Estudios cinéticos y estructurales de la glucosamina 6-fosfato isomerasa (desaminasa) de *Escherichia coli*.

Tesis

elaborada por

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Introducciòn

La enzima glucosamina 6-fosfato isomerasa (desaminasa) [E.C. 5.3.1.10] cataliza la reacciòn:

Glucosamina 6-fosfato (GlcN6P) → Fructosa 6-fosfato (Fru6P) + NH_4^+

En el la introducciòn del primer articulo incluido en esta tesis se da un panorama general de la importancia de esta enzima en el metabolismo de los aminoazùcares. Se purificò hasta homogeneidad para poder estudiar sus caracteristicas cinèticas y estructurales. Con el propòsito de estudiar la reacciòn en el sentido Fru6P + NH_4^+ → GlcN6P, en condiciones cinèticas adecuadas (con grado de avance bajo), se diseñó un mètodo potenciogràfico. Este procedimiento puede ayudar a establecer el mecanismo cinètico de esta enzima.

Como primer paso en la correlaciòn estructura funciòn, se determinò el estado de oxidaciòn y papel en la catàlisis de los residuos de Cys en esta enzima.

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PURIFICATION, MOLECULAR AND KINETIC PROPERTIES OF GLUCOSAMINE-6-PHOSPHATE ISOMERASE (DEAMINASE) FROM *ESCHERICHIA COLI*

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Glucosamine-6-phosphate isomerase (deaminase), (2-amino-2-deoxy-D-glucose-6-phosphate ketol isomerase (deaminating), EC 5.3.1.10) has been purified to homogeneity from *Escherichia coli* B as judged by several criteria of purity. The procedure included ammonium sulfate fractionation, anion-exchange chromatography and a biospecific affinity chromatography step with *N*-ε-amino-*n*-caproyl-D-glucosamine 6-phosphate bound to agarose as the ligand, the elution being performed with GlcNAc6*P*. The enzyme appears to be an hexamer of about 178 kDa, composed of six subunits of 29700 ± 300 Da; the isoelectric point was 6.0–6.1 and the sedimentation constant 9.0 S. The amino-acid composition of the enzyme was determined and a value for $E^{1\%}_{275}$ of 4.55 was calculated. The molecular activity was 1800 s^{-1} for the deamination reaction and 455 s^{-1} for the reaction of GlcN6*P* formation. A positive homotropic cooperativity was found for both sugar substrates; it was stronger for GlcN6*P* in the deamination reaction (Hill number 2.7 at pH 7.7). Ammonia behaved as a Michaelian substrate. Cooperativity was abolished by 0.1 mM GlcNAc6*P*; this allosteric modulator activated the reaction in both directions, with a positive K-effect upon both sugar phosphates, but had no effect on K_m for ammonia. The initial velocity patterns for the amination reaction were obtained under conditions of hyperbolic kinetics produced by GlcNAc6*P*; the K_m values for the allosteric substrates were determined under the same conditions, and their dependence upon pH was studied.

Introduction

The enzyme glucosamine-6-phosphate isomerase or glucosamine-6-phosphate deaminase, (2-amino-2-deoxy-D-glucose-6-phosphate ketol isomerase (deaminating), EC 5.3.1.10) catalyzes the first step in the catabolism of GlcN6*P*; it has been considered as an alternative route for the biosynthesis of aminosugars, at least in some species, but its synthetic function remains controversial. Although the equilibrium of the reaction favors the catabolic direction (1–3), in vitro glucosamine-6-phosphate isomerase coupled to glucosamine-6-phosphate acetyltransferase easily produces GlcNAc6*P* from fructose 6-phosphate (Fru6*P*), free ammonia and acetyl-CoA [3]. Furthermore, the enzyme forming GlcN6*P* from Fru6*P* and glutamine (glucosamine-6-phosphate isomerase (amino-transferring), EC 5.3.1.19) is absent in two species of diptera, at least in some stages of their development [4,5].

The reaction catalyzed by the deaminating isomerase is an aldo-keto isomerization coupled with an amination-deamination (Amadori rearrangement [6]), a mechanism that has not been described for any other biochemical reaction. The enzyme is present in several microorganisms and animal tissues and some partial purifications have

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been reported [1]. Midelfort and Rose purified the enzyme from *E. coli* and studied the chemical mechanism of the reaction [7]. Recently, Das and Datta reported the purification of the deaminase from *Candida albicans* [8].

The enzymes obtained from *E. coli* and animal tissues are allosteric and have GlcNAc6*P* as activator for both directions of their reaction. This property is not present in the enzyme from *Proteus vulgaris* [9] nor has it been reported in *C. albicans* [8]. The existence of an allosterically modulated enzyme catalyzing a functionally reversible reaction is an unusual property in a metabolic pathway. With all its interesting features, there is a considerable lack of information on the structural and kinetic properties of the enzyme.

In the present communication we describe an improved method for the purification of the glucosamine-6-phosphate isomerase (deaminase) from *E. coli* based on affinity chromatography; we report some molecular properties of the enzyme and describe the kinetics for the forward and backward reactions.

Materials and Methods

Chemicals. Enzymes, protein standards, ϵ-amino-*n*-caproic acid-agarose and carbohydrate derivatives were purchased from Sigma (U.S.A.). Other reagents were of the highest purity available. Gel filtration, ion-exchange media and electrofocusing ampholytes were products of Pharmacia Fine Chemicals (Sweden); Ultrogel AcA-34 was purchased from LKB (Sweden). GlcNAc6*P* was prepared by the chemical acetylation of GlcN6*P* and purified by ion-exchange chromatography according to the procedure of Leloir and Cardini [10]. The purity of the product was determined by thin-layer chromatography on microcrystalline cellulose plates.

The affinity matrix of *N*-ϵ-amino-*n*-caproyl-D-glucosamine-6-phosphate agarose was prepared by linking GlcN6*P* to ϵ-amino-*n*-caproic acid agarose (2 μequiv. amino caproate groups per ml packed gel). To a 2:1 water suspension of the agarose derivative GlcN6*P* (free acid) was added to a final concentration of 0.1 M, and pH was adjusted to 4.5 with NaOH. The reaction was made at room temperature adding 0.2 mmol of *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride for each milliliter of suspension and stirring it gently for 12 h. The gel was washed with 0.1 M Tris-HCl buffer (pH 7.25)/0.1 M NaCl. The affinity matrix was stored at −20°C, in the same buffer, adding glycerol to a final concentration of 50% (v/v).

