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Glucosamine-6-phosphate Synthase from *Escherichia coli*: Determination of the Mechanism of Inactivation by N^3-Fumaroyl-L-2,3-diaminopropionic Derivatives†

Nathalie Kucharczyk, Marie-Ange Denisot, François Le Goffic, and Bernard Badet*
Laboratoire de Bioorganique et Biotechnologies, UA CNRS 1389, ENSCP, 11 rue Pierre et Marie Curie, 75231 Paris Cedex 05, France

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ABSTRACT: A mechanistic investigation of the inactivation of *Escherichia coli* glucosamine-6-phosphate synthase by N^3-(4-methoxyfumaroyl)-L-2,3-diaminopropionate (FMDP) was undertaken. On the basis of the known participation of the *N*-terminal cysteine residue in this process [Chmara et al. (1986) *Biochim. Biophys. Acta 870*, 357; Badet et al. (1988) *Biochemistry 27*, 2282], the model reactions between FMDP and L-cysteine and between FMDP and the synthetic decapeptide Cys-Gly-Ile-Val-Gly-Ala-Ile-Ala-Gln-Arg, corresponding to the amino-terminal protein sequence, were studied. The results allowed us to propose a pathway that is in perfect agreement with the biochemical results: enzyme inactivation arose from Michael addition of glutamine binding site cysteine-1 on the fumaroyl double bond at the β-position of the ester group. Upon denaturation under slightly alkaline conditions, this adduct underwent cyclization to a transient succinimide adduct, which rearranged into the stable 2-substituted 1,4-thiazin-3-one-5-carboxylate involving participation of the cysteine amino group. The tryptic radiolabeled peptides purified from [^{3}H]FMDP-treated enzyme and resistant to Edman degradation coeluted with the products resulting from the model reaction between the synthetic decapeptide and the inhibitor.

Of all microbial infections in humans, the mycoses, particularly those affecting immunocompromised patients, are the most difficult to cure. Although real progress has been made in the development of antifungal drugs (Drouhet et al., 1987; Stevens, 1987; Graybill, 1989), only a few are active both in vitro and in vivo. The problems at present include the need for more effective agents, particularly with a novel mode of action. Glucosamine-6-phosphate synthase has been recognized only quite recently (Kenig et al., 1976; Chmara et al., 1984; Andruszkiewicz et al., 1984; Milewski et al., 1988) as a potential target for antibacterial and antifungal drug design.

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* To whom correspondence should be addressed.

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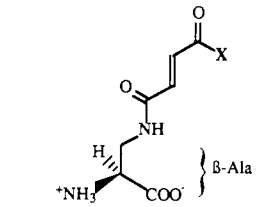
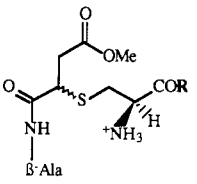
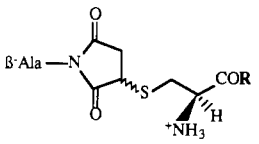
Chart I



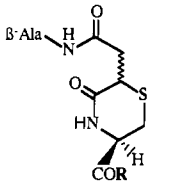
1 FMDP X= OCH_3
FCDP X= NH_2



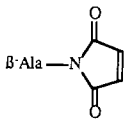
2 R= OH
12 R= GIVGAIAQR



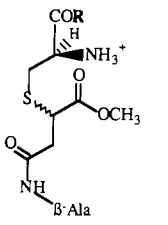
3 R= OH
13 R= GIVGAIAQR



4 R= OH
14 R= GIVGAIAQR



5



6 R= OH
16 R= GIVGAIAQR

This enzyme, which catalyzes the first reaction on the pathway of hexosamine biosynthesis (fructose-6P + L-glutamine → L-glutamate + glucosamine-6P), is indeed essential for the bacteria and fungi to build their protective cell wall of peptidoglycan and chitin, respectively. To date, four naturally occurring glucosamine synthase inhibitor precursors have been described. Anticapsin (Walker & Abraham, 1970) and chlorotetain (Rapp et al., 1988), produced respectively by *Streptomyces griseoplanus* and *Bacillus subtilis* strains, are dipeptides that contain, besides the N-terminal L-alanine, a β-(4-oxocyclohexyl)-substituted alanine; compounds A19009 (Molloy et al., 1972) and Sch 37137 (Cooper et al., 1988), isolated respectively from *Streptomyces collinus* and *Micronospora* strains, consist of an exotic amino acid, N^3-fumaramoyl-L-2,3-diaminopropionic acid (**1**, X = NH_2; Chart I) or its epoxide, coupled to L-alanine through the carboxyl or the amino group, respectively. These compounds exhibit good antifungal properties through inhibition of the glucosamine-6P-synthesizing enzyme of the host (Chmara et al., 1984; Chmara, 1985; Milewski et al., 1986) following permease-mediated internalization of the drug and cleavage by intracellular peptidases. However, the mechanism of action of the warhead is poorly understood. For the last 5 years investigations have been carried out in our group with synthetic derivatives of **1**. A systematic study of closely structurally related compounds conducted by Chmara and co-workers (Milewski et al., 1985; Chmara et al., 1986) has shown the corresponding methyl ester FMDP[1] (**1**, X = OCH_3) to be the most efficient inhibitor of partially purified glucosamine synthase from *Escherichia coli*, *Salmonella typhimurium*, and *Candida albicans*. The following structural requirements for enzyme inhibition have been demonstrated: (a) the carbon–carbon double bond is necessary, and substitution of the double bond by a methyl group resulted in a loss of activity; (b) the presence of a terminal carboxylic group or derivative is essential. For all the active compounds, time-dependent glutamine site directed inhibition has been observed, suggesting an irreversible modification of the enzyme.

Using the pure overexpressed glucosamine synthase from *E. coli*, our group recently demonstrated that irreversible inhibition stemmed from covalent modification of the catalytic amino-terminal cysteine; formation of a carbon–sulfur bond, albeit a tempting hypothesis (Badet et al., 1988), had to deal with the lack of reactivity of thiols with compounds **1** reported by our colleagues (Chmara et al., 1986).

In the present work, the study of the model reaction between cysteine and FMDP revealed Michael acceptor behavior of the inhibitor; the initial Michael adduct can undergo further transformation above pH 6 to give a substituted 1,4-thiazin-3-one. The synthetic thiazinone adduct between FMDP and the decapeptide CGIVGAIAQR is identical with the major tryptic peptide resulting from glucosamine synthase inactivation by radiolabeled FMDP.

Experimental Procedures

Materials

Silica gel thin-layer chromatography (TLC) plates $60F_{254}$ were purchased from Merck. TPCK-treated trypsin was from Cooper Biomedicals. Fumarase (EC 4.2.1.2) from porcine heart (380 units/mg in 3.3 M ammonium sulfate) and malic enzyme (EC 1.1.1.40) from chicken liver (14.8 units/mg in 2.9 M ammonium sulfate) were from Sigma. Lactate dehydrogenase (EC 1.1.1.27) from hog muscle (550 units/mg in 50% glycerol) was from Boehringer. Diaminopropionate ammonia lyase from *Salmonella typhimurium* was a kind gift of Dr. Tanizawa. The decapeptide CGIVGAIAQR was obtained from Neosystem Laboratories (Strasbourg, France).

