Glucosamine Synthetase from *Escherichia coli*: Kinetic Mechanism and Inhibition by N^3-Fumaroyl-L-2,3-diaminopropionic Derivatives†

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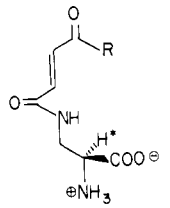
ABSTRACT: N^3-(4-Methoxyfumaroyl)-L-2,3-diaminopropionic acid (FMDP; **1**, R = OMe), a member of a new class of glutamine analogues, has been investigated as an inhibitor of pure *Escherichia coli* glucosamine synthetase. Product and dead-end inhibition studies indicate an ordered association to the enzyme with the sugar molecule binding prior to substrate or inhibitor. The inactivation exhibits pseudo-first-order kinetics, is irreversible, and occurs faster in the presence of fructose 6-phosphate, a behavior previously reported [Chmara, H., Andruszkiewicz, R., & Borowski, E. (1986) *Biochim. Biophys. Acta 870*, 357] for the partially purified enzyme from *Salmonella typhimurium*. The ratio k_{inact}/K_{irr} of 5500 makes compound **1** (R = OMe) one of the most efficient inhibitors of glucosamine synthetase to date. Inhibition occurs with partial covalent incorporation of L-FMDP into glucosamine synthetase. In the presence of fructose 6-phosphate, enzyme inactivation with [2-^{3}H]-DL-FMDP is associated with the incorporation of 0.75 equiv of inhibitor and with the modification of 0.78 thiol residue per enzyme subunit. This result is the first evidence for covalent entrapment of the entire inhibitor molecule following FMDP-mediated glucosamine synthetase inactivation. Preliminary inactivation with 6-diazo-5-oxo-L-norleucine, known to alkylate selectively the NH_2-terminal cysteine residue, completely prevents radioactivity incorporation. Therefore, this inhibitor is postulated to covalently modify glucosamine synthetase through direct addition of the thiol nucleophile from the terminal cysteine residue to the Michael acceptor **1**, so acting as an affinity label rather than a mechanism-based inhibitor.

The glucosamine 6-phosphate synthesizing enzyme glucosamine synthetase (L-glutamine:D-fructose-6-phosphate amidotransferase, EC 2.6.1.16) is an ubiquitous enzyme. The formation of D-glucosamine 6-phosphate can be considered as the first reaction on the pathway of hexosamine biosynthesis. Therefore, the inhibition of this catalytic step might have important repercussions on the synthesis of amino sugar containing macromolecules such as peptidoglycan or chitin. Two naturally occurring compounds, bacilysin (Abraham et al., 1946) and compound A 19009 (Molloy et al., 1972), have been shown to exhibit antibacterial and, more interestingly, antifungal properties. Their antimicrobial action was shown to be antagonized by D-glucosamine and *N*-acetyl-D-glucosamine (Molloy et al., 1972; Kenig & Abraham, 1976). It has been recognized that anticapsin [L-β-(*trans*-2,3-epoxy-4-oxocyclohexyl)alanine] was the inhibitory component of the dipeptide bacilysin and that its antimicrobial properties were the result of glucosamine synthetase inhibition. Both dipeptides bacilysin and A 19009 are transported into the cell by the dipeptide transport system and cleaved intracellularly by nonspecific peptidases to generate, besides L-alanine as the N-terminal amino acid in the former case and the C-terminal byproduct in the latter, anticapsin and N^3-fumaramoyl-L-2,3-diaminopropionic acid (**1**, R = NH_2). This illustrates the concept of illicit transport described by Ames and Gilvarg (Ames et al., 1973; Fickel & Gilvarg, 1973).

Since then, irreversible inhibition of the glucosamine synthetase activity by these two natural inhibitors has been reported in partially purified preparations of bacterial and yeast cells (Milewski et al., 1986; Chmara et al., 1986).

We recently reported (Badet et al., 1987) the purification and partial characterization of the enzyme from *Escherichia coli*. We have now studied the mechanism of inhibition of this important enzyme in order to elucidate the molecular details of the reactions occurring at the active site. This paper describes the kinetic behavior of compound **1** toward the pure bacterial glucosamine synthetase. The stoichiometry of incorporation of tritiated **1** (R = OMe) (position of label is shown by an asterisk in the structure) and the titration of thiol groups provide evidence for modification of an active site cysteine.



