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Glucosamine Synthetase from *Escherichia coli*: Purification, Properties, and Glutamine-Utilizing Site Location

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ABSTRACT: L-Glutamine:D-fructose-6-phosphate amidotransferase (glucosamine synthetase) has been purified to homogeneity from *Escherichia coli*. A subunit molecular weight of 70 800 was estimated by gel electrophoresis in sodium dodecyl sulfate. Pure glucosamine synthetase did not exhibit detectable NH_3-dependent activity and did not catalyze the reverse reaction, as reported for more impure preparations [Gosh, S., Blumenthal, H. J., Davidson, E., & Roseman, S. (1960) *J. Biol. Chem. 235*, 1265]. The enzyme has a K_m of 2 mM for fructose 6-phosphate, a K_m of 0.4 mM for glutamine, and a turnover number of 1140 min^{-1}. The amino-terminal sequence confirmed the identification of residues 2–26 of the translated *E. coli glmS* sequence [Walker, J. E., Gay, J., Saraste, M., & Eberle, N. (1984) *Biochem. J. 224*, 799]. Methionine-1 is therefore removed by processing in vivo, leaving cysteine as the NH_2-terminal residue. The enzyme was inactivated by the glutamine analogue 6-diazo-5-oxo-L-norleucine (DON) and by iodoacetamide. Glucosamine synthetase exhibited half-of-the-sites reactivity when incubated with DON in the absence of fructose 6-phosphate. In its presence, inactivation with [6-^{14}C]DON was accompanied by incorporation of 1 equiv of inhibitor per enzyme subunit. From this behavior, a dimeric structure was tentatively assigned to the native enzyme. The site of reaction with DON was the NH_2-terminal cysteine residue as shown by Edman degradation.

L-Glutamine:D-fructose-6-phosphate amidotransferase (EC 2.6.1.16) catalyzes the first reaction in hexosamine biosynthesis (eq 1). It belongs to the group of glutamine amidotransferases

L-glutamine + D-fructose 6-phosphate → D-glucosamine 6-phosphate + L-glutamate (1)

which utilize the amide of glutamine in the biosynthesis of amino acids, nucleotides, and coenzymes (Buchanan, 1973; Prusiner & Stadman, 1973). This enzyme is unique among this group in the fact it is the only one transferring the amide nitrogen to a keto group without the participation of a cofactor. The product, glucosamine 6-phosphate ($GlcNH_2$-6-P),[1] undergoes sequential transformations leading to the formation of UDP-*N*-acetylglucosamine, the major intermediate in the biosynthesis of all amino sugar containing macromolecules both in prokaryotic and in eukaryotic cells. Therefore, the

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[1] Abbreviations: APAD, acetylpyridine adenine dinucleotide; DABITC, 4-(*N*,*N*-dimethylamino)azobenzene-4′-isothiocyanate; DABTH, 4-(*N*,*N*-dimethylamino)azobenzene-4′-thiohydantoin; DON, 6-diazo-5-oxo-L-norleucine; DSP, dithiobis(succinimidyl propionate); DSS, disuccinimidyl suberate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FPLC, fast protein liquid chromatography; Fru-6-P, fructose 6-phosphate; $GlcNH_2$-6-P, glucosamine 6-phosphate; Gln, L-glutamine; IAAm, iodoacetamide; PMSF, phenylmethanesulfonyl fluoride; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; PTH, phenylthiohydantoin; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; PAGE, polyacrylamide gel electrophoresis; Cam, carbamoylmethyl; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; TFA, trifluoroacetic acid; kDa, kilodalton(s).

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inhibition of this enzyme might have important implications in chemotherapy.

Since the pioneering work reported by Gosh (Gosh et al., 1960), a few studies have been reported which dealt mainly with the regulation of the partially purified rat liver enzyme (Bates et al., 1966; Kornfeld, 1967; Winterburn & Phelps, 1971, 1973; Miyagi & Tsukui, 1971, 1979). More recent studies using a partially purified bacterial enzyme (Chmara et al., 1984; Chmara, 1985) reported the highest specific activity of 0.7 unit/mg with a native molecular weight of 90000.

Recently, the *glmS* gene, located downstream of the *unc* operon at minute 84 on the *Escherichia coli* chromosome (Bachman, 1983) and encoding glucosamine synthetase, was identified by homology with glutamine phosphoribosylpyrophosphate amidotransferase (Walker et al., 1984). From the work in this paper, we confirm this identification.

We report here the first purification to homogeneity of the *E. coli* glucosamine synthetase, the initial characterization of the protein, and the identification of the glutamine binding site by covalent modification with a radiolabeled glutamine analogue.

Experimental Procedures

Materials. Phosphoglucose isomerase (EC 5.3.1.9) from bakers' yeast (560 units/mg in 2.6 M ammonium sulfate), PMSF, Bistris–propane, Tris, DON, *p*-(hydroxymercuri)-benzoate–agarose, and alumina (type 305) were purchased from Sigma. Glutamate dehydrogenase (EC 1.4.1.3) from beef liver (120 units/mg in 50% glycerol), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) from yeast (140 units/mg in 3.2 M ammonium sulfate), APAD, Fru-6-P, and pepstatin were from Boehringer-Mannheim. Bistris, Q-Sepharose fast flow, and phenyl-Sepharose CL4B were from Pharmacia. (*N*,*N*-Dimethylamino)benzaldehyde, EGTA, and glutamine were from Serva. DSP, DSS, DABITC (recrystallized before use), and F1700 micropolyamide sheets were from Pierce. IAAm was from Fluka and Ultrogel AcA 44 from LKB. [^{14}C]Diazald (9.7 mCi/mmol) was purchased from Amersham. All other chemicals were of the highest quality available.

Assays. (*A*) *Colorimetric Assay.* Glucosamine-6-P was determined by using a modification of the Morgan–Elson procedure described by Gosh (Gosh & Roseman, 1962). A 1-mL solution containing 20 mM Fru-6-P, 15 mM Gln, 2.5 mM EDTA, 37.5 mM potassium phosphate, pH 7.5, and 0.2 mL of the enzyme solution was incubated for 20 min at 37 °C. The reaction was stopped by boiling for 4 min at 100 °C. The protein precipitate was removed by centrifugation, and 0.8 mL of the supernatant was used for assay of glucosamine-6-P (Zalkin, 1985). For each determination, a standard curve was constructed by using glucosamine as a standard. No correction was made for the lower response of glucosamine-6-P (factor 0.85). The standard plot gave a linear response between 0 and 200 nmol of glucosamine produced in a 20-min incubation.