Growth of bacteria. E. coli B was grown aerobically at 37°C in 14 l fermentors with 10 l of medium containing 0.05 M glucosamine as the only carbon and nitrogen source and supplemented with the mineral components of the medium of Comb and Roseman [3]. Cells were harvested at the late exponential growth and stored at −20°C until needed.

Enzyme assays. All kinetic studies were performed at 30°C. Routine enzyme determinations were made in the direction of Fru6*P* formation in a 200 μl reaction mixture containing 75 mM Tris-HCl buffer (pH 7.7), 0.85 mM GlcNAc6*P* and variable concentrations of the substrate GlcN6*P*. The reaction was initiated by addition of the enzyme and incubated for a fixed time. Reactions were brought to an end by the addition of 2.0 ml of 10 M HCl. Fru6*P* concentrations were determined by the resorcinol method of Roe [11] as described by Davis and Gander [12], but reading the absorbance at 512 nm. The progress of the reaction was always kept below 5% conversion.

In the direction of GlcN6*P* formation, velocity measurements were made determining the amount of product formed in a fixed time using the Morgan-Elson reaction [13], as described by Levy and McAllan [14]. Reaction mixtures of 200 μl contained 75 mM Tris-HCl buffer (pH 7.7), 0.85 mM GlcNAc6*P* and variable concentrations of the substrates, Fru6*P* and NH_4Cl. Reactions were stopped by heating for 1 min in a boiling-water bath. The amount of GlcN6*P* formed was corrected for the presence of GlcNAc6*P* by suitable blanks. The progress of the reaction was also kept as low as possible.

One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of Fru6*P* in 1 min at 30°C, at a pH of 7.7.

Treatment of kinetic data. For reactions following Michaelis-Henri kinetics, the data were fitted according to Johansen and Lumry [15] and presented as double-reciprocal plots. The Hill equa-

tion was adjusted by least squares.

Protein measurements. Proteins were determined by either the turbidimetric method of Bucher [16], or the Coomassie-G dye-binding technique of Bradford [17], with bovine serum albumin as standard. For the pure enzyme, concentrations were calculated from its absorbance at 275 nm in 50 mM Tris-HCl buffer (pH 7.7).

Polyacrylamide gel electrophoresis. Electrophoreses under non-denaturing conditions were performed in 80 × 80 × 2.7 mm polyacrylamide gel slabs with a linear pore gradient obtained by varying the monomers concentrations from 4 to 30 g/dl, with a constant cross-linkage of 4% [18]. Samples and six protein standards from 43 to 440 kDa were run at 10°C in 90 mM Tris-base/80 mM boric acid buffer (pH 8.35) at 125 V for 16 h. Electrophoreses under denaturing conditions were made after treating the enzyme with 1 g/dl sodium dodecyl sulfate and 0.15 M mercaptoethanol at 92°C for 5 min. Samples were run in 180 × 140 × 0.7 mm polyacrylamide gel slabs, using a discontinuous buffer system modified from Laemmli [18]. Seven proteins (14.5–68 kDa) were run simultaneously as molecular weight standards. Analytical electrofocusing in thin layers of polyacrylamide gels were run in 125 × 125 × 2 mm slabs in a Multiphor system (LKB Sweden) with ampholytes in the pH range 3–9 or 6–8 at a final dilution of 1 : 15. After focusing, pH was measured on the gel and then fixed and stained by standard methods.

Electrofocusing in denaturing conditions was made by a similar procedure, with the addition of 6 M urea and 2 g/dl Triton X-100 over a pH range 6–8. Samples were previously treated with the same denaturing agents at 92°C for 10 min.

Ultracentrifugation studies. Ultracentrifugation in sucrose density gradients were made as described by Martin and Ames [19] at 290000 × g and 5°C in 50 mM Tris-HCl buffer (pH 7.7) in a Beckman SW-50.1 rotor for 10 h. The content of the tubes was analyzed by puncturing the bottom of the tube and collecting the fractions. Density-gradient analyses were also carried out in presence of acrylamide monomers, according to the procedure of Jolley et al. [20]. Polymerization was done by photocatalysis and the gel formed was stained with Coomassie brilliant blue R. Six pure proteins (17–240 kDa) were run simultaneously as standards.

Analytical gel-exclusion chromatography. The molecular weight of the deaminating isomerase was estimated from its elution volume from a calibrated column of Ultrogel AcA-34 (1.6 × 100 cm) equilibrated with 75 mM Tris-HCl buffer (pH 7.7) at 4°C. Four proteins, from 17 to 240 kDa, were used as molecular weight standards. Elution profiles were determined recording the absorbance of the eluate at 280 nm.

Amino acid analyses. Amino acid analyses were carried out on a Durrum D-500 amino acid analyzer by Dr. Guillermo Ramirez, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México. Samples from the same enzyme preparation were dialyzed 12 h against 500 vol. water, and hydrolyzed at 110°C in 6 M HCl in sealed tubes under vacuum for 24, 48 and 90 h. Cysteine and half-cysteine were determined as cysteic acid from protein samples oxydized with performic acid [21] and hydrolyzed in 6 M HCl containing 0.5% phenol for 20 h at 110°C. Tryptophan was determined by amino acid analysis of the enzyme hydrolyzed 24 h at 110°C in 2-mercaptoethanesulfonic acid according to Penke et al. [22]. All results reported are the averages of two runs for each time of hydrolysis.

Results

Enzyme purification

All operations were carried out at 4°C with the exception of the affinity chromatography, which was done at 20°C. The harvested cells (100 g for a typical preparation) were washed twice in 0.15 M KCl and disintegrated by sonic oscillation in 50 mM potassium phosphate buffer (pH 7.0) using 3 ml of this solution per g of biomass (wet weight). The extract was centrifuged at 48000 × g for 1 h and the precipitate was submitted to a new cycle of sonic disruption and centrifugation. To the combined supernatants was slowly added a 5 g/l protamine sulfate solution to give a final concentration of 0.3 mg per mg protein, and the precipitate was removed by centrifugation at 48000 × g for 15 min. The supernatant was adjusted to pH 7.9 with 5 M ammonium hydroxide and fractioned with ammonium sulfate between 40 and 55% saturation at 4°C (225–315 g/l). The precipitate was dissolved in a minimal volume of

50 mM imidazole-HCl buffer (pH 7.4) and freed of ammonium sulfate by means of a Sephadex G-25 column (2.6 × 100 cm) equilibrated with the same buffer.