N^2-Boc-L-2,3-diaminopropionic acid was synthesized as described (Kucharckzyk et al., 1989); the synthesis of tritiated FMDP (9240 cpm/nmol) has been previously described (Badet et al., 1988). [^{14}C]Methanol (46 Ci/mol) was purchased from CEA. Glucosamine-6P synthase from *Escherichia coli* was assayed as previously described (Badet et al., 1987).

HPLC analyses were performed on a Perkin-Elmer 3B apparatus connected to a Spectraflow 757 (Kratos) detector with 5-μm Nucleosil C_{18} (Prolabo or Société Française de Chromatographie) columns. For kinetic experiments, peak areas were quantified on a Waters 745 data module; linear first-order or second-order replots were analyzed by the Cricket Graph program running on a MacIntosh. The peaks were characterized either by their retention time t_R or by their R_f, defined as the ratio $(t_R - t_o)/t_o$, where t_o is the retention time of the solvent. The purifications were performed by ion-exchange chromatography (AG1X4, acetate form; Bio-Rad) and/or by preparative HPLC on a Jobin-Yvon Miniprep using a 15–20-μm Licroprep C_{18} (Merck) as the stationary phase.

Biochemical Methods

Inactivation of Glucosamine-6P Synthase by [methoxy-^{14}C]FMDP. Enzyme (87 mg, 0.33 mM) was incubated for 10 h at room temperature in 20 mM KPO_4–1 mM EDTA, pH 7.2, in the presence of [^{14}C]FMDP (5.4 mM). The resulting solution (residual activity 11%) was loaded on a G-25 (su-

[1] Abbreviations: DON, 6-diazo-5-oxo-L-norleucine; FAB, fast atom bombardment; FCDP, N^3-fumaramoyl-L-2,3-diaminopropionic acid (**1**, R = NH_2); FMDP, N^3-(methoxyfumaroyl)-2,3-diaminopropionic acid (**1**, R = OCH_3); HPLC, high-pressure liquid chromatography; TFA, trifluoroacetic acid; KPO_4, a mixture of KH_2PO_4 and K_2HPO_4 at the same molarity.

perfine) column (50 × 2.5 cm) equilibrated in 100 mM NH_4HCO_3, pH 7.2. Complete separation of the protein from small molecules was accomplished at a flow rate of 25 mL/h. The protein peak (22 mL) was isolated and specific radioactivity (2600 cpm/nmol) determined by scintillation counting; the major radioactive peak corresponding to the small molecules was pooled and lyophilized. The inactivated protein (10% residual activity) released 98% of the associated radioactivity upon overnight dialysis at 4 °C against 100 volumes of 6 M guanidine, 0.3 M Tris, and 2 mM EDTA, pH 8.

Inactivation of Glucosamine-6P Synthase by [^{3}H]FMDP: Proteolysis and Purification of Radiolabeled Peptides. Inactivation with [^{3}H]FMDP was performed on 220 mg of enzyme (0.375 mM) as described above in the presence of 2 mM Fru-6P with a 12-fold excess of inhibitor. Following gel filtration, the enzyme exhibited 9% residual activity and a specific radioactivity of 5130 cpm/nmol. The inactivated enzyme (35 mL, 14.9 × 10^6 cpm) was dialyzed against 6 M guanidine, 0.3 M Tris, and 2 mM EDTA, pH 8 (3 × 1 L overnight), without loss of radioactive label; after reductive alkylation with iodoacetamide (Badet et al., 1984) the solution was extensively dialyzed against 100 mM NH_4HCO_3, pH 8. The resulting turbid solution (100 mL) was incubated at 37 °C with trypsin (1.5 mL of a 4 mg/mL solution) for 2 h and for a further 4 h following addition of the same amount of protease. After lyophilization the residue was dissolved in 50% acetic acid and the solution clarified by centrifugation. The supernatant (16 mL, 13.7 × 10^6 cpm) was loaded on a TSK HW-40F (Merck) gel filtration column (135 × 2.9 cm) running at 25 mL/h in 20% acetic acid. The broad radioactive peak eluting after 400 mL was concentrated under reduced pressure below 20 °C. The peptide mixture dissolved in 5 mL of 50% acetic acid (10.75 × 10^6 cpm) was further fractionated on a PepRPC HR 16/10 (Pharmacia) hydrophobic column with a 0–50% acetonitrile gradient (4 mL/min over 40 min). The broad radioactive peak observed by 214-nm detection was collected and further purified by HPLC with a preparative C_{18} column (SupRS Prep, 25 × 2 cm, Prolabo) and the same solvent system. Three radioactive peptides (A, B, and C) were isolated at this stage; they were ultimately purified on an analytical C_{18} column under isocratic conditions (acetonitrile/water containing 0.1% TFA, 0.8 mL/min): A (180000 cpm, 17.5% CH_3CN, t_R = 18.8 min), B (1.17 × 10^6 cpm, 20% CH_3CN, t_R = 13 min), and C (2.42 × 10^6 cpm, 20% CH_3CN, t_R = 15 min).

Determination of Fumarate. Fumarate was determined by the coupled assay with fumarase and malic enzyme (Burlina, 1983; Outlaw & Springer, 1983). Each assay contained in a 1-mL cuvette 100 mM Hepes, 4 mM $MnCl_2$, 2 mM NADP, 0.1 mM coenzyme A, and 12 units of fumarase. The reaction initiated by malic enzyme addition was followed at 340 nm; in our conditions the detection limit was estimated to be 5 nmol with no effect of added (up to 10 mM) diaminopropionate.

Determination of L-*2,3-Diaminopropionate.* Diaminopropionate was quantified by the determination of the amount of pyruvate produced during incubation with diaminopropionate ammonia lyase (Tanizawa et al., 1988). Each assay contained in a 1-mL cuvette 100 mM KPO_4, pH 7.5, 0.33 mM NADH, 0.2 unit of lactate dehydrogenase, and 0.12 unit of diaminopropionate ammonia lyase. The reaction was followed at 340 nm; under our conditions, 5 nmol of diaminopropionate could be easily detected with no effect of added (up to 10 mM) fumarate.

Peptide Sequencing. The amino acid sequences of the isolated peptides were determined by Dr. Bonicel (CNRS-CBM, Marseille, France) using an Applied Biosystem 470A gas-phase sequencer. The PTH-amino acids were identified on a Waters Associates HPLC equipped with an Applied C_{18} column.