(*B*) *Spectrophotometric Assay.* Glutamate determination was performed according to Shijo (Shijo & Ishi, 1969). Typically, a 1-mL cuvette equilibrated at 37 °C contained 10 mM Fru-6-P, 6 mM Gln, 0.3 mM APAD, 50 mM KCl, 100 mM potassium phosphate, pH 7.5, and 30 units of glutamate dehydrogenase. The activity after enzyme addition was followed at 365 nm. After a short lag period, the activity remained linear for at least 15 min. The enzyme activity was calculated by assuming ϵ_{365}(APAD) = 0.91 L·mm^{-1}·mmol^{-1} (Beutler & Supp, 1983).

For both assays, a unit of activity is defined as 1 μmol of product formed per minute.

(*C*) *Inactivation Assay.* For time-dependent inactivation assays, the enzyme solution was thoroughly dialyzed against 100 mM potassium phosphate, pH 7.5, and 1 mM EGTA to remove thiols. It was then preincubated at 0.5–3 μM concentration in the same buffer containing 10 mM Fru-6-P (except when noted) at room temperature. The reaction was initiated by addition of the desired compound; at different time points, 20-μL aliquots were removed and diluted 30-fold into the same buffer containing 5 mM 2-mercaptoethanol and 6 mM Gln on ice. The enzyme activity was then determined by using the spectrophotometric assay. In the conditions described, the ratio I_0/E_0 was kept above 10.

Glutaminase activity was checked by using glutamate dehydrogenase and NADH (Lund, 1985).

Reversal of the Reaction. Glucosamine deaminase activity was tested by coupling phosphoglucose isomerase and glucose-6-P dehydrogenase as described (Michal, 1984).

Methods. (*A*) *Electrophoresis* was performed on polyacrylamide slab gels in the presence of SDS according to Laemmli (1970). Protein bands were visualized by Coomassie blue R250 staining according to Vesterberg (1971).

(*B*) *Chromatofocusing* was performed on a Mono P HR 5/20 column (Pharmacia, FPLC system) using 25 mM Bis-tris-HCl, pH 6.3, as the starting buffer; a pH 6–4 pH gradient was generated during the elution (0.5 mL/min) by a 45-mL Polybuffer 74 (diluted 1:10 in water) solution, pH 4. Both the starting buffer and the elution buffer contained 20% ethylene glycol. Enzyme activity was tested on one-fifth of the 0.5-mL fractions by the Morgan–Elson procedure.

(*C*) *Gel Filtration.* Purified enzyme (50–200 μg) was injected on a Superose 12 HR 10/30 column (Pharmacia, FPLC) and eluted at 0.5 mL/min with one of the following buffers (pH 7.2): (buffer A) 50 mM potassium phosphate, 10 mM Gln, 1 mM EGTA, and 150 mM NaCl; (buffer B) buffer A without NaCl; (buffer C) buffer A + 10% acetonitrile; (buffer D) 6 M guanidine hydrochloride, 20 mM potassium phosphate, and 130 mM NaCl. Protein elution was followed at 280 nm and enzyme activity by colorimetric assay. The following protein markers were used: ferritin (M_r 450000), catalase (M_r 240000), aldolase (M_r 158000), BSA (M_r 68000), ovalbumin (M_r 43000), chymotrypsinogen A (M_r 25700), and cytochrome *c* (M_r 11700). BSA, ovalbumin, chymotrypsinogen A, and lysozyme (M_r 14300) were used in the run with guanidine.

(*D*) *Sedimentation Coefficient.* Glucosamine synthetase (about 250 μg) was applied to a preformed 4.5-mL linear gradient of 20–40% glycerol containing 50 mM Tris-HCl (pH 7.2), 100-mM KCl, 0.5 mM DTT, 0.5 mM EDTA, 10 mM 2-mercaptoethanol, and 1 mM PMSF. Centrifugation was carried out at 4 °C for 24 h at 47000 rpm in a Beckman SW 41 rotor; 270-μL fractions were collected from the bottom of the tube. The protein profile was determined by the 280-nm absorbance of a 1:10 diluted solution and the enzyme activity by the Morgan–Elson procedure. Catalase (11.3 S), aldolase (7.35 S), and chymotrypsinogen A (2.54 S) were used in separate gradients as molecular weight markers (Riou et al., 1985).

(*E*) *Cross-linking* was used to study subunit composition as described by Davies (Davies & Stark, 1970). Disuccinimidyl suberate (DSS) and dithiobis(succinimidyl propionate) (DSP) were used at 0.5, 1, 5, and 10 mg/mL in either 50 mM triethanolamine and 0.5 M NaCl, pH 8, or 50 mM potassium phosphate and 0.5 M NaCl, pH 7. The enzyme was 0.5 or 1 mg/mL. The reaction was performed at 4 °C and stopped by adding arginine to a final concentration of 0.2 M. SDS

was added to 0.6% and 2-mercaptoethanol to 10% unless otherwise specified. After a 1-h incubation at 37 °C, the samples were submitted to SDS electrophoresis.

(*F*) *Amino Acid Analysis.* The enzyme (carboxamidomethylated or not) was dialyzed against 50 mM NH_4HCO_3, pH 8, and lyophilized. Aliquots were hydrolyzed for 24, 48, and 72 h in twice-distilled 5.7 N HCl containing 0.1% phenol and analyzed on an LKB 4400 amino acid analyzer. Half-cystine was measured as its carboxymethyl derivative.

Tryptophan was determined by absorbance measurements in 6 M guanidine (Edelhoch, 1967) and by amino acid analysis after basic hydrolysis (Wilkinson et al., 1976). Tyrosine was determined from the spectra at two different pHs (Edelhoch, 1967).

(*G*) *Thiol Determination.* Spectrophotometric titration of thiols using Ellman's reagent was carried out following the recently reassessed protocol (Riddles et al., 1983).

(*H*) *Alkylation with [^{14}C]Iodoacetamide.* Glucosamine synthethase (0.42 mg) in 0.45 mL of 100 mM Tris-HCl (pH 7.5), 10 mM Fru-6-P, and 1 mM EDTA (A_{280} = 0.72) was treated with 0.25 mL of [^{14}C]IAAm solution (100 μCi), giving a final 2.55 mM concentration in alkylating agent. The solution was incubated at 25 °C for 1 h and then made 1% SDS or 4.2 M guanidine hydrochloride. Aliquots were removed from the incubation mixture and quenched by 5-fold dilution into cold buffer containing 50 mM 2-mercaptoethanol. The enzyme activity was determined spectrophotometrically vs. time. The radioactivity incorporated was analyzed by spotting 0.1 mL of the diluted primary solution onto fiber glass filters (GF/C, Whatman) and precipitating the enzyme into the filter by immersion into ice-cold 10% trichloroacetic acid (TCA) for 10 min. The filters were washed twice with 5% TCA at room temperature and then 3 times with 95% ethanol. The dried filters were then counted in 5 mL of scintillation cocktail. No significant background was observed in these conditions. The radioactivity incorporated into the protein was calculated after corrections for dilution and amino acid analysis.