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Experimental Procedures

Materials. D-Fructose 6-phosphate, EDTA,[1] L-glutamate, L-glutamine, and Norit A were purchased from Serva. For kinetic experiments, L-glutamine was further purified from contaminating L-glutamate by filtration through a short column of AG 50X8 (Cl^- form) resin obtained from Bio-Rad. Glucosamine 6-phosphate was from Sigma. Dimethyl fumarate, asparagine monohydrate, and di-*tert*-butyl dicarbonate were purchased from Janssen Chimica. Bis(trifluoroacetoxy)iodobenzene was obtained from Fluka. (1-Fluoro-2,4-dinitro-5-phenyl)-L-alanine amide (Marfey's reagent) was purchased from Pierce. Glutamate dehydrogenase from beef

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[1] Abbreviations: Boc, *tert*-butoxycarbonyl; DON, 6-diazo-5-oxo-L-norleucine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; FCDP, N^3-fumaramoyl-L-2,3-diaminopropionic acid (**1**, R = NH_2); FMDP, N^3-(4-methoxyfumaroyl)-L-2,3-diaminopropionic acid (**1**, R = OCH_3); Fru-6-P, fructose 6-phosphate; $GlcNH_2$-6-P, glucosamine 6-phosphate; TLC, thin-layer chromatography; TNB^-, 2-nitro-5-thiobenzoate anion.

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liver (120 units/mg in 50% glycerol) was from Boehringer Mannheim. Trypsin (TPCK-treated) was from Cooper Biomedicals, and *Staphylococcus aureus* V8 protease was from Miles Laboratories.

Thin-layer chromatography was carried out on Kieselgel GF_{254} plates (Merck); the compounds were visualized with UV light and ninhydrin reagent. Spectrophotometric assays were performed on a Uvikon 810 spectrophotometer coupled to a Uvikon 21 chart recorder (Kontron). A 1211 Rackbeta scintillation counter (LKB) was used for the determination of radioactivity in solution, and an automatic TLC linear analyzer coupled to an LB-511 chromatography data system (Berthold) was used for radioactive TLC scanning.

Enzyme Purification. Large-scale enzyme purification was performed following the previously described protocol (Badet et al., 1987). From 3270 g of cells (400-L culture), 100 mg of pure enzyme was isolated.

Steady-State Inhibition Studies. Initial velocity kinetic studies were carried out in 100 mM potassium phosphate, pH 7.5, and 1 mM EDTA at 37 °C. Enzyme concentrations ranging from 10^{-8} to 2×10^{-7} M were used for the glutamate dehydrogenase coupled spectrophotometric assay (Badet et al., 1987). L-Glutamate inhibition was followed with the discontinuous assay of $GlcNH_2$-6-P: following a 15-min incubation period at 37 °C of glucosamine synthetase (1.6×10^{-7} to 16×10^{-7} M) in 100 mM potassium phosphate–1 mM EDTA, pH 7.5, in the presence of glutamate concentrations ranging from 0 to 10 mM, the formed $GlcNH_2$-6-P was quantified by the Morgan–Elson procedure (Zalkin, 1985); each experiment was run in duplicate. The experiments using a nonsaturating concentration of one substrate were conducted at 0.6 mM Fru-6-P and 0.4 mM glutamine for glucosamine 6-phosphate inhibition and at 0.6 mM Fru-6-P and 0.3 mM glutamine for glutamate inhibition. Initial velocity data were analyzed manually. Double-reciprocal plots were drawn, but the slopes and intercepts were calculated according to the v^4 weighting method (Wilkinson et al., 1976).

Double-Inhibition Experiments. Double-inhibition experiments were carried out in 100 mM KPO_4^{2-}, 1 mM EDTA, and 10 mM Fru-6-P, pH 7.2, according to the spectrophotometric assay. Enzyme concentrations ranging from 1.6×10^{-8} to 8×10^{-8} M were used with various concentrations of glutamine (0.1–1 mM) and L-FMDP (0–2 μM). Double-reciprocal plots were drawn at different $GlcNH_2$-6-P concentrations (0.5, 1, 2, and 4 mM). Data from double-inhibition experiments at constant substrate concentrations were analyzed by eq 1 (Yonetoni & Theorell, 1964; Northrop & Cleland, 1974)

$$v_i = v_0/(1 + I/K_i + J/K_j + IJ/K_iK_j\beta) \qquad (1)$$

in which v_0 and v_i are the velocities in the absence and the presence of the two inhibitors at concentrations of I and J whose respective dissociation constants are K_i and K_j. The β term is an experimentally derived number that reflects the degree of cooperativity between the two inhibitors.

Inactivation Experiments. For time-dependent inactivation assays, the enzyme solution was extensively dialyzed against 100 mM KPO_4^{2-}, pH 7.2, containing 1 mM EDTA. It was then incubated at 0.3–1.3 μM concentration in the presence of inhibitor and Fru-6-P (10 mM) when necessary. At different time points, aliquots were removed and diluted 20–50-fold into a 1-mL cuvette for activity assay. Control experiments showed that this dilution completely stopped the inactivation reaction.