Chemical Methods

Synthesis of [methoxy-^{14}C]FMDP. [^{14}C]Methanol (3.24 mCi, 46 Ci/mol) was transferred under vacuum to a 10 × 100 mm vacuum hydrolysis tube (Pierce) containing recrystallized maleic anhydride (98 mg, 1 mmol) and methanol (40 μL, 1 mmol) maintained in liquid nitrogen. After return to room temperature, the vessel was heated at 50 °C for 12 h; 15 μL of oxalyl chloride was added, and heating was continued for 4 h. The crude product was purified by preparative TLC (silica gel; ether–acetic acid, 99:1) to give monomethyl fumarate (65 mg, 50%). A solution of this compound in tetrahydrofuran (2 mL) was added to *N*-hydroxysuccinimide (63 mg, 0.55 mmol) in the same solvent (1 mL). Dicyclohexylcarbodiimide (113 mg, 0.55 mmol) was added to the solution cooled in ice. After 10 h of stirring, the filtered solution was used directly for coupling with N^2-Boc-L-2,3-diaminopropionic acid as described (Andruszkiewicz et al., 1986). The crude product was purified on TLC (silica gel, AcOEt–MeOH–AcOH, 60:40:1) to afford N^2-Boc-FMDP (59 mg, 37%). Removal of the *tert*-butoxycarbonyl group was quantitatively performed by 2.9 N HCl in dioxane. The radiochemical purity of [^{14}C]FMDP solubilized in water (5.6 mL) was 80% as determined by radioactive TLC scanning; the FMDP concentration of the aqueous solution was calculated from its 213-nm absorbance (ϵ = 16.4 cm^2/μmol). These data allowed the determination of the specific radioactivity of the ^{14}C-labeled material, 4160 cpm/nmol.

*N-β-*L-*Alanylmaleimide* (**5**). Boc-diaminopropionic acid (1 g, 4.9 mmol) in acetic acid (10 mL) was stirred at room temperature with maleic anhydride (0.48 g, 4.9 mmol) for 4 h. After concentration, the residue was crystallized (acetone/pentane) to give N^2-Boc-N^3-[carboxy-(*Z*)-acryloyl]-L-2,3-diaminopropionate (1.21 g, 82%) as a white powder: mp 139 °C; TLC (silica gel, PrOH–H_2O, 80:20) R_f = 0.47; ^{1}H NMR (250 MHz, D_2O) δ 1.3 (s, 9 H), 3.4 (m, 2 H), 4.15 (m, 1 H), 6.19 (d, 1 H, J = 12.2 Hz), 6.36 (d, 1 H, J = 12.2 Hz).

This compound (500 mg, 1.65 mmol) was refluxed in 50 mL of toluene containing triethylamine (334 mg, 3.31 mmol) for 4 h. The oily residue was discarded. The solvent was removed in vacuo and the residue solublized in water; the aqueous solution was acidified to pH 2 with citric acid and extracted with ethyl acetate. The dried organic solvent was removed to give, after trituration with ether, N-protected compound **5** as a white foam (260 mg, 55%): mp 96 °C; ^{1}H NMR (250 MHz, acetone) δ 1.4 (s, 9 H), 3.8 (m, 2 H), 4.4 (m, 1 H), 6.1 (br s, 1 H), 6.9 (s, 2 H), 8 (br s, 1 H).

Compound **5** was obtained as the hydrochloride after treatment with HCl-saturated dioxane. The free amino acid was isolated in 80% yield by percolating the solution through AG1X2 resin (acetate form) and eluting with water: TLC (silica gel PrOH–H_2O, 70:30) R_f = 0.33; ^{1}H NMR (250 MHz, D_2O) δ 3.78 (dd, 1 H, J_{AX} = 7.3 Hz, J_{BX} = 3.6 Hz), 3.8 (dd, 1 H, J_{AB} = 15 Hz, J_{BX} = 3.6 Hz), 3.88 (dd, 1 H, J_{AB} = 15 Hz, J_{AX} = 7.3 Hz), 6.75 (s, 2 H).

Reaction of L-*Cysteine with FMDP.* The NMR spectra of the different products **2**–**4** resulting from the reaction between L-cysteine and FMDP at different pHs were analyzed by a computer program designed on an Atari 1040ST according to the resolution methods developed by Gunther (1985). All spectra are the superimposition of two ABX (cysteine and succinate parts) and one ABC systems (diaminopropionate

part). The ABC system was analyzed according to the results of Corrio (1960).

(*a*) *Experiment at pH 5.* FMDP (216 mg, 1 mmol) and L-cysteine (125 mg, 1 mmol) were suspended in 10 mL of water. The resulting solution (pH 5) clarified after 3 h of stirring at room temperature. The mixture was stirred overnight (no starting material could be detected after this period) and then frozen and lyophilized. HPLC purification (H_2O–0.1% TFA) gave **3** (147 mg, 0.48 mmol; $R_f = 0$) as a 60:40 mixture of diastereoisomers nonseparable by HPLC but distinguishable by NMR (Table I): TLC (silica gel, BuOH–AcOH–H_2O, 4:2:2) $R_f = 0.21$; MS (FAB) 306 (MH^+). Compound **2** [108 mg, 0.32 mmol, TLC (silica gel, BuOH–AcOH–H_2O) $R_f = 0.31$; MS (FAB) 338 (MH^+)] could be resolved into its diastereoisomers (28:72) under the HPLC conditions ($\mathbf{2_m}$, $R_f = 1.27$; $\mathbf{2_M}$, $R_f = 1.37$).

(*b*) *Experiment at pH 8.* The reaction was run under same conditions as above after adjustment of the pH to 8 with 1 N sodium hydroxide. The mixture was quenched after 5 h with acetic acid and lyophilized. HPLC analysis (H_2O–0.1% TFA) indicated a 98:2 mixture of thiazinones $\mathbf{4_m}$ and $\mathbf{4_M}$ (R_f = 4.13 and 3.91, respectively). The crude mixture was loaded on an anion-exchange AG1X4 (acetate form) column, which was eluted with a linear gradient of 0–2.5 M acetic acid. The ninhydrin-positive fractions were lyophilized. TLC (silica gel, BuOH–AcOH–H_2O, 4:2:2) $R_f = 0.28$; MS (FAB) 306 (MH^+); ^{13}C NMR (62.87 MHz, D_2O) δ 27.53 (CH_2), 39.83 (CH), 40.87 and 41.67 (CH_2), 56.39 and 59.10 (CH), 172.9, 173.58, and 176.2 (C=O).

Reaction of the Decapeptide CGIVGAIAQR with FMDP: Obtention of Thiazinone **14**. A total of 284 μL of a 20 mM FMDP solution (5.68 μmol) in 100 mM K_3PO_4, pH 8, was added to 5.6 mg of peptide (5.68 μmol) dissolved in 6 M urea. After 3 h of stirring, the solution turned into a gel. Then, 100 μL of 20% acetic acid was added and the crude product purified by HPLC (C_{18}, CH_3CN–H_2O, 25:75, containing 0.1% TFA, $R_f = 2.81$) to afford **14** (4 mg, 60%). ^1H NMR (250 MHz, D_2O/AcOD): (diaminopropionate) δ 3.98 (dd, H_A, J_{AB} = 15.2 Hz, J_{AC} = 3.7 Hz), 3.7 (dd, H_B, J_{AB} = 15.2 Hz, J_{BC} = 5.7 Hz), 4.23 (dd, H_C, J_{AC} = 3.7 Hz, J_{BC} = 5.7 Hz); (cysteine) δ 3.26 (dd, H_A, J_{AB} = 14.7 Hz, J_{AX} = 5 Hz), 3.10 (dd, H_B, J_{AB} = 14.7 Hz, J_{BX} = 4.4 Hz), 4.54 (dd, H_X, J_{AX} = 5 Hz, J_{BX} = 4.4 Hz); (succinate) δ 2.97 (dd, H_A, J_{AB} = 15.3 Hz, J_{AX} = 6.2 Hz), 2.85 (dd, H_8, J_{AB} = 15.3 Hz, J_{BX} = 8.4 Hz), 3.97 (dd, H_X, J_{AX} = 6.2 Hz, J_{BX} = 8.4 Hz). MS (FAB): 1171 (MH^+).