Alkylation in denaturing conditions was performed similarly with 6 M guanidine hydrochloride, 50 mM potassium phosphate, and 1 mM EDTA, pH 8, as the buffer; the concentration of IAAm was 2.34 mM.

(*I*) *Amino-Terminal Residue Analysis.* The amino-terminal residue was identified as the DABTH derivative according to Chang (1983). Authentic *S*-(carboxamidomethyl)cysteine was synthesized as described (Goodman et al., 1958).

(*J*) *Amino-Terminal Sequence.* Automatic Edman degradation was carried out in a Beckman 890C spinning-cup sequenator with 0.1 M Quadrol in the presence of Polybrene as described (Lederer et al., 1983) with the following modifications. The commercial Quadrol reagent (Pierce) was used as such, and peptides were dissolved in 20% acetic acid for loading. The HPLC system used for identification of PTH–amino acids consisted of two Waters Model 510 pumps, a Waters WISP/710B automatic injector, and a Kratos 773 spectrophotometer operating at 0.001 absorbance unit full scale (AUFS). The complete system was actuated by a Waters 840 data and control station. For the degradation of labeled samples, an aliquot of each PTH–amino acid was withdrawn before injection for radioactivity determination.

In addition, on one occasion, the benzene/ethyl acetate washes were systematically collected and counted after drying.

(*K*) *Synthesis of [^{14}C]DON.* *N*-(Trifluoroacetyl)-L-glutamic anhydride was synthesized as described (Weygand & Reiher, 1954) and hydrolyzed with anhydrous methanol (6 mL/10 mmol, 2 h, 25 °C). The 1-methyl ester was precipitated from a benzene solution at 4 °C using a 1.2-fold excess of dicyclohexylamine. It was washed with benzene and dried (33% yield, melting point = 191 °C). The acid chloride was formed as described (Weygand & Swodenk, 1957) and purified by sublimation at 70 °C under a vacuum of about 100 μm (57% yield, melting point = 60 °C). [^{14}C]Diazomethane was generated from [^{14}C]Diazald (20 mg, 2.62 mCi/mmol) in ether (2 mL) by addition of a 1-mL solution of sodium in octyl alcohol (10 mg/mL) according to the described procedure (Holcenberg et al., 1978); 0.5 mL of a solution of freshly sublimated *N*-(trifluoroacetyl)-L-glutamic acid 1-methyl ester 5-chloride in ether (73 μmol/mL) was added to the [^{14}C]-diazomethane/ether solution (about 2 mL) and incubated in the dark for 1 h at room temperature. [^{14}C]DON was deblocked and purified on a Norite/Celite (1:1) column eluted with 1% acetone in water (Hartman, 1963; Holcenberg et al., 1978). The DON-containing fractions (as determined by radioactivity and 274-nm absorption) were pooled, giving 3.5 μmol of labeled compound (10% yield). The specific radioactivity at this stage, as determined from the 274-nm absorbance and radioactivity determination, was 2.7 mCi/mmol. The same value was found after purification of an analytical sample on TLC (silica gel; 1-propanol/water 7:3).

(*L*) *Active-Site Labeling.* Enzyme (0.91 mL, A_{280} = 2.15) was reacted with 2.24 μmol of [6-^{14}C]DON in a reaction mixture containing 50 mM potassium phosphate, pH 7.5, and 10 mM Fru-6-P at 25 °C for 1 h. The residual activity after this period was 0.5%. Solid guanidine crystals were added to make a 6 M solution, and the pH was adjusted to 8.5 with a 2 M Tris base solution. The enzyme solution was then treated with 5 mg of $NaBH_4$ for 1 h prior to alkylation with 10 μL of freshly distilled 4-vinylpyridine for another 1 h. The protein was desalted by gel filtration on a Sephadex G-25 column (28 × 1.5 cm) in 9% formic acid. The protein-containing fractions were pooled (6.8 mL, A_{280} = 0.24), and the specific radioactivity was determined.

(*M*) *Cleavage at Methionyl Bonds.* The inactive protein remaining from the above experiment (86.5%) was lyophilized (Speed-Vac, Savant Instruments) and redissolved in 1 mL of 70% formic acid. Cyanogen bromide (10 mg) was added, and the resulting mixture was incubated under argon in the dark at room temperature for 24 h. After evaporation of the solution, the protein residue was washed with ether to remove the excess of CNBr and redissolved in 1 mL of 10% acetic acid for further purification.

(*N*) *Purification of Labeled Peptides.* The above mixture was fractionated by using a reverse-phase proRP/C HR5/10 column (Pharmacia, FPLC) and further purified by using a prepRP/C HR5/5 column under conditions described under Results. Peptide elution was followed by the absorption at 214 nm and scintillation counting.

(*O*) *E. coli Glucosamine Synthetase Purification.* *E. coli* 3000Hfr (ATCC 25257) was grown in 100-L batches to late exponential phase in CGPY medium (Lugtenberg & de Haan, 1971) at 37° C from a 10% culture inoculum. The cells were collected by using a Sharpless centrifuge, washed with 100 mM potassium phosphate and 2 mM EDTA, pH 7.5, frozen in liquid nitrogen, and stored at −20 °C until use. Typically, 500 g of cells (wet weight) was obtained from a 100-L culture. A crude extract was made by using either a Manton–Gaulin homogenizer or alumina disruption. The following steps were conducted at 4 °C except where noted. During the purification, glucosamine synthetase activity was followed by using the Morgan–Elson colorimetric assay.

Table I: Purification of Glucosamine Synthetase from *E. coli*

purification step	volume (mL)	protein (mg)[a]	act. (units)	sp act. (units/mg)	yield (%)	purification factor
crude extract	870	17870	350	0.019	100	1
Polymixin sulfate	1000	11250	350	0.031	100	1.6
Q-fast flow	350	1610	217	0.13	62	7.1
phenyl-Sepharose	200	226	153	0.68	44	35.8
organomercurial agarose	50	126	153	1.21	44	63.7
gel filtration	20	28.5	153	5.4	44	284
FPLC	3.3	9.6[b]	73	7.63	20	400

[a] Protein concentrations were determined by the method of Bradford. [b] Determined from A_{280} and corrected from amino acid composition.