Determination of Enantiomeric Purity of FMDP Isomers. A total of 200 μL of a 1% Marfey's reagent (Marfey, 1984) solution in acetone was added to 100 μL of a 50 mM FMDP aqueous solution; 20 μL of 2 M HCl was added to the cooled mixture. The derivatized amino acid was analyzed on a C_{18} column with a 45-min linear gradient running at 1 mL/min from 10 to 40% acetonitrile in 50 mM triethylammonium phosphate, pH 3. The compounds were detected by their absorbance at 340 nm.

Synthesis of [2-^{3}H]-DL-FMDP. Asparagine monohydrate (5.2 g, 34.6 mmol), aluminum sulfate–18-water (800 mg, 1.2 mmol), and pyridoxal hydrochloride (800 mg, 4 mmol) were suspended in 50 mL of tritiated water (140 Ci) and adjusted to pH 10. The mixture was magnetically stirred at 20 °C in the dark for 60 h before acidification to pH 5 with acetic acid. After evaporation to dryness ($t^0 < 35$ °C), the residue was dissolved in water (50 mL), ethanol (200 mL) was added, and the yellow solution was kept at −20 °C overnight. The yellow precipitate was recrystallized twice under same conditions and decolorized with Norit A in hot water (30 mL). A last crystallization at 4 °C afforded tritiated asparagine (2.87 g, 64% yield). Tritiated asparagine (2.87 g, 22 mmol) was solubilized in water (32 mL). To the cooled solution was added triethylamine (3.1 mL, 22 mmol) followed by dioxane (30 mL) and di-*tert*-butyl dicarbonate (6 g, 27.5 mmol) in dioxane (30 mL). The solution was allowed to warm up to 20 °C and evaporated to dryness under reduced pressure. The oily residue was redissolved in the minimum amount of water, and the pH was brought to about 2 with 2 M citric acid. The precipitate was collected, washed with cold water, and dried over P_2O_5 to give tritiated N^2-Boc-DL-asparagine (3.55 g, 70% yield). N^2-Boc-DL-2,3-diaminopropionic acid was obtained in 44% yield as an oil following the described protocol (Waki et al., 1981). N^3-(4-Methoxyfumaroyl)-DL-[2-^{3}H]-2,3-diaminopropionic acid was obtained from coupling *N*-succinimidoylfumaric acid methyl ester with tritiated N^2-Boc-DL-2,3-diaminopropionic acid on a 2-mmol scale as described (Andruszkiewicz et al., 1986). The crude product was deprotected in 2 N HCl in dioxane (10 mL) at room temperature. The precipitate was filtered off and washed with dry ether. Tritiated FMDP·HCl was dissolved in water and precipitated by careful neutralization with dilute ammonia. Crystallization from hot water afforded pure FMDP (139 mg, 32% yield): sp act. 9240 cpm/nmol; UV max (water) 213 nm (ϵ 16.4 $cm^2 \cdot \mu mol^{-1}$); proton NMR spectrum identical with reported data; ^{13}C NMR (D_2O, MeOH ext ref) δ 42.51 (CH_2), 55.20 (CH_3), 57.40 (CH), 132.60 and 138.10 (CH=CH), 169.84, 170.15 and 176.80 (C=O).

Results

Initial Velocity Kinetic Pattern of Glucosamine Synthetase. Initial velocity data, obtained by varying both fructose 6-phosphate and glutamine concentrations, indicated (data not shown) that the enzyme follows a sequential mechanism (Segel, 1975). The kinetic constants for Fru-6-P, K_a and K_{ia} (Michaelis and dissociation constants of the EA complex), and for glutamine, K_b and K_{ib}, were found to be 0.59 mM, 0.54 mM, 0.10 mM, and 0.60 mM, respectively. These values are consistent with the apparent kinetic constants that we previously obtained (2 mM for Fru-6-P, 0.4 mM for glutamine) at fixed, saturating concentrations of the second substrate (Badet et al., 1987). No catalytic activity could be detected in the reverse direction.

The kinetics of fructose 6-phosphate and glutamine saturation with the two substrates at nonsaturating concentrations did not show departure from classical Michaelis behavior. The data plotted according to the Hill equation, $\log (v/V_m - v) = n \log S - k$, gave straight lines (data not shown). When the Fru-6-P concentration was at saturation level (10 mM), the

Table I: Product Inhibition Pattern[a] for Glucosamine Synthetase

kinetic pattern	inhibitor	variable substrate: A, nonsat. B	A, sat. B	B, nonsat. A	B, sat. A
ordered bi-bi	P	N	U	N	N
	Q	C	C	N	*b*
random bi-bi	P	N	N	N	N
	Q	N	N	N	N
observed	L-Glu	N	U	N	N
	D-$GlcNH_2$-6-P	C	C	N	*b*

[a] The patterns are designed as competitive (C), noncompetitive (N), and uncompetitive (U) according to Cleland's nomenclature (Cleland, 1970); A and B are the substrates (A binding first in the ordered mechanism); P and Q are the products. [b] No inhibition observed.