The enzyme was applied on a column of DEAE-Sephacel (2.6 × 40 cm) equilibrated with the imidazole-HCl buffer. Elution was performed with a convex NaCl gradient in the 0 to 0.25 M range at constant buffer concentration. The enzyme eluted as a single activity peak at 0.095–0.100 M NaCl. Fractions with a specific activity greater than 20–25 units/mg were pooled and treated with ammonium sulfate (450 mg/ml) to concentrate the protein. The precipitate was dissolved in a minimal volume of 0.1 M Tris-HCl buffer (pH 7.25) and applied to a small column prepared with the affinity matrix of *N*-ε-amino-*n*-caproyl-D-glucosamine-6-phosphate agarose (1 ml of gel for each 10 mg of pure enzyme expected). The column was then washed with the buffer and the enzyme eluted with a 10 mM solution of GlcNAc6*P* prepared in the same buffer; the enzyme eluted as a single peak with a constant specific activity of 606.8 units per mg protein. The fractions containing the enzyme were dialyzed overnight against 100 vol. of 50% glycerol containing 50 mM Tris-HCl buffer (pH 7.7) and stored at −20°C. Compared with the initial extract, the enzyme was purified 1144-fold with a 49% yield. About 2.9 mg pure enzyme were obtained for each 100 g wet weight of *E. coli* cells. A summary of the purification procedure is given in Table I.

Homogeneity of the purified enzyme

The enzyme preparation gave a single band on SDS-polyacrylamide electrophoresis, acrylamide pore-gradient electrophoresis, and density-gradient ultracentrifugation. Chromatographic peaks from the affinity matrix during the preparation, or from Ultrogel AcA-34 are symmetrical, with constant specific activity. Electrofocusing in polyacrylamide gel gave a single sharp band, but some gels gave a faint line at both sides. Electrofocusing of the denatured enzyme in presence of 6 M urea and 2 g/dl Triton X-100 gave a single sharp band, but in the presence of 25 mM mercaptoethanol, a complex pattern of bands was observed. Fig. 1 shows some representative gels, corresponding to different techniques.

Stability

The purified enzyme was stable up to six months when stored at −20°C in 150 mM Tris-HCl buffer (pH 7.25) with 50% (v/v) glycerol. Some older preparations (12–18 months) showed a diminished activity and produced a heterogeneous pattern in pore-gradient polyacrylamide gel electrophoresis and gel filtration chromatography.

Molecular weight and subunit composition

A molecular weight of 29700 ± 300 was obtained for the protein subunit in SDS polyacrylamide gel electrophoresis; for the native enzyme a molecular weight of 190000 ± 2500 was estimated using polyacrylamide pore-gradient gel electro-

TABLE I

PURIFICATION PROCEDURE FOR GLUCOSAMINE-6-PHOSPHATE ISOMERASE (DEAMINASE) FROM *E. COLI*

Amounts are rated for 100 g biomass wet weight.

Procedure	Total protein (mg)	Total units	Specific activity (units/mg protein)	Yield (%)	Purification factor
Initial extract	6615	355	0.53		
Protamine sulfate	2187.5	341	1.552	96	2.9
Ammonium sulfate	388.5	31	7.97	87.3	15
DEAE-Sephacel	21.2	196	92.4	55.2	174.3
ε-Amino-*n*-caproyl-D-glucosamine-6-*P*-agarose	2.9	176	606.8	49.5	1144.9

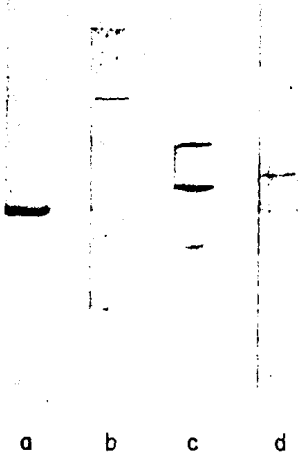

Fig. 1. Purity of glucosamine-6-phosphate isomerase preparation. (a) Polyacrylamide gel electrophoresis of the denatured enzyme in the presence of 0.1% SDS. Homogeneous gel, T = 11%, C = 2.6%. 4 μg protein were applied. (b) Pore-gradient gel electrophoresis in polyacrylamide (T = 4–30%; C = 4%). 4 μg protein applied. (c) Ultracentrifugation in acrylamide-containing linear sucrose density gradient, polymerized after the run (T = 8%, C = 3.6%). 25 μg enzyme. (d) Electrofocusing in polyacrylamide gel (pH 3 to 9) in the presence of 6 M urea and 2 g/dl Triton X-100. Cathode at the top. 6 μg protein applied.

phoresis, whereas a value of 185000 was determined by means of gel-filtration chromatography in Ultrogel AcA 34 (average of two determinations). Density gradient centrifugation by the method of Martin and Ames [20] showed an enzyme peak of 190 kDa; the sedimentation coefficient calculated was 9.0 S. These results were the average of three ultracentrifugations.

Amino acid composition

The amino acid composition of the enzyme is shown in Table II. For these results and according to the method of McMeekin et al. [23] a partial specific volume of 0.737 cm^3/g was estimated. From the mass of the enzyme calculated by amino-acid analysis, a molar absorptivity coefficient of $8.1 \cdot 10^4$ was estimated for the maximal absorbance of the enzyme at 275 nm (pH 7.7), and assuming a molecular weight of 178000. The value of $E^{1\%}_{275}$ was 4.55.

Isoelectric pH

The isoelectric pH of the enzyme estimated by analytical electrofocusing in polyacrylamide gel gave a value of 6.0–6.1 in two determinations.

Molecular activity

The molecular activity of the enzyme was determined for both directions of the reaction at pH 7.7 in the presence of 0.85 mM GlcNAc6*P* to obtain hyperbolic kinetics. In each case, true maximal velocities were used, obtained by extrapolation to infinite substrate concentration. The values found were 1800 s^{-1} for the deamination reaction, and 450 s^{-1} in the direct of GlcN6*P* formation.

Kinetic studies

In the direction of Fru6*P* formation, the enzyme presented a strong positive cooperativity with respect to GlcN6*P* (Fig. 2). The Hill number obtained at pH 7.7 was 2.7. This value declined under pH 7.4 and above pH 8.3, and approached unity at pH 7.0 and 8.8 (not shown).