Cells (500 g) were thawed overnight and disrupted in two batches by grinding with alumina using two weights of alumina per cell weight; 500 mL of buffer A (20 mM Bistris–propane, 10 mM Gln, 1 mM EGTA, 0.5 mM Fru-6-P, and 10^{-7} M pepstatin, pH 7.5, saturated in PMSF) was added to the white paste. Alumina was spun down (5000*g*, 10 min) and washed with 500 mL of buffer A. The combined supernatants were clarified by centrifugation (12000*g*, 1.5 h). The supernatant (870 mL) was made 0.6% in Polymixin sulfate by adding 100 mL of a 6% solution, pH 7. The white precipitate was removed, and the 2-fold-diluted supernatant was loaded at 400 mL/h on a Q-Sepharose fast-flow column (5 × 24 cm) equilibrated in buffer A. The column was washed with 500 mL of starting buffer and eluted with a 5-L linear gradient of 0–0.5 M NaCl in buffer A. Active fractions appearing at 0.35 M NaCl were pooled (350 mL), concentrated to 100 mL by ultrafiltration (Amicon, PM 10 membrane), and made 3 M in NaCl by slow addition of crystals. A 150-mL phenyl-Sepharose column was loaded with the enzyme solution (adjusted to pH 7.7) at a flow rate of 40 mL/h and directly eluted at 50 mL/h with a convex gradient of 250 mL of starting buffer B (20 mM potassium phosphate, 3 M NaCl, 10 mM Gln, 2 mM DTT, and 1 mM EGTA, pH 7.7) and 1 L of final buffer (buffer B without NaCl containing 20% glycerol). Active fractions (200 mL) were pooled, concentrated to 30 mL (Amicon, PM 10 membrane), and dialyzed against buffer C (20 mM potassium phosphate, 0.5 M sucrose, and 1 mM EGTA, pH 7). A 50-mL *p*-(hydroxymercuri)benzoate–agarose column was then loaded overnight at 2 mL/h. The column was washed with buffer C (50 mL) at 10 mL/h and eluted at 20 mL/h with a 200-mL linear gradient of buffer C to 20 mM DTT and 10 mM Gln in buffer C. The pooled active fractions (50 mL) were concentrated to 4 mL and loaded on an AcA 44 gel filtration column (95 × 1.6 cm) previously equilibrated in 20 mM potassium phosphate, 10 mM Gln, 2 mM DTT, and 1 mM EGTA, pH 7 (buffer E). The activity eluting just after the void volume was pooled (20 mL), concentrated to 5 mL, and dialyzed against 30 mM Tris-HCl, 10 mM Gln, 2 mM DTT, and 1 mM EGTA, pH 8 (buffer F). The 30% pure protein was finally purified at room temperature on a Mono Q HR5/5 FPLC column using a linear gradient of 150–500 mM NaCl in buffer F. Active fractions were pooled, dialyzed against buffer E, pH 7.25, and frozen in liquid nitrogen. Table I summarizes the purification.

(*P*) *Protein concentration* was estimated from the absorbance at 280 nm or by the method of Bradford (1976) or Lowry (1951) using BSA as the standard. The values for pure protein were corrected from amino acid analysis.

Results

Purification of Glucosamine Synthetase from E. coli. The *E. coli* glucosamine synthetase was purified as summarized in Table I. Among the several cell disruption techniques used, alumina grinding gave the best results, the method being restricted, however, to medium-scale purification. The Manton–Gaulin homogenizer would undoubtedly be suitable for larger scale purification. Partial precipitation of the enzyme precluded the use of protamine sulfate, and we followed for this reason the original method described by Gosh (Gosh et al., 1960). The rapidity in carrying out the ion-exchange step was a determining factor for a successful purification. Using classical ion-exchange gels, we observed as much as 75% loss in activity during this step. The addition of protease inhibitors was of some use, especially in stabilizing the activity in the crude extract, but did not noticeably increase the activity recovered after the ion-exchange step. At this step, however, the use of high flow rates (Q-Sepharose fast flow) allowed a 60% recovery in enzyme activity. Glucosamine synthetase bound very tightly to a hydrophobic matrix; when the phenyl-Sepharose column was used as the first step, no enzyme activity was recovered which explains the use of a convex gradient at this stage. Thiol affinity chromatography turned out to be quite efficient in enzyme reactivation by thiols as we observed in a few cases an increase of total recovered activity. Moreover, phosphoglucose isomerase activity which did not bind to the gel under these conditions (Hosoi et al., 1978) was removed. After gel filtration, the 25–30% pure enzyme was further purified by using ion-exchange FPLC at pH 8. Higher pHs resulted in significant losses in activity.

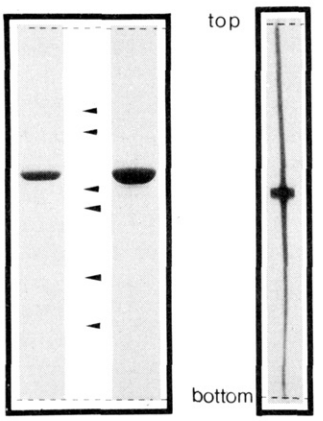

FIGURE 1: Electrophoresis of purified glucosamine synthetase. (Left panel) SDS–PAGE loaded at 10 and 30 μg; the following markers (arrows) were used: β-galactosidase (135 kDa); phosphorylase *a* (92 kDa); BSA (68 kDa); catalase (57.5 kDa); ovalbumin (43 kDa); lactate dehydrogenase (36.5 kDa). (Right panel) 8% native gel.

Purification of the glucosamine synthetase from 500 g of *E. coli* cells gave almost 10 mg (corrected value, see below) of more than 90% pure enzyme (Figure 1), the specific activity of which was 7.63 units/mg. Such a purification could easily be carried out in less than 2 weeks.

Amino Acid Composition. The composition was determined on two different enzyme preparations. Table II gives the average value of these analyses and, for comparison, the translated DNA sequence of the recently assigned *glmS* gene believed to encode glucosamine synthetase (Walker et al., 1984; see Discussion). From amino acid analysis, we calcu-

Table II: Amino Acid Composition of *E. coli* Glucosamine Synthetase

amino acid	no. of residues per subunit: predicted[a]	found[b]
Asp	30	
Asn	20	51.3[f]
Thr	28	26.3
Ser	39	35.5
Gln	20	
Glu	53	81.6[g]
Pro	21	25.3
Gly	48	55.9
Ala	59	57.0
Val	44	38.4
Met	14	10.5
Ile	43	38.3
Leu	70	70.9
Tyr	18	19.0[c]
Phe	13	16.4
His	24	20.8
Lys	22	23.0
Arg	36	37.2
Trp	3	3.7[d]
Cys	4	5.2[e]
total residues	609	616
mol wt	66900	66900

[a] From DNA sequence (Walker et al., 1984). [b] Normalized to 66.9 kDa. [c] Average value from spectrophotometric (18.8) and amino acid analysis (19.2) determinations. [d] Average value from spectrophotometric (4.0) and basic hydrolysis (3.4) determinations. [e] Determined as (carboxymethyl)cysteine. [f] Determined as Asx. [g] Determined as Glx.

lated a protein concentration of 1.3 mg/mL per A_{280} unit and estimated Lowry determination to reflect accurately the concentration of pure enzyme.