Hill plot gave an n value of 0.96 for glutamine; when the Fru-6-P concentration was at nonsaturating level (0.4 mM), the data, expressed as a Hill plot, gave an n value of 0.89 for glutamine.

Product and Dead-End Inhibition Studies. The kinetic patterns obtained from product inhibition studies, as well as the predicted patterns for ordered and random bi-bi kinetic mechanisms, are summarized in Table I. The inhibition by glucosamine 6-phosphate was monitored with the spectrophotometric assay; this method, based on continuous L-glutamate determination by glutamate dehydrogenase, was of course unsuited to L-glutamate inhibition analysis. The glutamate inhibition pattern was therefore determined by the colorimetric detection of $GlcNH_2$-6-P produced during a 15-min incubation in the presence of the required amount of inhibitor. The weak sensitivity of this method compared to the spectrophotometric assay required higher enzyme concentrations but gave the reproducible pattern presented in Table I. Table II summarizes the inhibition constants of L-glutamate and D-glucosamine 6-phosphate, where K_{is} and K_{ii} are the dissociation constants derived from the slope and the intercept, respectively.

Unambiguous results were also obtained from dead-end inhibition patterns in which D-$GlcNH_2$-6-P and the affinity label DON were used as analogues for Fru-6-P and glutamine. Under initial velocity conditions, at saturating substrate concentrations, DON behaves as a dead-end inhibitor binding to the enzyme–Fru-6-P complex (K_i = 1.36 μM; competitive with respect to glutamine; uncompetitive with respect to Fru-6-P). No irreversible inhibition of the enzyme occurred during the course of the experiment. We previously showed that, under irreversible conditions, only the glutamine binding site amino-terminal cysteine residue of the protein is alkylated. These results indicate that glucosamine synthetase obeys an ordered bi-bi mechanism.

Inactivation of Glucosamine Synthetase. On incubation of glucosamine synthetase with FDP derivatives (see structure **1**), time-dependent inactivation ensued (data not shown). In both cases, gel filtration of inactive enzyme did not promote regain in catalytic activity. The inactivation parameters for the two inhibitors are given in Table III together with the kinetic constants of the affinity label DON for comparative purpose. The higher affinity of the enzyme for an inhibitor in the presence of fructose 6-phosphate would suggest prior binding of the latter. This was confirmed by reversible inhibition experiments under initial velocity conditions using amounts of inhibitor which did not affect irreversibly the enzyme in the presence of saturating substrate concentrations.

As predicted by the dead-end inhibition pattern for an inhibitor binding to the E–A complex in an ordered bi-bi mechanism (Segel, 1975), DON and FDP derivatives bind preferentially to the enzyme–Fru-6-P complex (Table III). This point was confirmed in double-inhibition experiments using both FMDP and $GlcNH_2$-6-P. In one set of experiments, the dead-end inhibition pattern of FMDP vs glutamine was determined in the presence of increasing concentrations of $GlcNH_2$-6-P. Under initial velocity conditions, a double-reciprocal plot of velocity vs glutamine concentration should be competitive, a pattern which was indeed observed (Table III). However, in the presence of exogenous $GlcNH_2$-6-P, such a plot would remain competitive only if L-FMDP binds preferentially to E·Fru-6-P. The formation of the tight complex E·$GlcNH_2$-6-P·FMDP would contribute to an intercept effect resulting in a noncompetitive pattern. The patterns remained strictly competitive (data not shown) upon addition of exogenous amino sugar at concentrations sufficient to ensure that a substantial concentration of enzyme–$GlcNH_2$-6-P complex was present in the steady state (as checked by the corresponding decrease in the value of V_{max}). Double-inhibition experiments at constant substrate concentration can be described by eq 1. The β term represents the degree of cooperativity between the two inhibitors I and J. If $\beta < 1$, the binding of I and J is synergistic; if $1 < \beta < \infty$, there is negative cooperativity in the binding of an inhibitor in the presence of the second; if $\beta = \infty$, the binding of the two inhibitors is not cooperative. A plot of $1/v_i$ vs I or J at different concentrations of J or I will yield a set of intersecting lines if $\beta < \infty$ or of parallel lines if $\beta = \infty$. The enzyme activity was therefore determined at fixed concentrations of the substrates (2 mM glutamine, 10 mM Fru-6-P) and variable concentrations of $GlcNH_2$-6-P (0, 0.5, 1, and 2 mM) and L-FMDP (0, 0.25, 0.5, 1, and 2 μM). Parallel lines were indeed observed (data not shown) from the plot 1/velocity versus $GlcNH_2$-6-P concentration, indicating that the binding of L-FMDP and $GlcNH_2$-6-P is mutually exclusive.