In the direction of GlcN6*P* formation, there was a slight positive homotropic cooperativity with

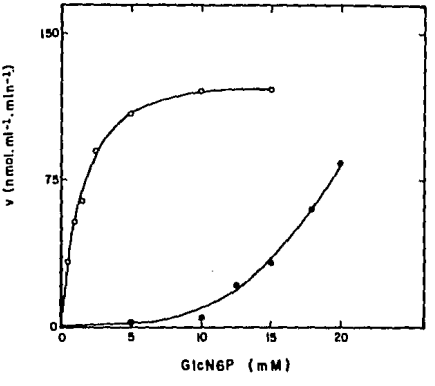

Fig. 2. Velocity of the reaction versus substrate concentration for the deamination reaction, in the presence (○) or absence (●) of 0.85 mM GlcNAc6*P*. The reaction mixture contained 0.2 M Tris-HCl buffer (pH 7.7) at 30°C.

TABLE II

AMINO ACIDS COMPOSITION OF GLUCOSAMINE-6-PHOSPHATE ISOMERASE (DEAMINASE) FROM *E. COLI*

Amino acid	Residues per 29.7 kDa			Average [a]	Nearest integer
	24 h	48 h	90 h		
Asx	28.48	27.62	27.07	27.62	28
Thr [b]	15.81	15.71	15.06	16.18	16
Ser [b]	11.27	10.80	9.72	11.96	12
Glx	25.03	26.38	26.14	25.85	26
Pro	9.84	8.77	10.19	9.60	10
Gly	18.46	19.48	19.64	19.19	19
Ala	21.21	22.42	22.32	21.98	22
Val [c]	18.80	20.86	20.90	20.90	21
Met	9.35	9.61	9.59	9.52	10
Ile [c]	12.84	14.00	13.30	14.00	14
Leu	24.40	24.39	24.33	24.37	24
Tyr	6.94	7.57	7.83	7.45	7
Phe	10.49	11.42	11.65	11.19	11
His	11.14	11.88	12.00	11.67	12
Lys	15.20	15.26	15.58	15.35	15
Arg	10.42	11.52	11.48	11.14	11
CyS [d]	4.65	–	–	4.65	5
Trp [e]	2.64	–	–	2.64	3

Total number of amino acids: 266

[a] Unless otherwise indicated.
[b] Extrapolated to zero time, assuming first order kinetics.
[c] Maximum values taken.
[d] Determined in a separate hydrolysis as cysteic acid, taking leucine as internal reference.
[e] Determined in a separate hydrolysis, and taking leucine as internal reference.

respect to Fru6*P*, but ammonia behaved as a Michaelian substrate. The Hill number found for Fru6*P* at pH 7.7 was 1.4, and did not vary with ammonia concentration (not shown).

GlcNAc6*P* activated the reaction in the deamination direction, as shown in Figs. 2 and 3; it diminished the apparent K_m for GlcN6*P*, without modifying the maximal velocity (positive K-effect). In the absence of GlcNAc6*P*, the double-reciprocal plots were concave upwards, and at high substrate concentration converged to the same maximal velocity as that obtained in the presence of the activator (Fig. 3). The effect was observed in relation to Fru6*P* for the opposite direction of reaction, but the apparent K_m for ammonia did not change with the addition of the activator. At saturating concentrations, GlcNAc6*P* abolished all cooperativity, as shown in Fig. 4 for the deamination reaction; under these conditions the enzyme displays Michaelis-Henri kinetics. The K_m for GlcN6*P* at 0.2 mM GlcNAc6*P* was 2.2 ± 0.4 mM.

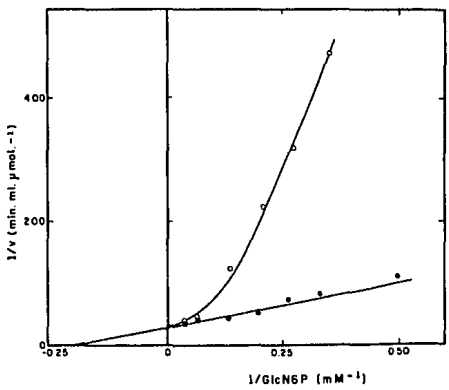

Fig. 3. Deamination reaction at pH 7.2. Double-reciprocal plot of velocity of reaction versus substrate concentration in 0.2 M Tris-HCl buffer in presence (●) or absence (○) of 0.85 mM GlcNAc6*P*. The Hill number at this pH was 1.7.

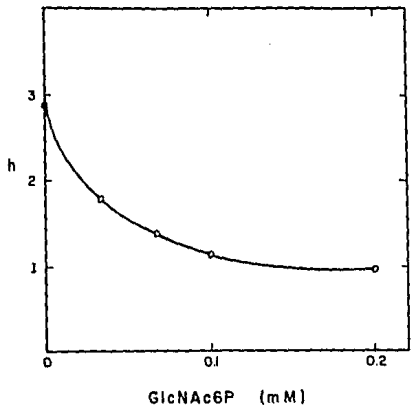

Fig. 4. Variation of the Hill number as a function of GlcNAc6*P* concentration for the deamination reaction. The conditions were similar to those in Fig. 3; *h* was also determined as the slope of the Hill plot, taking V_{max} from an experiment with a high concentration (0.85 mM) of GlcNAc6*P*.

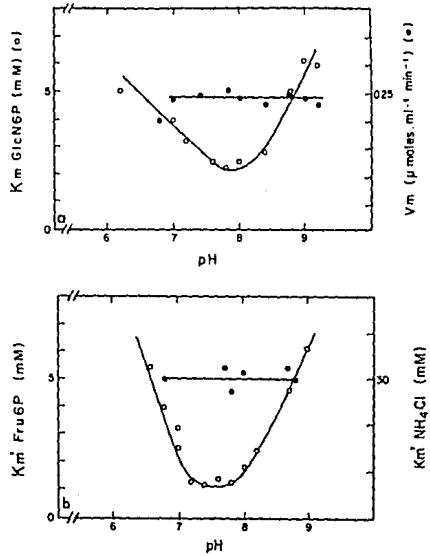

Fig. 5. Effect of pH on the reaction: (a) K_m (○) and maximal velocity (●) for the deamination reaction versus pH. Buffers used were: Tris-HCl above pH 7.2, and Pipes (1,4-piperazinediethanesulfonic acid) below pH 7.2. (b) Apparent K_m for Fru6*P* (○) and for NH_4Cl (●) versus pH; the buffers used were the same as in (a).