Results

Inactivation of Glucosamine-6P Synthase with Radiolabeled FMDP. Incubation of the enzyme with a 16-fold excess of [*methoxy*-^{14}C]FMDP for 10 h resulted in 90% enzyme inactivation together with the incorporation of 0.64 equiv of inhibitor. The radiolabeled complex released most of its counts (>98%) upon dialysis under slightly alkaline denaturing conditions (6 M guanidine, pH 8). Under the same conditions, enzyme inactivation with the α-tritiated compound resulted in the formation of a radiolabeled protein–inhibitor complex with a stoichiometry of 0.55; the associated radioactivity was perfectly stable in the denaturing dialysis solution.

Assay for Fumarate or Diaminopropionate Formation. The solution resulting from enzyme (3.125 μmol) inactivation with [^3H]FMDP was gel filtrated as described under Biochemical Methods; the excess of inhibitor and possible small molecules were recovered as the second radioactive peak eluting after the protein. After lyophilization, the residue dissolved in water (2 mL) was brought to pH 12 with 1 N KOH and incubated for 30 min at room temperature; under these conditions the monomethyl fumarate is quantitatively converted into fumarate. The resulting neutralized mixture was assayed for the presence of fumarate as follows: malate, produced from the fumarase action, was converted into pyruvate in an NADP-dependent reaction catalyzed by the malic enzyme. With 100 μL (5%) of the alkali-treated solution, no increase in the 340-nm absorbance could be detected. The same solution was also checked for the presence of diaminopropionate by determination of the amount of pyruvate produced during incubation with diaminopropionate ammonia lyase (pyruvate-forming enzyme; Tanizawa et al., 1988). Again, no increase in the 340-nm absorbance could be detected by the NAD-dependent lactate dehydrogenase with 100 μL of the same solution. Under the conditions of these analyses 10 mM FMDP did not interfere with the enzymatic activities. Assuming a detection threshold of 5 nmol in each test, less than $5 \times 20/3125 = 0.03$ mol of fumarate or diaminopropionate was produced per mole of glucosamine-6P synthase (M_r = 70 000) during inactivation.

Proteolysis of [^3H]*FMDP-Inactivated Enzyme: Peptide Purification and Sequencing.* The radioactive complex resulting from enzyme inactivation with the tritiated inhibitor was digested with trypsin after reductive alkylation. Multistep HPLC purification afforded, in 25% overall yield, three radiolabeled peptides, A, B, and C (5:31:64), homogeneous on analytical C_{18} and phenyl columns. The amino acid sequence of peptide A was XGIVGAIAQR, corresponding to the amino-terminal sequence of native glucosamine synthase. However, most of the radioactivity was released during the preliminary washing steps, and only 5% of the radioactivity loaded in the sequencer was associated with the first residue (cysteine), which could not be identified. Both peptides B and C were refractory to degradation.

Model Reaction between FMDP and Cysteine. The reaction of stoichiometric amounts of L-cysteine and FMDP at slightly alkaline pH (8) resulted in rapid [$t_{1/2}$(FMDP) = 15 s] and quantitative formation of cyclic adduct **4** identified by mass and NMR spectra. Analytical HPLC revealed a 98:2 mixture of diastereoisomers, which could be separated and analyzed by NMR: both compounds exhibited almost identical spectra except for the methylenic protons (H_A and H_B) of the succinic part (Table I).

When the same experiment was performed without pH adjustment (pH of the solution about 5), the resulting mixture consisted of two different products: major (60%) cyclic adduct **3** nonretained on C_{18} HPLC and minor (40%) acyclic adduct **2** as a mixture of two HPLC-resolved diastereoisomers (60:40). $\mathbf{2_M}$ and $\mathbf{2_m}$ exhibited very similar NMR spectra as the two methylenic protons (H_A and H_B) of the diaminopropionate part have the same chemical shift; they slightly differed however in the ABX cysteine part.

NMR analysis of compound **3** revealed a 72:28 diastereomeric mixture of succinimide derivatives: as in **2**, the two methylenic protons of the diaminopropionate part have the same chemical shift, but the 0.6 ppm differences between the two methylenic protons of the succinic part is characteristic of a cyclic derivative. The authentic compound was quantitatively synthesized (as a 55:45 mixture) from the reaction between cysteine and *N*-β-L-alanylmaleimide (**5**) (pH 4, 5 h).

Evolution of the Reaction and of Purified Compounds **2**–**4** *at pH 8.* The reaction of stoichiometric amounts of L-cysteine and FMDP was followed under pH-controlled conditions (100 mM KPO_4, pH 8). At different time points aliquots were withdrawn, quenched with 20% phosphoric acid to pH 2, and

Table I: 250-MHz ^{1}H NMR Spectra (D_2O) of Adducts between Cysteine and FMDP[a]

		diamino-propionate		cysteine		succinimide	
		ppm	J (Hz)	ppm	J (Hz)	ppm	J (Hz)
2[b]	$\mathbf{2_m}$	3.61		3.07	14.7	2.82	17.0
		3.61	5.1	3.02	4.4	2.69	9.1
		3.96	5.1	3.94	6.7	3.68	5.9
	$\mathbf{2_M}$	3.63		3.10	14.8	2.83	17.1
		3.63	3.8	2.90	4.0	2.68	8.6
		3.99	3.8	4.02	7.9	3.65	6.5
3	$\mathbf{3_m}$	3.89		3.37	15.1	3.17	19.0
		3.89	5.3	3.06	4.5	2.56	9.2
		4.09	7.8	4.13	7.9	3.98	4.4
	$\mathbf{3_M}$	3.89		3.27	15.5	3.17	19.0
		3.89	4	3.17	4.2	2.56	9.5
		4.09	4	4.20	7.8	3.95	4.6
4	$\mathbf{4_m}$	3.69	14.9	3.12	13.2	2.73	15.3
		3.48	3.7	2.89	4.2	2.49	6.6
		3.78	6.9	4.20	7.2	3.62	7.7
	$\mathbf{4_M}$	3.69	14.8	3.07	13.9	2.74	15.4
		3.43	3.7	2.92	4.1	2.66	5.7
		3.79	6.2	4.27	5.4	3.65	8.4

[a] The chemical shifts of the methylene protons (H_A and H_B in two ABX and one ABC systems) and of the methine proton (H_X or H_C) were determined from the spectra as described under Chemical Methods. [b] 3.51 ppm (s, OCH_3).

analyzed by isocratic reverse-phase HPLC. As shown in Figure 1 the use of 2% MeOH in water allowed a very good separation of the starting FMDP from the Michael adducts **2** and the thiazinones **4**; separation of $\mathbf{2_m}$ from $\mathbf{2_M}$ could be performed in pure water. The succinimide derivatives, nonretained under these conditions, could not be quantified during the reaction. The evolution in the reaction mixture composition (Figure 1, insert) showed a rapid disappearance of FMDP ($t_{1/2}$ = 15 s) concomitant with the formation of the Michael adducts **2** and the slower formation of $\mathbf{4_M}$ ($t_{1/2}$ = 300 s).