Molecular Weights of Enzyme and Subunit. The purified protein is homogeneous by the criteria of native PAGE and SDS–PAGE as shown in Figure 1. From the plot log molecular weight vs. migration distance in denaturing conditions, a molecular weight of 70 800 was obtained by regression analysis, a value in reasonable agreement with the one deduced from the DNA sequence. Glucosamine synthetase behavior in gel filtration conditions gave reproducibly a molecular weight ranging from 170 000 (buffer C) to 193 000 (buffer A). These values were affected neither by the presence of NaCl nor by 10% acetonitrile. As the protein behavior on gel filtration columns is more precisely related to Stokes radius than to molecular weight (Siegel & Monty, 1965), sedimentation in a glycerol gradient was carried out. A sedimentation constant of 5.4 S was deduced from this experiment. From this value and the K_{av} values calculated from the gel filtration experiments (buffer A), we assigned to the enzyme a Stokes radius of about 57 Å (data not shown). Using these two values, we calculated a molecular weight of 127 000 assuming a viscosity $\eta = 0.01$, a density $\rho = 1$, and a partial specific volume $\bar{v} = 0.725$.

Cross-Linking Experiments. Native enzyme was exposed to DSP or DSS at pH 7 or 8 (Davies & Stark, 1970; D'albis & Gratzer, 1976), and cross-linked products were analyzed by SDS–PAGE. Figure 2 presents typical results. Prior to exposure to the cross-linking agent, only the monomer band was seen. After 30-min reaction at 4 °C, one main band appeared. At longer reaction times (1.5 and 3 h), there was a slight modification in the band patterns. As expected for a disulfide-containing cross-linking agent, thiol addition reduced the DSP cross-linked protein without restoring, however, the original electrophoretic profile. As seen from the gel, the cross-linking is much less efficient at low pH. The same result was observed by using a 2-fold higher protein concentration

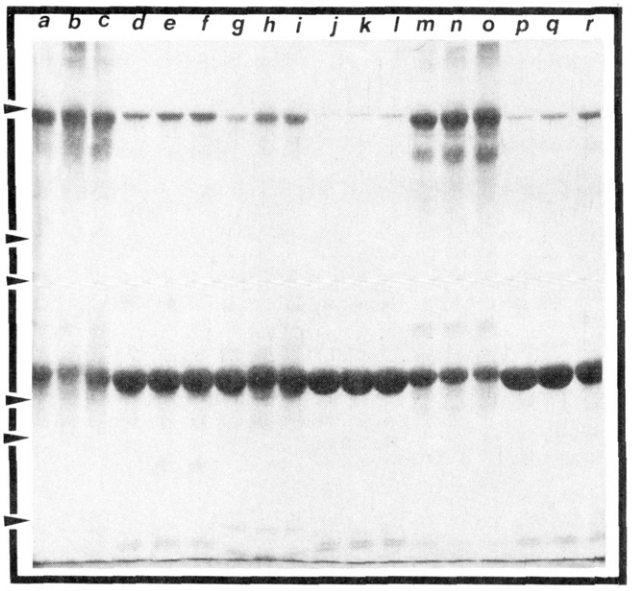

FIGURE 2: Chemical cross-linking of glucosamine synthetase. Enzyme (0.65 mg/mL) was cross-linked at pH 8 (lanes a–f and m–o) or pH 7 (lanes g–l and p–r) with DSP (lanes a–l) or DSS (lanes m–r) at 1 mg/mL at 4 °C. Reaction mixtures were incubated for 0.5 h (lanes a, d, g, j, m, and p), 1.5 h (lanes b, e, h, k, n, and q), or 3 h (lanes c, f, i, l, o, and r) and stopped by arginine addition to 0.2 M. Samples were diluted with the same volume of buffer containing 1.2% SDS and 20% 2-mercaptoethanol except in lanes a–c and g–i and incubated at 37 °C for 1 h prior to SDS electrophoresis. Ferritin (220 kDa), β-galactosidase (135 kDa), phosphorylase *b* (94 kDa), BSA (68 kDa), catalase (57.5 kDa), and ovalbumin (43 kDa) were used as markers (arrows). The control for enzyme without cross-linking displayed only one band (lane not shown).

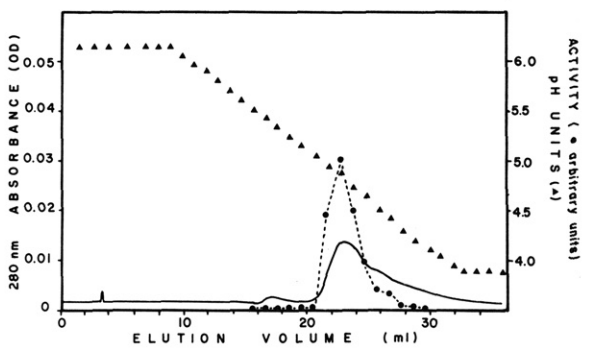

FIGURE 3: Chromatofocusing of glucosamine synthetase. The purified protein (100 μg) in 25 mM Bistris-HCl and 20% ethylene glycol, pH 6.3, was applied to a MonoP HR 5/20 column (Pharmacia). The column was washed with the same buffer, and a linear pH gradient (6.3–4) was generated by elution with 45 mL of Polybuffer 74 (diluted 1:10 in water) containing 20% ethylene glycol. Enzyme activity was tested on one-fifth of the 0.5-mL fractions by the Morgan–Elson procedure.

or a 2-fold lower cross-linking agent concentration. A molecular weight of 210 000 was attributed to the major cross-linked species. Unexpectedly, no species at apparent molecular weight 140 000, expected for the dimer, was detectable.

Isoelectric Point. Chromatofocusing of the purified glucosamine synthetase using a pH 6–4 pH gradient gave the profile shown in Figure 3. There was obviously partial enzyme denaturation even in the presence of 20% ethylene glycol as stabilizing agent. The activity profile was, however, sharp enough that an isoelectric point of 4.9 could be attributed to the enzyme.