In the direction of GlcN6*P* formation, and under hyperbolic kinetics in the presence of 0.25 mM GlcNAc6*P* (pH 7.7) when Fru6*P* was varied at different concentrations of NH_4Cl and the results were presented as double-reciprocal plots, a family of converging lines was found, all intersecting on the abscissa.

When NH_4Cl was the variable substrate, a similar pattern was obtained, with or without GlcNAc6*P*, but in the latter case the slope or intercept replots are not linear.

K_m values at pH 7.7 for NH_4Cl were 31.4 ± 4.7 mM in the presence of 0.25 mM GlcNAc6*P*, and 35.4 ± 2.7 mM without the activator. The apparent K_m for Fru6*P* was dependent upon GlcNAc6*P* concentrations, and a value of 1.7 ± 0.3 mM was obtained in the presence of a high concentration (0.5 mM) of GlcNAc6*P* and constant concentration (50 mM) of ammonium chloride (pH 7.7).

For the deamination reaction in the presence of 0.25 mM GlcNAc6*P*, the maximal velocity was found independent of pH in the range from 7.0 to 9.0, but the K_m for GlcN6*P* showed minimal values between pH 7.6 and 8.2 (Fig. 5a). For the opposite direction of the reaction, also in the presence of the same concentration of activator, the apparent K_m for Fru6*P* at 50 mM NH_4Cl was minimal over the range pH 7.2 to 7.7. At fixed Fru6*P* concentration (3.75 mM), the apparent K_m for NH_4Cl is approximately constant over the pH range studied (Fig. 5b).

Equilibrium constant

The equilibrium constant of the reaction was determined by allowing it to proceed in the direction of GlcN6*P* formation until a constant concentration of product was obtained. The value found was 0.22 M at pH 7.7, defining K_{eq} as $[Fru6P][NH_4Cl]/[GlcN6P]$.

Discussion

In this article we describe a new procedure for purifying the enzyme glucosamine-6-phosphate isomerase (deaminase) from *E. coli* that includes

an affinity-chromatography step based on the specific interaction between the enzyme and an affinity matrix of *N*-ε-amino-*n*-caproyl-GlcN6*P* bound to agarose, and its elution by GlcNAc6*P*. This immobilized ligand can be considered as a structural analog of the allosteric activator of the enzyme. Leloir and Cardini observed that a *N*-propionyl group can be replaced for the *N*-acetyl group in the molecule of the activator with no loss of its properties [2]. Our results suggest that the length of the *N*-acyl substituent in the GlcN6*P* molecule is not critical for its activity as an allosteric modulator.

The purified enzyme has been shown to be homogeneous by means of different criteria of purity, and to have a specific activity of about 607 units/mg, the highest value so far reported. In this connection, it may be emphasized that our data are based on true maximal velocities, determined in the presence of GlcNAc6*P* to ensure hyperbolic kinetics, and that the last purification step by affinity chromatography could eliminate all inactive enzyme molecules possibly present.

Results on the molecular weight of the native and denatured enzyme indicate that the deaminating isomerase is most probably an hexamer of similar molecular mass subunits of 29.7 kDa. The electrofocusing experiments of the enzyme in media containing 6 M urea and 2 g/dl Triton X-100 gave a single band, a result that suggests an homopolymeric enzyme.

A very different molecular weight for the enzyme from *C. albicans* (17500) has been reported by Das and Datta and this enzyme seems to be monomeric [8]. A molecular weight similar to the enzyme from *E. coli* has been reported by Benson and Friedman [4] for the deaminase of *Musca domestica* (154000, estimated by sucrose gradient ultracentrifugation). This enzyme also has allosteric properties.

The enzyme prepared by our procedure exhibits an intense homotropic positive cooperativity for the deamination reaction, maximal at pH 7.6 and 8.2, with a Hill number of 2.7. At pH 7.7 the $S_{0.5}$ for the substrate GlcN6*P* is 15 mM. The same kind of cooperativity can be observed with respect to Fru6*P* at the opposite direction of the reaction, but it is less intense according to the Hill number found. Furthermore an hyperbolic or quasi-hyperbolic kinetics can be easily obtained at high Fru6*P* concentration. Ammonia does not seem to be a cooperative ligand, anyway.

This cooperative behavior of the glucosamine-6-phosphate isomerase (deaminase), was not observed by other authors in any of the animal or microbial species studied [1] including *E. coli* [3,7]. Cooperativity for the deamination reaction is so conspicuous that this discrepancy cannot be easily explained. In may be relevant to take into account that our preparation is based on mild purification procedures.

The known positive allosteric modulator of this enzyme, GlcNAc6*P*, acts in both directions of reaction, decreasing the $S_{0.5}$ for the substrates GlcN6*P* or Fru6*P*. According to these results, the enzyme can be described as a K system, as defined by Monod et al. [24]. The apparent K_m for the ammonium salt is not modified by this modulator. The complete transformation of the cooperative kinetics to hyperbolic at high activator concentration is consistent with an exclusive binding model for GlcNAc6*P*.

In the biosynthetic direction, initial velocity patterns presented as double-reciprocal plots appear as a family of straight lines intersecting on the abscissa. The pattern is symmetric for both substrates of the backward reaction, and corresponds to a particular case of the sequential bi-uni mechanism, for which it is possible to describe the initial velocity kinetics in the absence of the product (GlcN6*P*) with one kinetic constant for each substrate. Our results mean, according to the nomenclature proposed by Cleland [25], that $K_{ia} = K_a$, and $K_{ib} = K_b$. This result does not imply a random mechanism in a steady-state approach and could be the consequence of a fortuitous coincidence of kinetic constants [26]. The kinetic mechanism for this reaction should be determined by the usual kinetic or equilibrium methods.

The study of the kinetic parameter variation in the deamination reaction as a function of pH in the presence of activator shows an optimum range between pH 7.6–8.2 due to the pH-dependence of the K_m for GlcN6*P*, because V_{max} is constant over this range.

The molecular activity for the deamination reaction is nearly 4-times the value for the amination reaction. This result makes plausible the pos-

tulated role of this enzyme in glucosamine biosynthesis by means of the coupled reaction with the acetyl-CoA glucosamine-6-phosphate acetyl transferase. Nevertheless, the K_m for ammonia is very high and, as far as is known, it is not modified by any modulator. Hence, the velocity of the amination reaction could be of minimal consequence at physiological concentrations of ammonia.