Compound **2**. Both diastereoisomers of the Michael adducts were separately incubated under the same conditions, and the composition of the mixture during the reaction was followed as described above. Each compound disappeared with first-order kinetics (Table II) to give in both cases the same 98:2 diastereoisomer mixture of **4**. The formation of compounds **4** followed complex kinetics but was obviously slower than disappearance of the starting material (Table II). In each experiment using one of the two isomers of **2**, no formation of the other could be detected during the time course of the reaction.

Compound **3**. When compound **3** (as a 60:40 mixture of diastereoisomers) was incubated in 100 mM K_3PO_4, pH 8, thiazinone **4** was the only compound to be detected (as a 98:2 mixture of isomers).

Compound **4**. The thiazinone adducts $\mathbf{4_m}$ and $\mathbf{4_M}$ did not epimerize into each other when incubated overnight at room temperature in 100 mM KPO_4, pH 8.

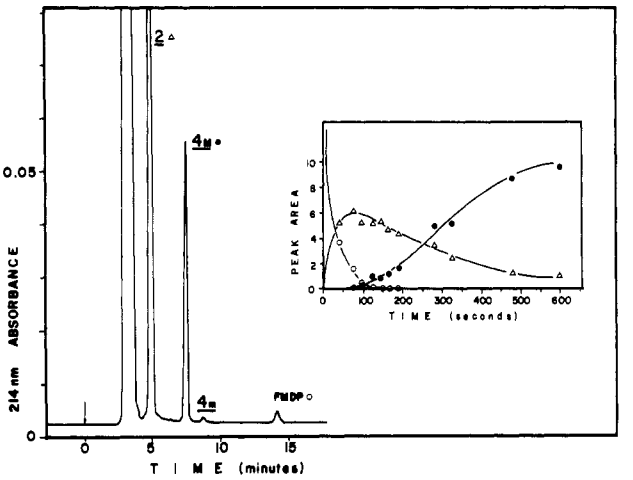


FIGURE 1: Reverse-phase HPLC profile (2% MeOH in water containing 0.1% TFA, isocratic) at t = 145 s of the reaction between FMDP and L-cysteine in KPO_4 buffer, pH 8. (Insert) composition of the mixture as a function of the reaction progress: (○) FMDP; (△) $\mathbf{2_M}$ + $\mathbf{2_m}$; (●) $\mathbf{4_M}$ (peak integrations are given on the Y axis).

Do the Succinimides $\mathbf{3_m}$ *and* $\mathbf{3_M}$ *Equilibrate under the Reaction Conditions?* (*a*) *Competition with* D-*Cysteine*. A 60:40 mixture of succinimides **3** (7.54 μmol) was added to a solution of D-cysteine (75.4 μmol, 10 equiv) dissolved in 0.3 mL of 100 mM KPO_4, pH 8. The time course of the reaction was followed by quenching 20-μL aliquots with 80 μL of 20% H_3PO_4. HPLC analysis exhibited only the peaks corresponding to thiazinones **4**. Under the conditions of analysis, the corresponding adducts with D-cysteine were perfectly separated from their L counterparts ($\mathbf{4_m^L}$, R_f = 5.93; $\mathbf{4_m^D}$, R_f = 7.70; $\mathbf{4_M^L}$, R_f = 4.65; $\mathbf{4_M^D}$, R_f = 3.86).

(*b*) *Experiment in* D_2O. A total of 22 mg of **3** (77 μmol) was reacted for 3 h in 3 mL of 100 mM KPO_4 buffer in D_2O. The residue after lyophilization was dissolved in water, acidified to pH 2 with phosphoric acid (0.5 mL), and purified by HPLC. The recovered 19.5 mg was analyzed by ^{1}H NMR. The disappearance of the characteristic quadruplet due to the H_X proton of the succinimide part at 3.65 ppm together with the suppression of two coupling constants were revealing of quantitative incorporation (at the NMR sensitivity) of deuterium at the α-position to the sulfur. As expected, the only modification in the ^{13}C NMR spectrum was the transformation of the methine singlet at 39.83 ppm into a triplet (J = 41.8 Hz).

Model Reaction of Decapeptide CGIVGAIAQR and FMDP. The reaction between the synthetic decapeptide and FMDP was performed at pH 8 in the same way as with cysteine except for the presence of 1.5 M urea to ensure solubilization (urea did not affect the evolution of the reaction between cysteine and FMDP). As shown in Figure 2 compound $\mathbf{14_M}$ isolated in 60% yield after 3 h of reaction and

Table II: Kinetic Parameters for the Reaction of Compounds **2**, **12**, and **13** at pH 8 or 6

	disappearance of X			formation of thiazinone **4** or **14**		
compd X	k_1 (min^{-1})	k_2 (M^{-1} min^{-1})	$t_{1/2}$ (min)	k_1 (min^{-1})	k_2 (M^{-1} min^{-1})	$t_{1/2}$ (min)
pH 8						
FMDP		nd	0.25	complex	kinetics	5.0
$\mathbf{2_m}$	0.287		2.41	complex	kinetics	3.8
$\mathbf{2_M}$	0.485		1.43	complex	kinetics	3.6
$\mathbf{12_m}$	0.135		5.14	complex	kinetics	nd
pH 6						
CGIVGAIAQR		nd	1.9	complex	kinetics	nd
$\mathbf{13_m}$	0.083		84	0.09		77
$\mathbf{13_M}$	0.087		80			

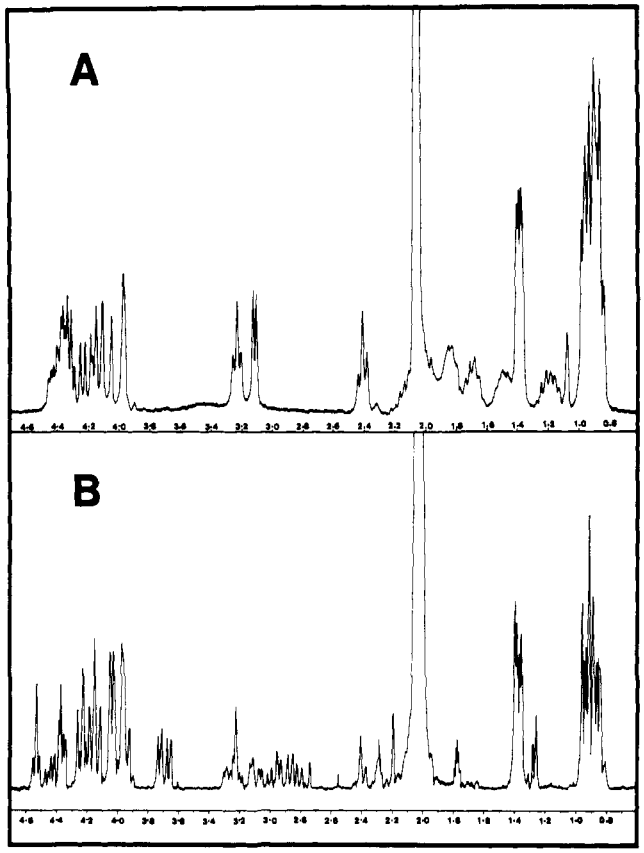


FIGURE 2: 250-MHz ^1H NMR spectra (D_2O/AcOH, 90:10) of (A) decapeptide CGIVGAIAQR and (B) adduct **14** with FMDP (projection of the 2D COSY spectrum).