The next question we addressed was the stoichiometry of the fixation of the studied compound to the enzyme. With substoichiometric amounts of inhibitor, glucosamine synthetase was incubated at 20 °C until a constant activity was observed (about 2 h). The extrapolation of the curve of enzyme activity vs molar ratio (inhibitor/enzyme subunit) gave the number of inhibitor molecules which are necessary to fully inactivate one enzyme subunit. Figure 1 illustrates the method in the case of DON and L-FMDP. As previously described for DON (Badet et al., 1987), the stoichiometry was raised from 0.5 after incubation in the absence of Fru-6-P to 1 when Fru-6-P was present in the incubation mixture. Table IV summarizes

Table II: Kinetic Constants (mM) for Inhibition of Glucosamine Synthetase by L-Glutamate and D-Glucosamine 6-Phosphate

inhibitor	variable substrate: Fru-6-P, sat. Gln K_{is}	sat. Gln K_{ii}	nonsat. Gln K_{is}	nonsat. Gln K_{ii}	glutamine, sat. Fru-6-P K_{is}	sat. Fru-6-P K_{ii}	nonsat. Fru-6-P K_{is}	nonsat. Fru-6-P K_{ii}
L-glutamate			10.8	5.9	7.4	4.4	17.7	6.6
D-$GlcNH_2$-6-P	0.38		0.35		*a*	*a*	0.93	0.59

[a] No inhibition.

Table III: Kinetic Constants for Reversible and Irreversible Inhibition of Glucosamine Synthetase by DON and FDP Derivatives

	reversible		irreversible		
compd	K_i (μM)[a]	K_m/K_i	K_{irr} (μM)[b]	k_{inact} (min^{-1})	k_{inact}/K_{irr} ($M^{-1}\cdot s^{-1}$)
DON	1.4	71	2.8 (13.7)	0.17 (1.31)	997
FCDP	55.1	1.8	32.9 (53.7)	0.15 (0.50)	76
FMDP	0.35	285	6.4 (6.7)	2.14 (1.39)	5550

[a] Competitive inhibition with respect to glutamine and uncompetitive inhibition with respect to Fru-6-P were observed. [b] The K_{irr} values, resulting from a replot of $1/k_{obsd}$ vs $1/[I]$, were obtained for inactivations carried out in the presence of Fru-6-P. The data resulting from inactivation experiments performed in its absence are given in parentheses.

Table IV: Influence of Fructose 6-Phosphate on the Stoichiometry of Alkylation of Glucosamine Synthetase with DON, FCDP, and FMDP

	stoichiometry (mol of inactivator/mol of enzyme subunit)[a]	
inactivator	-Fru-6-P	+Fru-6-P
DON	0.51	1.03
FCDP	*b*, *c*	0.50[b]
FMDP	0.68	0.87
	0.41[b]	0.56[b]

[a] Determined from titration of residual activity after a 2-h incubation period with substoichiometric amounts of inhibitor (see Figure 1). [b] Determined as in footnote *a* following an 18-h incubation period. [c] Too slow to be determined.

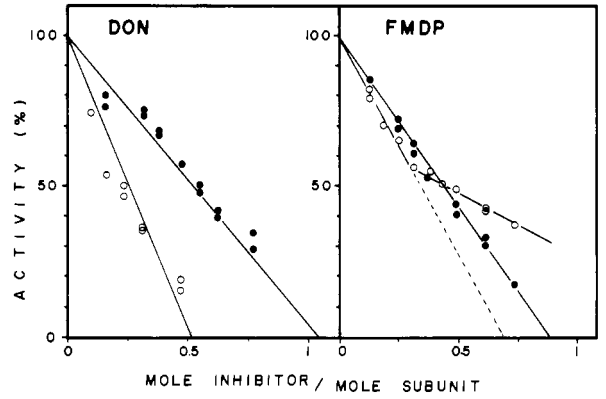

FIGURE 1: Stoichiometry of alkylation of glucosamine synthetase with DON and FMDP. Homogeneous enzyme (0.82 μM in subunit) was incubated at room temperature in 100 mM KPO_4^{2-} and 1 mM EDTA, pH 7.3, with various amounts of inhibitor (0–0.6 μM); Fru-6-P, when present, was added to 10 mM. The residual activity, determined after 120 min with the spectrophotometric assay, was plotted against the molar ratio of inhibitor to enzyme subunit (M_r 70 000). Full symbols, Fru-6-P added; open symbols, no Fru-6-P added.

the inactivation stoichiometries obtained for each inhibitor studied. In the case of L-FMDP, the plot is clearly biphasic, and the value given in Table IV, which corresponds to extrapolation of the first five points, is the result of duplicate experiments. When the mixture was incubated for 18 h before assay for residual activity, the data fit a straight line from which the stoichiometries of 0.41 (no Fru-6-P) and 0.56 (added Fru-6-P) were obtained (data not shown). The inactivation with FCDP turned out to be so slow in the absence of the first substrate that almost no enzyme inactivation occurred after incubation for 18 h. In each case, the remaining activity was corrected from the spontaneous inactivation of the enzyme on incubation conditions (10–20% over 20 h).