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pHMETRICAL DETERMINATION OF THE GLUCOSAMINE-6-PHOSPHATE ISOMERASE DEAMINASE REVERSE REACTION

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Summary: In the reverse direction, the reaction catalyzed by glucosamine 6-phosphate isomerase deaminase consumes ammonia and forms GlcN6P. As a consequence of the formation of a product with a lower pK than the substrates, a measurable pH drop in the reaction medium is produced. This property can be used to follow potentiometrically the course of the reaction. The usefulness of the method is demonstrated obtaining the inhibition pattern by GlcN6P when Fru6P is the varied substrate.

INTRODUCTION

The reaction catalyzed by the enzyme glucosamine 6-phosphate isomerase (deaminase) (2-amino-2-deoxy-D-glucose-6-phosphate ketol isomerase (deaminating), E.C. 5.3.1.10) is reversible, and the equilibrium constant, defined as [Fru6P] [NH_4Cl]/[GlcN6P], has been estimated in 0.22 M, at 30°C, pH 7.7 (1). Initial velocities of the reverse reaction were generally determined by measuring colorimetrically the GlcN6P formed in a fixed time by means of the Elson-Morgan reaction or some of its modifications (2,3).

Employing this procedure, some kinetic data have been published (1,4), although it is always difficult to maintain a reasonably low reaction progress to obtain good initial velocity measures at different substrate concentrations.

As part of our studies on this enzyme from *Escherichia coli*, we were interested in determining the product inhibition patterns of the enzyme reaction in the glucosamine-forming direction under conditions of hyperbolic kinetics. This study requires a different assay method, compatible with variable concentrations of GlcN6P and saturating concentrations of GlcNAc6P, the allosteric activator of the enzyme. Both amino sugars interfer with the colorimetrical assay of the enzyme. The kinetics measurements in the

presence of the reaction product, in the thermodynamically unfavorable direction of the reaction, also require a sensitive method to obtain reliable initial velocity values.

In the course of the reaction, catalyzed by glucosamine 6-P isomerase, an ammonium ion (pK = 9.3) disappears from the reaction mixture, and a 2-amino group with a pK = 8.1 (5) is formed on the sugar-phosphate. On the other hand, the pK of the phosphoric acid ester at C-6 in both sugar-phosphates remains practically unchanged; indeed, values of 6.08 and 6.11 have been reported for GlcN6P and Fru6P, respectively (5). The change in the buffer capacity of the incubation mixture produced by the enzyme reaction determines a measurable release of protons. In this paper, we describe an enzyme assay that takes advantage of this fact to obtain a continuous and sensitive record of the reaction, using a potentiograph. We also demonstrate the value of the method to obtain the product inhibition pattern, when Fru6P is the variable substrate.

MATERIALS AND METHODS

Materials. Fru6P and GlcN6P were products of Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Other reagents were of the best quality available. GlcNAc6P was prepared by chemical acetylation of GlcN6P, according to Leloir and Cardini (6), and the purity of the product was controlled by thin layer chromatography on microcrystalline cellulose plates, developed in ethyl acetate: acetic acid: water: ammonia, 3:3:2:1.

Glucosamine-6-phosphate isomerase deaminase was purified from *E. coli* B (ATCC 11303) as already described (1), omitting the protamine sulfate step.

Ensyme assay. The activity of glucosamine-6-P-isomerase deaminase was measured in standard 1.5 ml polypropylene microcentrifuge tubes under continuous magnetic stirring in a contolled-temperature bath at 30°C. Changes of pH were recorded with a Metrohm E-536 potentiograph, using a small combined glass calomel electrode. The span of the recorder was used at the maximal sensitivity of the instrument, i.e., 0.04 pH unit/cm.

The reaction mixtures contained the substrates, (Fru6P and NH_4Cl), an excess (2.5 mM) of the allosteric activator, GlcNAc6P, and when indicated, variable amounts of the reaction product, GlcN6P. Each mixture was titrated to a pH near 7.7 with 0.1 M KOH, and the volume was completed to 490 µl with water. The reaction was started with the addition of 10 µl of enzyme solution in water, usually 0.2 - 0.8 µM. The reaction was recorded for a few minutes, and the velocities were calculated from the H^+ concentration difference between the first and second minute. The buffering capacity of each reaction mixture was measured by means of a similarly prepared sample, to wich 2.5 µl of 40 mM HCl was added instead of the enzyme. The pH change was recorded to calculate the actual release of hydrogen ions during the enzyme reaction.

Treatment of kinetic data. Michaelis-Henri kinetics was fitted and the corresponding parameters calculated according to Wilkinson (7); inhibition data were also processed according to Canela (8). In both cases, a Commodore 128 microcomputer was used, with the necessary modifications of the original programs.

RESULTS AND DISCUSSION

Fig. 1 A shows a typical potentiograph record for the glucosamine-6-phosphate isomerase deaminase reverse reaction. The initial rates were read at the recorded curve over a pH range smaller than 0.1 unit. We have already reported (1) that Km values for Fru6P and ammonium are nearly constant for this enzyme over the pH range 7.2 to 7.7; the maximal velocity for the reaction is also constant over a wider range. So, the measures allow the determination of the kinetic parameters without a significant effect of the pH drop during the reaction.

The linearity of the time course of the reaction is apparent when hydrogen ion concentration (the actual concentration measured, or the calculated amount of H^+ ions released per liter) is plotted as a function of time. (Fig. 1 B).

The value of the H^+/GlcN6P ratio was determined experimentally in a reaction mixture containing 10 mM Fru6P and 50 mM NH_4Cl, under conditions similar as described in Methods. Simultaneously with the pH recording, 10 µl samples of the reaction mixture were taken every 30 seconds for colorimetric analysis of GlcN6P according to Levy and Mc Allan (3). (Fig. 1B). This experiment gave a H^+/GlcN6P ratio of 0.11 (average of two separate determinations).

Taking into account the concentration change of GlcN6P and ammonia, its pK values and the pH change, a theoretical H^+/GlcN6P ratio of 0.10 was calculated for the same extent of reaction.

When the proton release rate was measured as described at fixed substrate and allosteric activator concentrations, first-order kinetics with respect to the enzyme concentration over a wide range was obtained. (Fig. 2).

The GlcN6P inhibition pattern with variable Fru6P and fixed ammonium concentrations is show in Fig. 3. The Km value for Fru6P is 1.07 mM. The inhibition by GlcN6P is competitive, according to the statistical procedure of Canela (8); apparent Ki is 0.17 mM.