HPLC purification could be clearly analyzed by comparison with the simpler adduct $\mathbf{4_M}$. The cysteine methylene group initially present in the decapeptide as a doublet (Figure 2A) at 3.2 ppm appeared as an AB part of an ABX system on both sides of the CH_2 of arginine, resonating at about 3.25 ppm. The AB part of the succinimide ABX system appeared between 2.8 and 3.05 ppm whereas the H_B proton of the diaminopropionate ABC system exhibited a characteristic quadruplet centered at 3.7 ppm. COSY (data not shown) and FAB analyses confirmed the structure. In contrast with the model reaction with cysteine, the composition of the mixture could be easily determined at any extent of the reaction by HPLC analysis.

Identification of Intermediates during the Reaction between Decapeptide and FMDP. In order to identify the reaction intermediates that transiently appeared during the formation of thiazinone **14**, the reaction was performed under conditions disfavoring the final cyclization. In phosphate buffer, pH 6, the disappearance of the starting peptide obeyed second-order kinetics with a $t_{1/2}$ of 1.9 min (Figure 3). Under these conditions the formation of the six-membered adduct **14** did not noticeably occur. Therefore, for preparative purposes, decapeptide (20 mg) was reacted at 13 mM concentration with an equal amount of FMDP at pH 6 and the reaction stopped after 48 min when maximum amounts of intermediates have accumulated. HPLC purification of the first four peaks (Figure 3) allowed, by comparison of the spectra of derivatives **2** and **3**, identification of compounds $\mathbf{12_m}$, $\mathbf{12_M}$, $\mathbf{13_m}$, and $\mathbf{13_M}$ isolated in 12, 12.5, 9, and 6.5% yields, respectively.

Evolution of Purified Compounds **12** *and* **13**. Incubation of $\mathbf{12_M}$ in phosphate buffer; pH 6, resulted in slow formation of diastereomeric succinimides **13**. At pH 8 $\mathbf{12_m}$ reacted fairly rapidly according to first-order kinetics (Table II) to give the

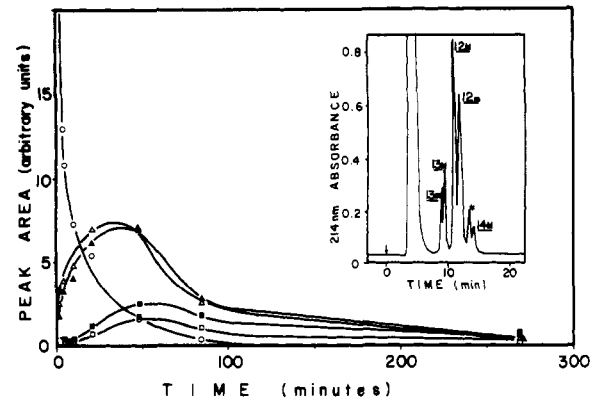
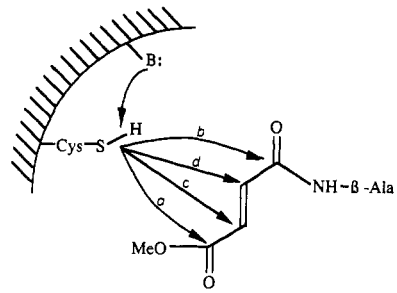


FIGURE 3: Evolution of the reaction between decapeptide and FMDP in KPO_4, pH 6: (○) initial peptide, (▲) $\mathbf{12_m}$; (△) $\mathbf{12_M}$; (□) $\mathbf{13_m}$; (■) $\mathbf{13_M}$. (Insert) HPLC profile after 48 min (isocratic conditions, 20% CH_3CN in water containing 0.1% TFA); (*) initial peptide.

Scheme I



transient succinimides that rearranged to a single isomer, $\mathbf{14_M}$. No isomerization of the starting Michael adduct was observed.

Because of the fast interconversion between $\mathbf{13_m}$ and $\mathbf{13_M}$, the evolution of the reaction was followed on the diastereoisomeric mixture generated from addition of decapeptide on compound **5** at pH 6. Both succinimide adducts were formed quantitatively in less than 1 min; they disappeared then with very similar first-order rate constants (Table II) to afford $\mathbf{14_M}$, which appeared at about the same rate.

Discussion

Pioneering work on glucosamine-6P synthase inhibition by Chmara et al. (1984) clearly demonstrated the potential utility of the synthetic compound N^3-(methoxyfumaroyl)-L-2,3-diaminopropionate (FMDP, **1**, X = OCH_3) as an antibacterial and more interestingly as an antifungal agent. From the time-dependent irreversible loss in activity observed following incubation of FMDP with semipurified enzyme from various microorganisms together with the lack of reactivity of **1** toward thiols, the authors postulated a mechanism-based behavior: incubation of glucosamine-6P synthase and **1** would generate, by cleavage of the central amide bond (Scheme I, pathway b), a fumaroyl thiol ester which upon further reaction with an active site nucleophile would result in covalent enzyme modification. More recently (Badet et al., 1988), using the pure overexpressed *E. coli* enzyme, we have reported that enzyme inhibition with FMDP was associated with the modification of the amino-terminal cysteine residue involved in the catalytic mechanism at the glutamine binding site. Therefore, the reactivity of **1** allowed postulation of two different mechanisms (Scheme I): an acylation reaction where the active site thiol would form an acyl-enzyme intermediate able to react further (pathways a and b) or an alkylation reaction with formation of a Michael adduct between Cys-1 and the fumaroyl double bond (pathways c and d). Pathways a and b are similar to the reaction that is believed to take place in

all the glutamine-dependent amidotransferases (Buchanan, 1973; Zalkin, 1985), that is, generation of free ammonia with concomitant formation of a γ-glutamyl thiol ester, and that occurs in cysteine proteinases such as papain and cathepsin (Brocklehurst et al., 1987). Pathways c and d are much more common since they result from the known propensity of α,β-unsaturated compounds to nucleophilic attack (Silverman, 1988). Inhibition according to route a would result in methanol production whereas route b would result in diaminopropionate formation and possibly monomethyl fumarate by hydrolysis of the corresponding fumaroyl thiol ester.