Quantitative Studies of Glucosamine Synthetase Inhibition by FMDP. Direct racemization of FMDP in alkaline tritiated water using pyridoxal and Al^{3+} turned out to be of no synthetic use due to the formation of unidentified FMDP derivatives.

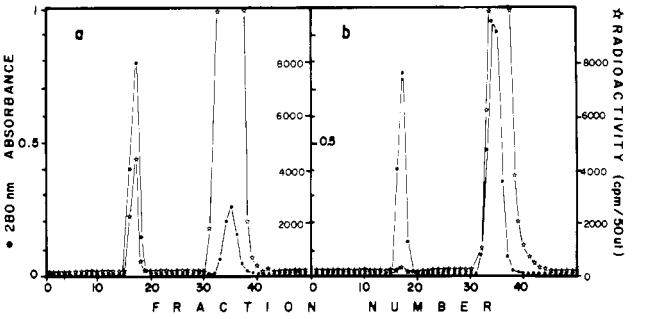

FIGURE 2: Gel filtration of [^{3}H]-DL-FMDP-treated glucosamine synthetase. Enzyme (3.1 mg) was incubated in 0.515 mL of 50 mM KPO_4^{2-}, 1 mM EDTA, and 10 mM Fru-6-P, pH 7.2, without (a) or with (b) 1.1 mM DON. The residual activities after 15 min were respectively 100% (a) and 4% (b). Tritiated FMDP was added to a 2 mM final concentration and the solution further incubated for 2 h at room temperature. The residual activites were 8% (a) and 2% (b). The samples were loaded on a G-25 gel filtration column (1.5 × 50 cm) running in the same buffer without Fru-6-P at 5 mL/h. Fractions of 1.88 mL were collected.

[2-^{3}H]-DL-FMDP was therefore synthesized by known chemical procedures. Asparagine racemization in tritiated water in the presence of Al^{3+} and pyridoxal (Townsend et al., 1983) afforded the tritiated racemic amino acid in 63% yield. The N-protected derivative underwent Hofmann rearrangement through treatment with bis(trifluoroacetoxy)iodobenzene (Waki et al., 1981) to give tritiated N^3-Boc-2,3-diaminopropionic acid in 44% yield; the latter compound was subsequently coupled with succinimidoylfumaric acid methyl ester to give after deprotection the expected [2-^{3}H]-DL-FMDP in 10% yield from asparagine. The radioactive compound (9240 cpm/nmol) exhibited a single radioactive spot by TLC (*n*-BuOH/AcOH/H_2O, 4:2:2) which coincided with the ninhydrin spot (R_f 0.4). A single peak was moreover detectable at 214 nm on a C_{18} HPLC column (10% acetonitrile in water). Proton and ^{13}C NMR of the radiolabeled compound confirmed identity with the unlabeled material.

Unlabeled D- and L-FMDP were synthesized in the same way starting from D- or L-asparagine. The enantiomeric purity of each was determined by generating a mixture of diastereoisomers through reaction with (1-fluoro-2,4-dinitro-5-phenyl)-L-alanine amide (Marfey, 1984). A good separation between the two diastereoisomers (data not shown) allowed us to calculate a 4.6% contamination of the D isomer by the L isomer; the purity of L-FMDP was found to be greater than 99.5%.

Before running any inactivation experiment with the radioactive compound, it was of importance to determine the behavior of D isomer toward glucosamine synthetase. D-FMDP exhibited a competitive behavior with respect to glutamine with a K_i of 141 μM, 100-fold higher than its enantiomer. Moreover, the enzyme inactivation by 3 μM L-FMDP in the presence of 3 μM D-FMDP did not alter the pseudo-first-order inactivation rate constant previously determined for L-FMDP alone (data not shown). Therefore, we could conclude that the presence of D isomer in equimolar amount to L did not modify the behavior of the latter during inactivation experiments.