Thiol Determination. Protein titration using Ellman's reagent (Riddles et al., 1983) in denaturing conditions gave 4.2 reactive cysteine residues per subunit. Two of them reacted

Table III: Reaction of Glucosamine Synthetase with Sulfhydryl Reagents

sample analyzed	method	groups reacted per subunit
native	DTNB for 1 min	2.0
native	DTNB for 2 h	3.1
native	IAAm for 1 h	0.5
enzyme in 1% SDS	IAAm for 13 h	1.6
enzyme in 6 M guanidine hydrochloride	IAAm for 13 h	2.5
enzyme in 6 M guanidine hydrochloride	DTNB for 1 min	4.2

instantaneously when DTNB (0.7 mM final concentration) was added to the native enzyme solution (3.23 μM) in potassium phosphate buffer (pH 7.3). The third one reacted in the next 2 h (Table III). Alkylation of native protein (8.5 μM) with [^{14}C]IAAm (2.55 mM) was followed as a function of time (data not shown). Under these conditions, the enzyme activity dropped to less than 10% of the original value after alkylation of only 0.5 cysteine. This residue is believed to be the glutamine binding site thiol residue as shown by protection experiments using the substrate (see below). SDS addition after 1 h allowed a total of 1.6 cysteine residues to react over 13 h. Under the same conditions, addition of guanidine hydrochloride to 4.2 M allowed titration of 2.2 residues. A stoichiometry of 2.5 cysteines/subunit, close to the preceding value, was reached over the same time period when alkylation was carried out in 6 M guanidine hydrochloride at the same pH (Table III).

Amino-Terminal Sequence. The identification of the NH_2-terminal amino acid in the isolated enzyme was crucial as phosphoribosylpyrophosphate amidotransferase uses this one as the active-site cysteine. For the 25 cycles run in the sequenator, 21 were positively identified (Table IV). Amino-terminal cysteine was confirmed by using the DABITC manual end-group method (Chang, 1983). The DABTH–amino acid, extracted from the reaction of 2 nmol of S-carboxamidomethylated protein with DABITC, comigrated on a micropolyamide sheet with authentic DABTH–Cam-cysteine synthesized separately (Goodman et al., 1958). This experiment definitely established cysteine as the amino-terminal residue.

Kinetic Parameters. The *E. coli* glucosamine synthetase exhibits a narrow pH optimum centered at pH 7.2 (data not shown). Among a large variety of tested buffers, phosphate, Hepes, Bistris, and Tes appeared to be the most suitable for highest enzymatic activity. At pH 7.5, the synthetase has a K_m of 2 mM for fructose 6-phosphate, a K_m of 0.4 mM for L-glutamine, and a V_{max} in the range 4–8 units/mg depending on the enzyme batch used. The dose–response curve for the spectrophotometric assay exhibited linearity in the range of 0.5–6.6 nmol of glutamate formed/min.

Inactivation Studies. (*A*) *Iodoacetamide.* As an active-site thiol protein, glucosamine synthetase is sensitive to thiol modifying reagents. Iodoacetamide was selected in a first approach as a thiol active-site titration method. Alkylation of native enzyme with various IAAm concentrations (about 1000-fold in excess) in the presence of Fru-6-P resulted in time-dependent inhibition of the glucosamine synthetase activity. The inactivation followed pseudo-first-order kinetics and was irreversible as 30-fold dilution did not promote reactivation. From the double-reciprocal plot 1/inactivation rate vs. 1/[IAAm], an inhibition constant K_i = 12.6 mM and a maximum inactivation rate k_{inact} = 1.34 min^{-1} were calculated (data not shown). The rate of inactivation at 2 mM IAAm was slowed down (1.75-fold) by the addition of glutamine (10 mM) which argued for alkylation by IAAm of a cysteine residue of the glutamine binding site.

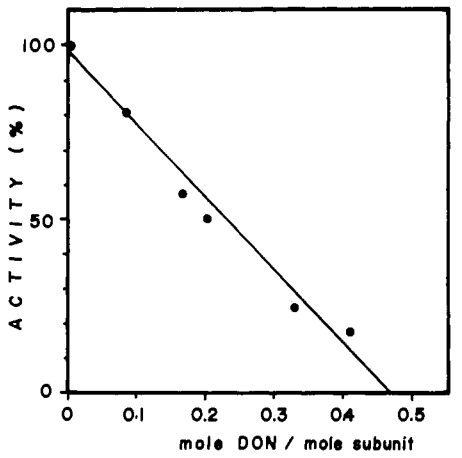

FIGURE 4: Stoichiometry of alkylation of glucosamine synthetase with DON in the absence of Fru-6-P. The enzyme was inactivated with limiting amounts of DON. The activity is plotted against the molar ratio of DON to enzyme subunit (M_r 70.8K). Homogeneous enzyme (1.2 μM in subunit) was incubated at room temperature in 100 mM potassium phosphate and 1 mM EGTA, pH 7.5, for 130 min with various amounts of DON (0.1–0.7 μM). The line was calculated by linear regression.

(*B*) *6-Diazo-5-oxo-L-norleucine.* The affinity label, DON (Pinkus, 1977), behaved also as a time-dependent inhibitor: using 10 mM Fru-6-P in the incubation mixture resulted in linear time-dependent activity plots from which a K_i of 2.8 μM and a k_{inact} of 0.176 min^{-1} were calculated (data not shown). The stoichiometry of alkylation of glucosamine synthetase by unlabeled DON was determined initially by measuring the extent of partial inactivation using substoichiometric amounts of this inhibitor (Mantsala & Zalkin, 1976) in the absence of fructose-6-P. The data in Figure 4 show that only 0.47 mol of DON/mol of subunit (i.e., 0.94 DON/dimer) was required for 100% inactivation. A 1/1 stoichiometry was reached, however, in the same conditions but in the presence of 10 mM Fru-6-P. Table V summarizes the influence of added fructose-6-P on the stoichiometry of IAAm and DON alkylation.

Active-Site Labeling with [6-^{14}C]DON. Glucosamine synthetase (2.52 mg, 36 nmol) was reacted with 6-^{14}C-labeled DON in the presence of Fru-6-P to inactivate the glutamine-dependent glucosamine synthetase. The 99% inactivated protein was reduced with sodium borohydride to stabilize an acid-labile DON–enzyme adduct (Holcenberg et al., 1978) and to reduce disulfide bonds and was alkylated with 4-vinylpyridine. Measurement of radioactivity of the protein

Table IV: Amino-Terminal Sequence of *E. coli* Glucosamine Synthetase[a]

from DNA	NH_2-Met-Cys- Gly-Ile-Val-Gly- Ala-Ile-Ala-Gln-Arg- Asp-Val-Ala-Glu-Ile- Leu- Leu-Glu- Gly-Leu- Arg- Arg-Leu- Glu-Tyr-
from Edman degradation	NH_2-Cys^1-Gly-Ile-Val-Gly^5-Ala-Ile-Ala-Gln-Arg^{10}-Asp-Val-Ala-Glu-Ile^{15}-XXX-Leu-XXX-Gly-Leu^{20}-XXX-Arg-XXX-Glu-Tyr^{25}-

[a] 1014 pmol was submitted to an automatic degradation. The average repetitive yield was 88.3%; the initial coupling yield was 55%. Positions 4 and 12 were identified as Val or Met, because the PTH identification system did not allow a distinction between those two residues. In addition, in the particular run presented, an ambiguity between Ile and Phe was present at cycle 15.