Incubation of *E. coli* glucosamine-6P synthase with an excess (12-fold) of α-tritiated FMDP resulted in incorporation of 0.55 inhibitor equiv per enzyme molecule (M_r 70000) inactivated to 90%. This stoichiometry increased to 0.92 when the molar ratio of inhibitor/enzyme increased to 70 (data not shown). A similar experiment performed with [*methoxy*-^{14}C]FMDP (16-fold in excess) resulted in incorporation of 0.64 inhibitor equiv. The stoichiometry close to 0.5 is reminiscent of half-of-the-sites reactivity behavior previously noticed with the affinity label DON (Badet et al., 1987), which might result from a conformational change occurring during the formation of the adduct between the dimeric protein and FMDP. Since this value increased close to unity on prolonged incubation with a large excess of inhibitor, the irreversible inhibition of the enzyme is probably mainly associated with the fixation of the uncleaved inhibitor.

In order to check for possible turnover of the FMDP molecule by the enzyme and for participation of mechanisms a and b in the inactivation process, the small molecules resulting from treatment by the tritiated inhibitor were separated from the protein; HPLC analysis of the mixture revealed that more than 99% of the recovered radioactivity coeluted with FMDP (data not shown), ruling out noticeable participation of route a. Assays for the presence of fumarate or diaminopropionate were performed as described under Results with fumarase/malic enzyme and diaminopropionate ammonia lyase/lactate dehydrogenase coupled reactions, respectively: none of these byproducts were detected in the mixture. Assuming a detection threshold of 5 nmol, less than 0.032 × 90% = 3.5% of fumarate or diaminopropionate was produced (by pathway b) during enzyme inactivation. From these analyses it can be concluded that neither mechanism a or mechanism b could account for enzyme inhibition, which then must arise from one of the two remaining possibilities, c or d (Scheme I). Since the regioselectivity of Michael addition on fumaramide monomethyl ester has not been reported, the model reaction between L-cysteine and FMDP was investigated.

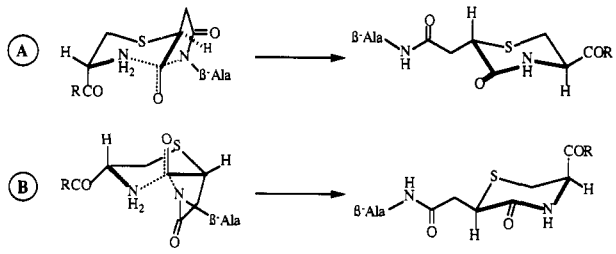
Reaction of stoichiometric amounts of L-cysteine and FMDP at pH 8 afforded in nearly quantitative yield the thiazinone adduct **4** as a 98:2 mixture of diastereoisomers. When the reaction was run at lower pH however, a 40:60 mixture of Michael adducts **2** and succinimides **3** was isolated in 80% yield. Both diastereoisomers (70:30) of the Michael adducts quantitatively formed the thiazinones **4** (98:2 mixture) when incubated separately at pH 8. Since under these conditions no epimerization of the starting isomer was observed, the diastereomeric ratio of the Michael adducts must result from some diastereoselection during their formation. In buffered medium (pH 8) they both disappeared with first-order rate constants (Table II) whereas the cyclic compound **4** formed slower with more complex kinetics. This result, which is typical of consecutive reactions (Fersht, 1985), implied the existence of a reaction intermediate on the pathway leading to the thiazinone **4**; we were unfortunately unable to follow the formation of succinimide derivatives **3** during this experiment since they were not retained on the C_{18} column. However, a 60:40 mixture of $\mathbf{3_M}$ and $\mathbf{3_m}$ gave at pH 8 quantitatively the same 98:2 mixture of isomeric thiazinones **4**; the latter did not epimerize under the reaction conditions. Taken together, these experiments suggest a global mechanism with initial formation of the Michael adducts **2** followed by intramolecular cyclization to the succinimides **3** stable at pH 5; under slightly alkaline conditions compounds **3** can be ring opened by the free amino group to generate the six-membered heterocycle **4**; this reaction has been used previously to synthesize potential nephrotoxic compounds (Augustin & Muller, 1985; Shih & Rankin, 1988).

Compound **6** instead of **2** was also susceptible to undergo the series of transformations described above. Since physical data of the isolated compound did not allow a clear-cut attribution, synthesis of authentic compound(s) by unambiguous routes might appear necessary. However, assuming **3** as an obligatory intermediate in the formation of **4** (which was demonstrated in the decapeptide case, see below), the presence of structure **6** was ruled out for the following reasons: (a) An acrylic ester is 20–30-fold more reactive as a Michael acceptor than acrylamide; therefore, the carbon adjacent to the amide group is likely to be prone to nucleophilic addition (Shenhav et al., 1970). (b) If **6** were the adduct to be generated, it had to undergo succinimide formation rather than direct cyclization into **4** (direct cyclization of **2** is impossible). The huge difference in basicities (10 orders of magnitude; Arnett, 1963) between amide and amino groups makes this process unlikely.

The second question that arose from the study described above was the origin of the high diastereomeric excess (96%) observed in the final adduct **4**. As neither Michael adducts $\mathbf{2_m}$ and $\mathbf{2_M}$ nor thiazinones $\mathbf{4_m}$ and $\mathbf{4_M}$ isomerize at pH 8, the conversion of succinimides **3** to **4** must be responsible for this diastereoselectivity; that is, a rapid equilibrium between $\mathbf{3_m}$ and $\mathbf{3_M}$ must occur before preferential cyclization of one of them. Two different mechanisms could rationalize this epimerization: (a) a retro-Michael reaction would generate *N*-β-L-alanylmaleimide (**5**) and L-cysteine, which could reattack randomly the maleimide carbon–carbon double bond; (b) a simple deprotonation at the α-position to the sulfur would generate a β-alkylthio-substituted enolate able to protonate on either side back to a 50:50 diastereomeric mixture.

The first hypothesis was tested by running the reaction **3** → **4** at pH 8 in the presence of a 10-fold excess of D-cysteine; if a retro-Michael reaction was involved in the process, the D isomer should compete with its enantiomer for the addition to **5** to give the corresponding thiazinone with the opposite configuration at the α-carbon of the amino acid. No trace of this adduct (quantitatively formed upon reaction between FMDP and D-cysteine) was detected by HPLC, ruling out hypothesis a. Incubation of the same mixture of succinimides in D_2O at pH 8 resulted in formation of **4** with one deuterium atom at C_2 as analyzed by NMR. This result confirmed total equilibration of the succinimides **3** by enolization. The conversion of **3** to **4** has to go through a bicyclic transition state where the cis junction is highly favorable. Two transition states can therefore be considered (Scheme II): in (A) both the carboxyl group and the carbonyl oxygen are in an axial position, maximizing electronic and steric repulsion, whereas in (B) no such unfavorable interaction exists. On the basis of these considerations, one can expect (B) to cyclize more rapidly to the (2*R*)-thiazinone $\mathbf{4_M}$. This predicted stereochemistry will require determination of the absolute configuration of the isolated thiazinone by NMR and/or X-ray.