Incubation of pure glucosamine synthetase with 2 mM [2-^{3}H]-DL-FMDP in 50 mM KPO_4^{2-}, 10 mM Fru-6-P, and 1 mM EDTA at room temperature for 2 h inactivated the enzyme to more than 95%. After gel filtration, the determinations of both radioactivity and protein profiles (Figure 2a) gave a stoichiometry of 0.75 mol of inhibitor/mol of subunit. This value decreased to 0.047 when the enzyme was previously incubated with 1 mM DON for 15 min (4% remaining ac-

Table V: Influence of FMDP Treatment on Titrable Thiol Groups of Glucosamine Synthetase under Denaturing Conditions

sample[a]	thiol concn (μM)	titrable groups per subunit
native		
t = 0	66.7	
t = 4 h	66.4	4.44
native + 2 mM FMDP		
t = 20 s	56.8	3.78
t = 3 h	54.4	3.63
t = 4 h	54.9	3.66

[a] Glucosamine synthetase (15 μM in subunit, M_r 70000) in 100 mM KPO_4^{2-}, 1 mM EDTA, and 10 mM Fru-6-P, pH 7.2, was incubated at 20 °C with 2 mM L-FMDP. Thiol titration was performed by diluting 150 μL of the solution into a cuvette containing 1 mM DTNB in 6.82 M guanidine hydrochloride, 100 mM KPO_4^{2-}, and 1 mM EDTA, pH 7.2, as described (Riddles et al., 1983). Thiol concentration was determined from the increase in absorbance at 412 nm. No reaction occurred between DTNB and FMDP in the absence of enzyme.

tivity) and then treated with 2 mM tritiated FMDP as above (Figure 2b). A similar result (stoichiometry 0.036) was observed when the experiment was repeated with DON-inactivated, gel-filtrated enzyme. This result strongly suggests that the amino-terminal cysteine, responsible for covalent binding of DON (Badet et al., 1987), is involved in glucosamine synthetase inactivation by FMDP. Further evidence was obtained from the titration of thiol groups, before and after FMDP treatment, with Ellman's reagent under denaturing conditions (Table V). The modification of 0.66 thiol residue occurred almost instantaneously, as expected from the k_{inact} previously determined, following inhibitor addition. After 3 h no significant TNB^- release was noticeable. The overall modification of 0.78 (4.44–3.66) SH group, close to the observed stoichiometry of 0.75 for FMDP incorporation, argues for the modification of the glutamine binding site cysteine during the inactivation by FMDP.

Stability of the Adduct to Various Conditions. The stability of the [^{3}H]FMDP–enzyme adduct was tested under different conditions. No tritium release occurred on dialysis against 50 mM KPO_4^{2-} at pH 4.4 or 10 in the presence of 1 M NH_2OH or 0.1 M 2-mercaptoethanol. Enzyme precipitation with trichloroacetic acid, conditions used to release L-glutamate from the glutamyl–thiol ester CTP synthetase adduct (Levitzki & Koshland, 1971), trapped 98% of the radioactivity in the precipitate. However 15% of the radioactivity was released by overnight dialysis against 1 M ammonia, pH 12; this value increased to 45% when the protein was initially in 6 M urea.

Discussion

In our attempts to develop new inhibitors of glucosamine synthetase, it appeared necessary to clarify the behavior of the natural compounds anticapsin and L-FMDP toward the title enzyme. Pioneering work of Chmara et al. (1984) on inactivation of the partially purified enzyme from *S. typhimurium* by L-FCDP (**1**, R = NH_2) facilitated our approach with the pure *E. coli* enzyme. Anticapsin, the most effective inhibitor of *E. coli* enzyme (k_{inact}/K_{irr} = 12000 $M^{-1}\cdot s^{-1}$; Badet et al., unpublished data), would certainly have been the best choice to study this interaction. Its availability is however still a problem as it is so far produced by fermentation. We turned then our attention to the more accessible compound **1** (R = OMe), FMDP.

Prior to addressing the mechanism of enzyme inhibition by these compounds, it was necessary to better understand the kinetic order of the glucosamine synthetase as determined by steady-state analysis. The sequential nature of the reaction was confirmed through initial velocity studies. To determine the order of substrate binding in the formation of the ternary complex, the product inhibition pattern was determined. The correspondence of the results with the predicted patterns was consistent with an ordered binding to the enzyme in which the sugar binds prior to the amino acid substrate. Subsequent dead-end inhibition experiments using DON as a competitive inhibitor of glutamine confirmed the ordered bi-bi mechanism. An ordered binding of the substrates has been proposed (Winterburn & Phelps, 1971) for the highly regulated glucosamine synthetase from rat liver; the complexity of the data did not allow a distinction between the ordered bi-bi and bi-bi ping-pong mechanisms. More recently, an ordered mechanism was suggested for the bacterial enzyme on the basis of uncompetitive inhibition vs fructose 6-phosphate observed with anticapsin and FMDP (Chmara et al., 1986).

We initially hypothesized that, in the mechanism of glucosamine synthetase inhibition by FMDP, the inhibitor would bind preferentially with one enzyme form resulting in a ternary complex composed of fructose 6-phosphate and inhibitor within the glutamine binding site of the enzyme as occurred with DON. The results of inhibition under initial velocity conditions verified this proposal. Moreover, double-inhibition experiments corroborated the fact that the binding of glucosamine 6-phosphate has no cooperative effect on the binding of FMDP.