Table V: Influence of Fru-6-P on the Stoichiometry of Alkylation of Glucosamine Synthetase with IAAm and DON

inactivator	Fru-6-P	inactivation stoichiometry (mol of inactivator/mol of subunit)
IAAm	+	0.5[a]
IAAm	-	0.5[a]
DON	+	0.87[a,b]
DON	-	0.47[b]

[a] Determined from radioactivity incorporation. [b] Determined by titration of residual activity after incubation with substoichiometric amounts of inhibitor (see Figure 4).

Table VI: Identification of Phenylthiohydantoin–Amino Acids Sequentially Released by Automated Edman Degradation of [6-^{14}C]DON-Labeled Glucosamine Synthetase

cycle	PTH–amino acid major (nmol)	PTH–amino acid minor (nmol)	radioactivity[a] (cpm)
1	XXX (-)	Asp (0.8)	2110
2	Gly (2.1)	Val/Met (1.6)	100
3	Ile (2.1)	Glu (1.4)	100
4	Val/Met (1.8)	Gly (0.5), Ile (0.7)	100
5	Gly (1.5)	Ala (0.9)	100
6	Ala (1.6)	Ser (-)	100
7	Ile (1.4)	Glu (0.7)	100

[a] 6815 cpm (3.4 nmol) were loaded in the sequenator cup. In addition to the radioactivity associated with the first cycle, 590 cpm were found in the benzene/ethyl acetate wash at the end of the second coupling step; 80 cpm remained after the seventh cycle.

after gel filtration indicated the incorporation of 0.87 mol of DON/mol of subunit. The stoichiometry for incorporation of DON supports the view that alkylation of a single residue per enzyme subunit completely inactivates the enzyme under the conditions described. It seems, however, puzzling that half-of-the-sites reactivity was not observed in the presence of Fru-6-P.

Automated Edman degradation of [6-^{14}C]DON-labeled enzyme released most of the radioactivity in the first cycle. The results summarized in Table VI show that two sequences were obtained during Edman degradation over seven cycles. The major sequence which accounted for two-thirds of the two PTH–amino acid peaks matched only with the amino-terminal protein sequence as indicated by a computer search. The minor one, assuming valine at the second cycle and isoleucine at the fourth, matched exactly with the translated DNA sequence encompassing residues 324–330. Even if the minor peptide resulted from some uncontrolled protease cleavage of enzyme on storage, uncertainty did exist since the first residue of the minor peptide, located at position 324, was an aspartate residue. We therefore carried out protein cleavage in order to purify the peptide(s) carrying radioactivity.

Active-Site Peptide(s) Isolation and Sequence Determination. The [^{14}C]DON-labeled glucosamine synthetase was cleaved with cyanogen bromide and the resulting peptide mixture directly chromatographed, after elimination of excess CNBr, on a reverse-phase C_4–C_8 column using a linear gradient of 25–40% acetonitrile in water (both solvents contained 0.1% TFA). The resulting radioactive fractions were further purified on a C_{18} column with a linear gradient of 25–45% 2-propanol in water (0.1% TFA added). Finally, the two radioactive peaks were resolved in the latter conditions using 1-propanol instead of 2-propanol to give respectively 3000 dpm for peptide 1 and 4100 dpm for peptide 2 (2.2% and 3% yield, respectively, based on recovered radioactivity); the two peptides were submitted to Edman degradation. Sequence data in Table VII established that the two DON-labeled peptides were NH_2-terminal CNBr peptides with unexpected cleavage to generate a longer and a shorter peptide of the same initial sequence. These results definitely ascertain the NH_2-terminal cysteine as the glutamine active-site residue which is likely to be involved in glutamyl thiol ester formation, a common intermediate in all amidotransferase-catalyzed reactions (Buchanan, 1973).

Discussion

The *E. coli* glucosamine synthetase has been purified 400-fold to homogeneity. This is the first total purification reported for this enzyme; its lability had precluded previous isolation. The final specific activity is 30-fold higher than the original preparation described by Gosh (Gosh et al., 1960) and 11-fold higher than the one more recently described by Chmara (Chmara et al., 1984). The purified enzyme did not exhibit the lability previously reported and could be kept at 4 °C for 1 week without significant loss in activity. During enzyme manipulation, we did not notice any significant inactivation resulting from oxidation. Moreover, anaerobic incubation with or without DTT did not affect catalytic efficiency. This result forms sharp contrast to *E. coli* phosphoribosylpyrophosphate amidotransferase (Messenger & Zalkin, 1979) for which the aerobic inactivation in the presence of 0.5 mM DTT was so rapid that activity was lost before the enzyme assay could be completed. Thiol oxidation must, however, occur to some extent to explain the slight increase in activity observed during the organomercurial agarose–thiol elution step. The purified enzyme is stable on storage at −20 °C or in liquid nitrogen for several months.

The absorbance spectrum of highly active pure glucosamine synthetase did not exhibit any absorption in the region 300–500 nm (data not shown): the enzyme is colorless at a concentration of 5 mg/mL. This observation suggests this amidotransferase is not an iron-containing protein in contrast to the glutamine-utilizing phosphoribosylpyrophosphate amidotransferase from *Bacillus subtilis* (Wong et al., 1977). Attempts to increase catalytic activity by treatment with divalent cations (Fe, Zn, Mn, Cu, and Mg) failed. X-ray fluorescence spectrometry will be required to draw a definite conclusion.

Table VII: Identification and Quantitation of Phenylthiohydantoin–Amino Acids Sequentially Removed by Automated Edman Degradation of the [6-^{14}C]DON-Labeled CNBr Peptides

cycle	peptide 1[a] residue	peptide 1[a] yield (pmol)	peptide 1[a] radioactivity (cpm)	peptide 2[b] residue	peptide 2[b] yield (pmol)	peptide 2[b] radioactivity (cpm)
1	XXX		265	XXX		585
2	Gly	660	115	Gly	680	0
3	Ile	580	0	Ile	590	0
4	Val	420	0	Val	190	0
5	Gly	190	0	Gly	490	0
6	Ala			Ala	410	0
7	Ile			Ile	500	0

[a] 40% of the isolated peptide 1, i.e., about 1010 cpm, was loaded. [b] 40% of the isolated peptide 2, i.e., 1600 cpm, was loaded in the sequenator cup.