The procedure described in this article is simple, reliable, and has the additional advantage of providing a continuous record of the reaction. Its main value resides in that it allows measuring the enzyme activity in the presence of considerable amounts of GlcN6P or GlcNAc6P.

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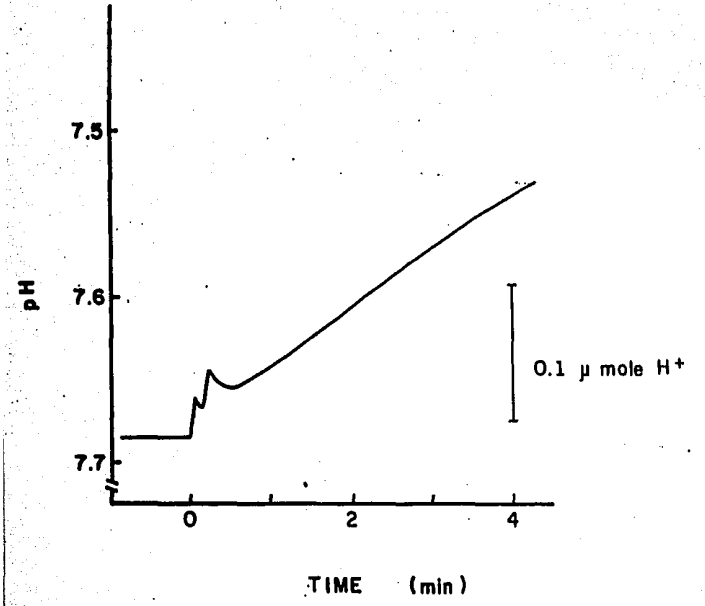
Abbreviations: GlcN6P , D-glucosamine 6-phosphate; GlcNAc6P, N-acetyl-D-glucosamine 6-phosphate; Fru6P, D-Fructose 6-phosphate.

LEGENDS FOR FIGURES

Figure 1. A: A typical potentiograph record of the glucosamine-6-phosphate isomerase reverse reaction. The reaction mixture contained 10 mM Fru6P, 50 mM NH_4Cl, 2.5 mM GlcNA6P and 30 nM enzyme. The vertical line indicates the pH change produced by the addition of a calibrating amount of HCl solution. B: (●) Data from the same experiment expressed in actual H^+ concentration. Reaction rate is 2.13×10^{-9} M min$^-$, and corresponds to a release of H^+ ions of 8.05×10^{-5} M min^{-1} , according to the buffer capacity determined from calibration. (o) Time course of the same reaction, determined by the simultaneous colorimetrical assay of GlcN6P, as described in Methods. ($vo = 7.92 \times 10^{-4}$ M min^{-1}).

Figure 2. Initial reaction rates (expresed in concentration of released H^+ ions per minute) as a function of enzyme concentration. The reaction mixture contained 10 mM Fru6P, 50 mM NH_4Cl, 2,5 mM GlcNAc6P and variable enzyme concentrations, as indicated. From these data and a H^+/GlcN6P ratio of 0.10 (see the text), a molecular activity of 447 s^{-1} can be calculated.

Figure 3. Inhibition pattern by GlcN6P with variable concentration of Fru6P, 50 mM NH_4Cl and 2.5 mM GlcNAc6P. Reaction rate is expressed in concentration of released H^+ ions per minute. (●) without GlcN6P; (o) 0.5 mM GlcN6P; (▲) 0.75 mM GlcN6P. The inhibitor concentrations were respectively 30 and 20% less than the equilibrium concentrations calculated with the lowest concentration of Fru6P used. The values of the parameters, evaluated according to Canela (8), were: (●) Vm = 16.38 ± 0.77 µM/min, Km = 0.96 ± 0.18 mM; (o) Vm = 18.10 ± 0.22 µM/min, Km = 1.13 ± 0.12mM Ki = 0.16 ± 0.04 mM (▲) Vm = 16.50 ± 0.72 µM/min, Km = 1.06 ± 0.19 mM, Ki = 0.17 ± 0.09 mM. Points represent experimental data; lines were drawn with the parameters obtained by the statistical procedure.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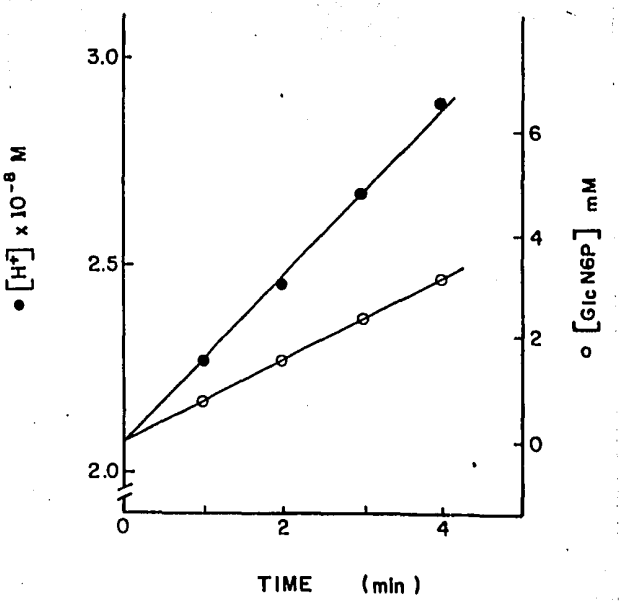
A