L-FMDP behaved as a time-dependent inhibitor of *Escherichia coli* glucosamine synthetase. The same behavior has been previously characterized with a 7-fold less highly purified preparation from *S. typhimurium* (Chmara et al., 1986). The positive intercept of the double-reciprocal plot indicates that inactivation involves a saturable enzyme–inhibitor complex prior to the onset of inactivation (Tipton, 1980). The apparent second-order rate constant k_{inact}/K_{irr}, reflecting the efficiency of the inhibitor under study (Fersht, 1985), is 5-fold higher for FMDP than for DON. As depicted by the ratio K_m/K_i (Table III), FMDP exhibits the highest affinity to the glutamine binding site. Chmara et al. (1986) reported similar values for the *S. typhimurium* enzyme. They observed however a much lower reactivity of DON since, from comparison of second-order rate constants, FMDP (k_{inact}/K_{irr} = 6100 $M^{-1}\cdot s^{-1}$) was about 40-fold more efficient than DON (k_{inact}/K_{irr} = 160 $M^{-1}\cdot s^{-1}$). They noticed, as we did in this study, the dramatic drop of the inhibition constants through modification of the ω-carboxyl group of the molecule.

The stoichiometry of the binding was first estimated from partial inactivation experiments using substoichiometric amounts of inhibitor. With DON, glucosamine synthetase clearly exhibited half-of-the-sites reactivity in the absence of the first substrate. L-FMDP however did not give such a clear-cut result (see Results). There is at the moment no complete explanation for half-of-the-sites reactivity phenomena; a conformational change occurring on fixation of the first inhibitor molecule, preventing the next one to bind, is a possible explanation which has been proposed for DON-mediated CTP synthetase inactivation (Levitzki et al., 1971).

The value of n (0.89) for glutamine obtained from the Hill plot at nonsaturating Fru-6-P concentration (data not shown) indicates a weak negative cooperativity behavior for the binding of glutamine. The substrate analogues DON and FMDP might so enhance the phenomenon that the second inhibitor molecule is prevented from binding by the first covalent label. With FMDP however, these results must be taken with caution. A partial processing of the molecule would actually decrease the theoretical stoichiometry. FMDP in this case would partially act as an alternate substrate, substituting for

glutamine to yield different products (fumarate methyl ester and diaminopropionate are obvious candidates); the expected competitive behavior vs glutamine would remain unchanged and the expected noncompetitive inhibition with respect to Fru-6-P nondetectable. At this point it is worth mentioning that no effect on enzyme activity was seen on incubation of the enzyme with a 1:1 mixture of fumarate methyl ester and diaminopropionate up to a concentration of 4 mM with saturating substrate concentrations.

The synthesis of labeled FMDP seemed necessary to track the culprit. We decided for expediency to introduce the radioactivity on the α-position of the amino acid function. The use of tritiated DL-FMDP confirmed the covalent binding of the inhibitor to the enzyme with a stoichiometry of 0.75. The possible influence of the D isomer on L-mediated enzyme inactivation was ruled out after showing that the former had almost no affinity for glucosamine synthetase.

The selectivity of the compound for the glutamine binding site cysteine is strongly suggested by (1) the complete abolition of radioactivity incorporation through preliminary incubation with DON, an affinity label known to alkylate selectively the amino-terminal cysteine residue and (2) the decrease of 0.78 titrable thiol residue in denatured, FMDP-inactivated protein. Much effort has been devoted to localize the modified residue on the purified radiolabeled peptides resulting from protein cleavage (data not shown); even if a peptide containing the active site cysteine was systematically identified from the sequence, the lability of the radioactivity to sequencing conditions (most of the tritium was released before the first cycle) precluded unambiguous attribution.

The covalent association of the radioactivity with the protein suggests that inactivation occurs mainly from trapping the whole inhibitor molecule by an active site nucleophile. Therefore a nucleophilic attack of the SH group on the central amide carbonyl leading to the formation of a fumaroyl thio ester and hence to enzyme inactivation, a mechanism originally proposed by Chmara et al. (Chmara et al., 1986), cannot be taken as the main event responsible for inactivation (but might be part of it). We suggest rather a Michael addition of the thiol nucleophile at the β-position of the ester generating a saturated inhibitor–enzyme adduct. This adduct, when exposed to the solvent, would undergo easily the reverse reaction under alkaline conditions (as the trimethylamine treatment during the gas-phase sequencing) to regenerate phenyl isothiocyanate modified inhibitor. The lack of activity reported by Chmara et al. (1986) for compounds related to **1** in which the double bond has been modified (saturated or substituted) might support such a mechanism. More work will be necessary to unravel the mechanistic details of this unexpectedly complex inhibition.

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