Most of the 13 known glutamine-dependent amidotransferases exhibit glutaminase activity and are able to use NH_3 instead of glutamine (Buchanan, 1973). Using large enzyme amounts, no hydrolytic activity could be detected by the standard spectrophotometric assay. This reaction, however, might require prior binding of Fru-6-P, and therefore further investigations, using sugar analogues, to inhibit the overall reaction, will be necessary to draw a definite conclusion. The enzyme was unable to substitute NH_3 for glutamine as no activity was detected with NH_4Cl as cosubstrate under conditions where the free ammonia concentration was 20 mM (150 mM NH_4Cl, pH 8.5), thus confirming the initial results on 3% pure enzyme reported by Gosh (Gosh et al., 1960; Gosh & Roseman, 1962); the glutamine-hydrolyzing activity and sugar-aminating activity must be tightly coupled. Also, the enzyme does not catalyze detectably the reverse reaction. In contrast to mammalian glucosamine synthetase (Bates & Handshumacher, 1968; Winterburg & Phelps, 1973), UDP-GlcNAc does not affect enzyme activity, as previously shown by Kornfeld in crude extracts from *E. coli* and *B. subtilis* (Kornfeld, 1967).

The glucosamine synthetase amino-terminal sequence agrees well with that predicted from the DNA sequence of the *glmS*-encoded protein (Walker et al., 1984) except for the first methionine residue which is lacking from the purified enzyme (Table IV). This residue is therefore removed in vivo to yield the functional enzyme having an NH_2-terminal cysteine, a process also observed in the case of glutamine phosphoribosylpyrophosphate amidotransferase (Tso et al., 1982a,b; Vollmer et al., 1983).

The 32% identity over the 25 residues in the amino-terminal region between *E. coli* glucosamine synthetase and phosphoribosyl amidotransferase led us to investigate the behavior of the glutamine analogue DON. This glutamine analogue has been shown (Tso et al., 1982a,b) to alkylate the amino-terminal cysteine residue required for the glutamine amide transfer function of the phosphoribosyl amidotransferase. Titration of enzyme activity in the absence of fructose-6-P showed that loss of activity resulted in the incorporation of 0.5 mol of DON/mol of subunit, thus exhibiting half-of-the-sites reactivity. A similar behavior was reported for CTP synthetase inhibition with DON (Levitzki et al., 1971). To our knowledge, half-of-the-sites reactivity behavior has so far been characterized only in multimeric enzymes with an even number of subunits (Levitzki et al., 1971; Seydoux et al., 1974). Therefore, a dimeric structure of the native enzyme is a simple hypothesis to suit to our results. Thus, in the absence of Fru-6-P, DON would promote a conformational change large enough to prevent the binding of a second DON molecule on the other subunit; Fru-6-P, when present, would prevent this conformational change, leaving it accessible to a second inhibitor molecule. The difference with iodoacetamide alkylation, giving a 0.5/1 stoichiometry whether the Fru-6-P is present or not, remains unexplained.

The radioactivity in [6-^{14}C]DON-inhibited glucosamine synthetase was located on the terminal cysteine residue as shown by direct protein degradation or after isolation of the radiolabeled peptide resulting from CNBr cleavage. This observation strengthens the previously noted analogy, from DNA sequence data, with phosphoribosyl amidotransferase.

At this point, it has to be noted that, in some glutamine amidotransferases, the glutamine and NH_3 binding sites are on separate subunits (e.g., anthranilate synthase) whereas in other enzymes a single subunit bears both functions (e.g., cytidinetriphosphate synthetase). Of interest is the question whether gene fusion may account for a bifunctional active site when the glutamine hydrolysis and the Fru-6-P → $GlcNH_2$-6-P conversion are closely coupled. This active site may be in addition buried such that exogenous NH_3 cannot get to the active site. The Fru-6-P effect on stoichiometry of DON alkylation suggests substantial conformational changes which may be crucial in catalytic coupling and intermediate sequestration.

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Registry No. DON, 157-03-9; [^{14}C]DON, 106947-55-1; Fru-6-P, 643-13-0; EC 2.6.1.16, 9030-45-9; L-Gln, 56-85-9; L-Cys, 52-90-4; $CF_3COGlu(Cl)OMe$, 107033-39-6; $CF_3COGluOMe$, 23403-47-6; $(CF_3COGlu)_2O$, 106947-56-2; ^{14}C-labeled $N_2{=}CH_2$, 13204-27-8.

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Dissociation of the Octameric Bifunctional Enzyme Formiminotransferase–Cyclodeaminase in Urea. Isolation of Two Monofunctional Dimers†

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ABSTRACT: Partial denaturation of the circular octameric bifunctional enzyme formiminotransferase–cyclodeaminase in increasing urea concentrations leads to sequential dissociation via dimers to inactive monomers. In potassium phosphate buffer, dissociation to dimers in 3 M urea coincides with loss of both activities and a major decrease in intensity of intrinsic tryptophan fluorescence. In the presence of folic acid, these dimers retain the deaminase activity, but with folylpolyglutamates, both activities are protected and the native octameric structure is retained. The protection profiles with polyglutamates are cooperative with a Hill coefficient greater than 2, suggesting that binding of more than one folylpolyglutamate per octamer is required to stabilize the native structure. In triethanolamine hydrochloride buffer, transferase-active dimers that retain the intrinsic tryptophan fluorescence can be obtained in 1 M urea and stabilized at higher urea concentration by the addition of glutamate. Deaminase-active dimers are obtained by the protection of folate in 3 M urea. Proteolysis of the two kinds of dimers by chymotrypsin leads to very different fragmentation patterns, indicating that they are structurally different. We propose that the two dimers retain different subunit–subunit interfaces, one of which is required for each activity. This suggests that the native octameric structure is required for expression of both activities and therefore for "channeling" of intermediates.

The folate-dependent bifunctional enzyme formiminotetrahydrofolate:glutamate formiminotransferase (EC 2.1.2.5)–formiminotetrahydrofolate cyclodeaminase (EC 4.3.1.4) catalyzes two sequential reactions of the histidine degradation pathway in mammalian liver:

formiminoglutamate + $H_4PteGlu_n$ ⇌ formimino-$H_4PteGlu_n$ + glutamate

formimino-$H_4PteGlu_n$ ⇌ methenyl-$H_4PteGlu_n$ + NH_4^+

With longer polyglutamate derivatives $H_4PteGlu_n$ ($n \geq 4$)[1] direct transfer of the formimino intermediate from the transferase site to the deaminase site has been observed, with complete "channeling" of the pentaglutamate (Paquin et al., 1985). The channeling requires noncovalent attachment of the intermediate to the enzyme, possibly to a separate polyglutamate binding site that "anchors" the substrate while the pteroyl moiety is transferred from the transferase site to the deaminase catalytic site (MacKenzie & Baugh, 1980). There appears to be a steric requirement for optimal channeling since the efficiency of channeling does not correlate directly with either binding affinity or kinetic efficiency (V_m/K_m) as polyglutamate chain length increases (Paquin et al., 1985).

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[1] Abbreviations: $H_4PteGlu_n$, tetrahydropteroylpolyglutamate with n glutamates; PteGlu, folic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; TEA·HCl, triethanolamine hydrochloride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

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