaline and nitrogen as the products of hydrolysis of 5-diazo-4-oxonorvaline by *E.* coli asparaginase (Jackson and Handschumacher, 1970). Hartman (1968) reported that glutaminase A from *E. coli* reacted with [6-^{14}C]DON in two ways. Most of the [^{14}C]DON was hydrolyzed to glutamic acid and [^{14}C]methanol. This hydrolysis probably followed a nucleophilic attack by the enzyme on the carbonyl group of DON. The remainder of the [^{14}C]DON progressively covalently bound to the enzyme and inactivated it. The amino acid residue that binds [^{14}C]DON has not been identified but is probably not a cysteine moiety since sulfhydryl reagents like iodoacetate and *N*-ethylmaleimide did not inhibit the enzyme (Hartman, 1968).

The following evidence suggests that the DON binding site on *Acinetobacter* and *Pseudomonas 7A* glutaminase–asparaginase enzymes is also part of the catalytic site for glutamine and asparagine: (1) DON is hydrolyzed by both enzymes under the reaction conditions used for binding; and (2) asparagine can block both the binding and enzyme inhibition of DON with the *Acinetobacter* enzyme. The *Acinetobacter* enzyme catalyzes the formation of hydroxamates from glutamine and asparagine (Roberts et al., 1972). An acyl derivative between the carbonyl group of the substrates and the threonine hydroxyl could be an intermediate in this hydroxamate formation.

The serine proteases like chymotrypsin, trypsin, and subtilisin also utilize a hydroxyl group for catalysis of peptide cleavage and esterase activity. The attack by the hydroxyl group of the serine proteases is promoted by a histidine group located at another portion of the primary sequence. The *Acinetobacter* enzyme resembles these proteases in its inhibition by methanesulfonyl fluoride, its rapid photoinactivation, and a pH dependence curve that resembles the ionization of imidazole (Roberts et al., 1972). The latter properties suggest cooperation of a histidine residue in the catalytic mechanism. In contrast, *Acinetobacter* glutaminase is not inactivated by the protease inhibitors, diisopropyl phosphorofluoridate, and phenylmethanesulfonyl fluoride. Serine esterases exhibit great differences in reaction rates with these inhibitors (Gold, 1967). Nevertheless, inhibition by only methanesulfonyl fluoride appears to be unique. Methanesulfonyl fluoride is more soluble in aqueous solutions and can be used in much higher concentrations than the other inhibitors. It appears to have much less specificity toward trypsin in comparison with trypsinogen than diisopropyl phosphorofluoridate (Morgan et al., 1974). The smaller reactive site in the zymogen may not accommodate the larger inhibitor as well as methanesulfonyl fluoride. Perhaps only this smaller inhibitor can fit in the active site of the *Acinetobacter* enzyme. Further experiments are planned to identify the products of photoinactivation and compare the amino acid sequences with that of the serine proteases.

The following glutamyl transferase enzymes have been shown to have a reactive cysteine at the catalytic site by binding studies with DON or related inhibitors or by specific reaction with iodoacetate: the phosphoribosyl transferase component of anthranilate synthetase in *Salmonella typhimurium* (Nagano et al., 1970), component II of anthranilate synthetase of *Pseudomonas putida* (Goto et al., 1976), glutamate synthetase (Mantsala and Zalkin, 1976), xanthine 5′-monophosphate–amidotransferase (Patel et al., 1977), cytidine triphosphate synthetase (Long et al., 1970), formylglycinamide ribonucleotide amidotransferase (Dawid et al., 1972), and carbamoyl phosphate synthetase (Pinkus and Meister, 1972). These enzymes catalyze the hydrolysis of glutamine and the transfer of ammonia to an acceptor. They are complex proteins with multiple subunits. After blockade of the glutamine site with inhibitors like DON, many of these amidotransferase enzymes can still function with ammonium salts. It is intriguing to postulate that all the amidotransferases utilize a sulfhydryl group for nucleophilic attack on glutamine while the purely hydrolytic glutaminases and asparaginases utilize a hydroxyl group. *Acinetobacter* and *Pseudomonas 7A* glutaminase–asparaginase have no cysteine residues. *E. coli* asparaginase has internal cystine residues which are not required for catalysis (Todokoro et al., 1975). It remains to be seen whether glutaminase or asparaginase enzymes with free sulfhydryl groups catalyze the hydrolysis with a hydroxyl group. Recently, Tate (1977) described the binding of [^{14}C]DON to glutamyl transpeptidase. This enzyme transfers the γ-glutamyl moiety of glutathione to amino acids and, therefore, may utilize an entirely different group for binding of the DON or γ-glutamyl moiety.

E. coli asparaginase has an 8 amino acid residue segment identical with one containing the reactive threonine in *Acinetobacter* and *Pseudomonas 7A* glutaminase–asparaginase (Figure 7). Nevertheless, *E. coli* asparaginase does not bind 5-diazo-4-oxonorvaline to this region but to a threonine or serine residue at residue 117–119 (Peterson et al., 1977; Maita et al., 1974). The N-terminal sequences of *E. coli* asparaginase and *Acinetobacter* glutaminase–asparaginase show that about 50% of the residues are identical. Thus, the difference in the hydroxyl groups involved in binding is surprising. There are many possible explanations. Perhaps, the threonine in the N-terminal segment and hydroxyl group at positions 117–119 act cooperatively as part of the active site of all three enzymes. The smaller analogue, 5-diazo-4-oxonorvaline, may react preferentially with the latter site. *E. coli* asparaginase hydrolyzes 5-diazo-4-oxonorvaline at a rapid rate and readily binds the compound only in high concentration of dimethyl sulfoxide (Lachman and Handschumacher, 1976). The *E. coli* enzyme used in our experiments was reacted in 50% dimethyl sulfoxide. This solvent may have changed the binding from threonine-12 to the hydroxyl group at residues 117–119. Finally, *E. coli* asparaginase is a poor catalyst for hydrolysis of glutamine and is not inhibited by DON. Perhaps, this variation in specificity is produced by use of two different active sites or different part of the same active site. Solution of this problem will require further sequencing of the *Acinetobacter* and *Pseudomonas 7A* enzymes, evaluation of the site of 5-diazo-4-oxonorvaline binding to *E. coli* asparaginase in the absence of dimethyl sulfoxide, and eventually x-ray crystallographic comparison of these enzymes (Wlodawer et al., 1977).

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