Scheme II



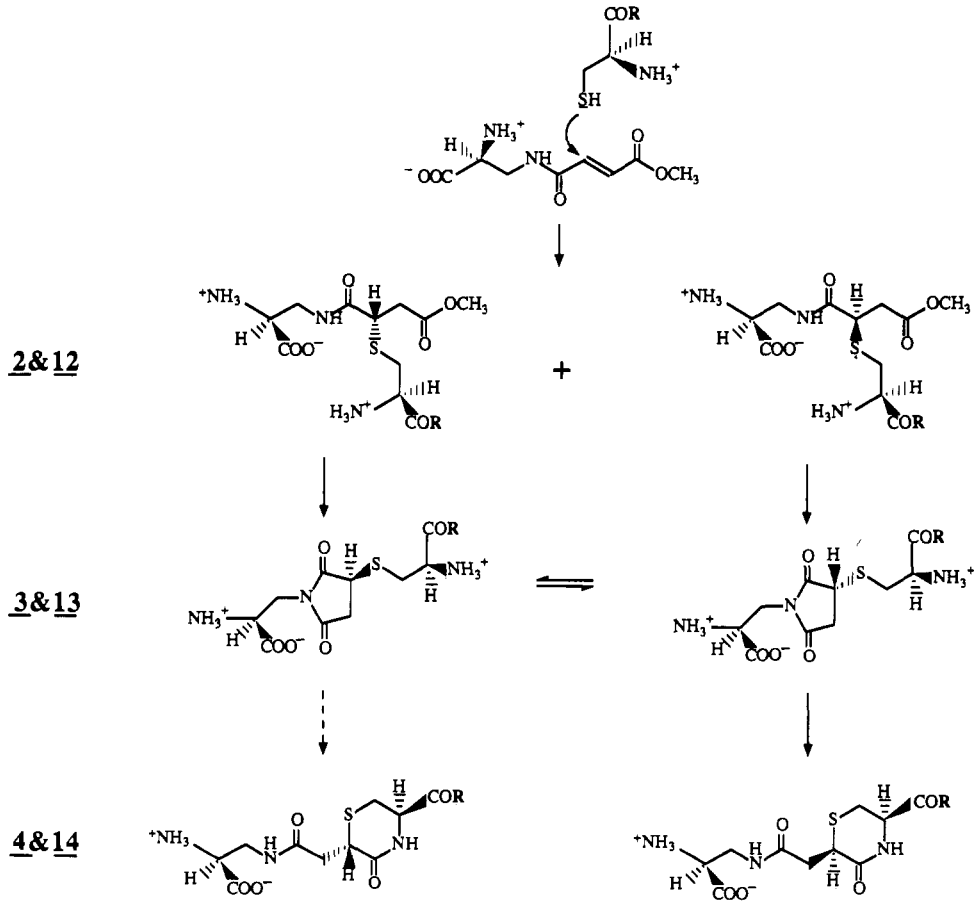
In light of the results presented above, the reaction between synthetic decapeptide CGIVGAIAQR, corresponding to the tryptic amino-terminal fragment of the enzyme, and FMDP was also investigated. The evolution of the reaction could be clearly analyzed by HPLC, and trapping of all the intermediates was possible. A reaction pathway very similar to what was found from the reaction with L-cysteine could be deduced: the starting peptide rapidly disappeared ($t_{1/2}$ = 1.9 min at pH 6) with second-order kinetics to generate the two Michael adducts $\mathbf{12_m}$ and $\mathbf{12_M}$ (45:55). Each adduct underwent ring closure to diastereomeric succinimides **13** (35:65); at pH 6 both isomers disappeared with first-order kinetics ($t_{1/2}$ = 82 min) to generate the final thiazinone at the same rate (within experimental error), suggesting a rapid equilibrium between the two diastereoisomers **13**.

Taken together, the two model reactions are consistent with the unifying mechanism depicted in Scheme III: FMDP reacts as an electrophile at the β-position of the ester with the amino-terminal cysteine located in the glutamine binding site. The formation of the corresponding covalent adduct **12** is responsible for enzyme inactivation as demonstrated by stoichiometric incorporation of tritiated inhibitor (0.92 with a large excess of FMDP). The half-time for enzyme inactivation at pH 7.2 (first-order reaction, $t_{1/2}$ = 0.5 min; Badet et al., 1988) is in reasonable agreement with the half-time of the model reaction (second-order reaction, $t_{1/2}$ = 1.9 min) at pH 6. Denaturing conditions promote cyclization to succinimides **13** with release of methanol (as demonstrated with ^{14}C-labeled inhibitor) and further conversion into a substituted 1,4-thiazin-3-one derivative. Tryptic digestion of FMDP-treated enzyme afforded three radioactive peptides (A/B/C, 5:31:64). The two major components, C and B, coeluted with thiazinones $\mathbf{14_M}$ and $\mathbf{14_m}$, explaining a posteriori the failure of Edman degradation. The surprising lack of diastereoselection during the formation of B and C (33:67) compared to that in the model reaction (>2:98) can be rationalized in light of the following experiments. When the reaction between FMDP and cysteine was run at pH 8.5, a net decrease in the diastereomeric excess of compound **4** was noticed ($\mathbf{4_M/4_m}$ = 70:30); under strongly alkaline conditions (1 N NaOH), $\mathbf{14_M}$ epimerized rapidly (1 min) before degradation into unidentified products (data not shown). Therefore, the epimerization of a unique compound resulting from enzyme inactivation and proteolysis is certainly a more tempting hypothesis than a lack of diastereoselectivity during the cyclization of the 600 amino acid substituted intermediate. The minor product (A) coeluted with succinimide $\mathbf{13_M}$ but also with one of the degradation products of $\mathbf{14_M}$ under alkaline conditions. Identification of compound C awaits additional information from mass spectrometry sequencing actually under investigation.

Conclusions

The study described in this paper demonstrates that irreversible inhibition of glucosamine-6P synthase by FMDP arises

Scheme III



from nucleophilic attack of the catalytic thiol residue on the double bond of the fumaroyl moiety. This result is reminiscent of papain inactivation by Ep-475, an epoxysuccinyl-containing compound, where the active site thiol reacts in a high regioselective manner with the epoxide ring (Yabe et al., 1988). An NMR study of [^{13}C]FMDP-inactivated enzyme, as described with papain, will certainly give definitive proof of the regioselectivity of the attack in the glucosamine synthase case.

The currently described mechanism is nevertheless in good agreement with the inhibitory properties of fumaroyldiaminopropionate derivatives, which can be ranked according to the second-order rate constant in enzyme inactivation (relative rates at pH 7.2 for X of **1**: OMe, 160; CN, 40; NH_2, 4; OH, 1). Although the molecular parameters for glucosamine synthase inhibition are certainly more complex than those described by Michael acceptor ability, the mentioned values correlate remarkably well with the reactivity of nucleophiles toward acrylate derivatives [relative rates at pH 8.1: methyl acrylate, 26; acrylonitrile, 6.6; acrylamide, 1 (Shenhav et al., 1970)].

Finally, the proof for the existence of a succinimide intermediate might be helpful in our effort to improve the efficiency of enzyme inhibition. As an indicative example, compound **5**, with a second-order rate constant of 2000 M^{-1} s^{-1} for enzyme inactivation, is almost as efficient as FMDP, the most efficient glucosamine synthase affinity label analyzed to date.

ACKNOWLEDGMENTS

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