• [H+] x 10-8 M
o [Glc N6P] mM
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2.0
6
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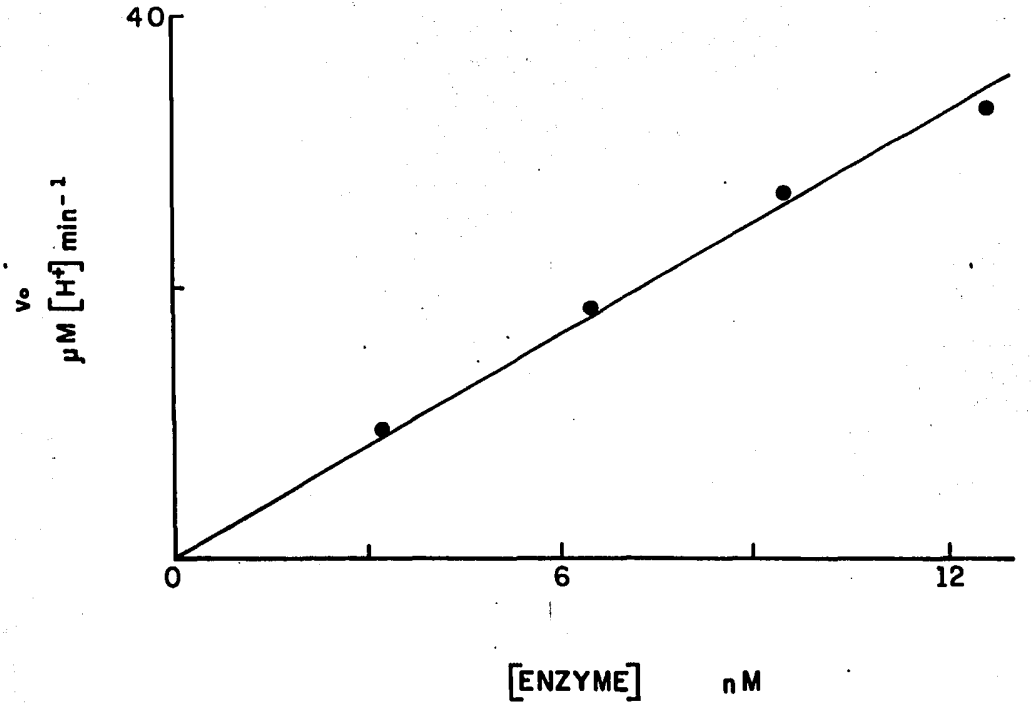
0
2
4
TIME (min)

B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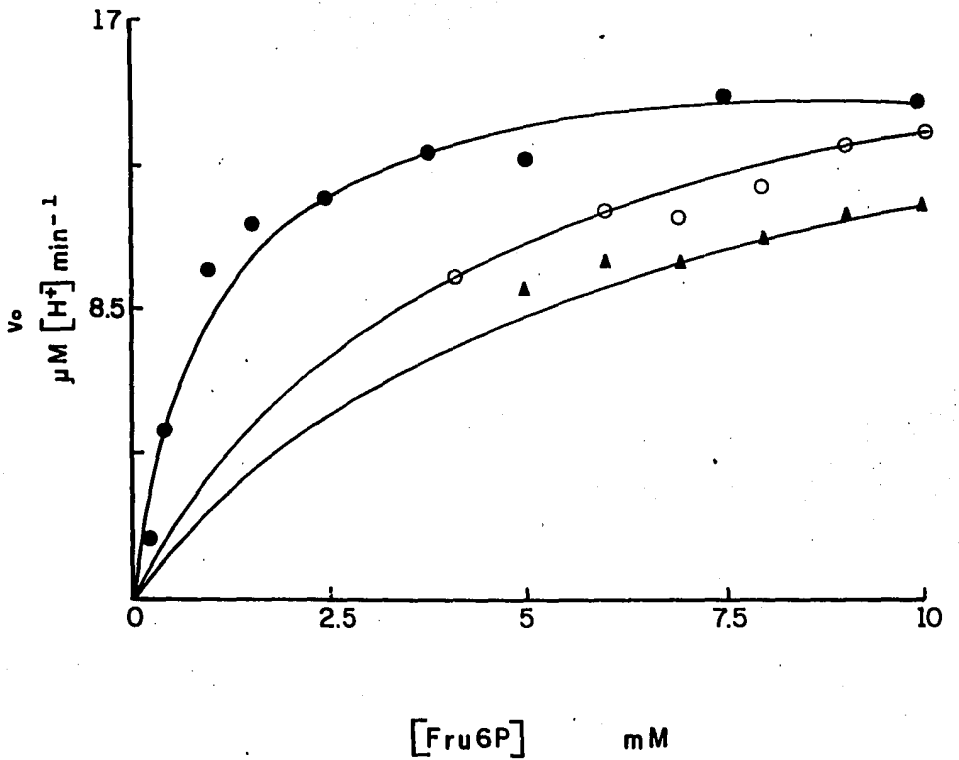
[ENZYME] nM


17
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$\mu M\ [H^+]\ min^{-1}$
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5
7.5
10
[Fru6P] mM

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SULFHYDRYL GROUPS OF GLUCOSAMINE-6-PHOSPHATE ISOMERASE-DEAMINASE FROM ESCHERICHIA COLI.[1]

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Running Title : Sulfhydryl Groups of Glucosamine-6-phosphate deaminase.

SUMMARY.

Glucosamine-6-phosphate isomerase deaminase, (2-amino-2-deoxy-D-glucose-6-phosphate ketol isomerase (deaminating), E.C. 5.3.1.10) from Escherichia coli is an hexameric homopolymer that contains 5 half cysteines per chain. The reaction of the native enzyme with 5'5'-dithiobis-(2-nitrobenzoate) or methyl iodide revealed two reactive SH groups per subunit, whereas a third one reacted only in the presence of denaturants. Two more sulfhydryls appeared when denatured enzyme was treated with dithiothreitol, suggesting the presence of one disulfide bridge per chain.

The enzyme having the exposed and reactive SH groups blocked with 5'-thio-2-nitrobenzoate groups was inactive, but the corresponding alkylated derivative was active and retained its homotropic cooperativity towards the substrate, D-glucosamine-6-phosphate, and the allosteric activation by N-acetyl-D-glucosamine-6-phosphate. Studies of SH reactivity in the presence of enzyme ligands showed that a change in the availability of these groups accompanies the allosteric conformational transition. The results obtained show that sulfhydryls are not essential for catalysis or allosteric behaviour of glucosamine-6-phosphate deaminase.

Sulfhydryl groups play an important role either in ligand binding or in catalysis of several enzymes acting upon phosphorylated substrates, and Rippa et al. (1) have found evidence for the involvement of vicinal thiols near the phosphate ester binding sites of seven enzymes studied.

Recently we purified and characterized the glucosamine-6-phosphate isomerase deaminase (Glucosamine-6-phosphate deaminase, 2-amino-2-deoxy-D-glucose-6-phosphate ketol isomerase (deaminating), E.C. 5.3.1.10) from Escherichia coli B. This enzyme catalyzes the conversion of GlcN6P in Fru6P and ammonia, and is allosterically activated by GlcNAc6P. It is composed of six apparently identical subunits, and the amino acid analysis reveals five cysteine residues per polypeptide chain (2). The enzyme from E. coli has been reported to be inhibited by p-hydroxy-mercuribenzoate (3). The deaminase purified by Das and Datta (4) from Candida albicans, which seems to be a different protein, is also inhibited by sulfhydryl reagents.

The present study was undertaken to elucidate the status of cysteine residues in the glucosamine-6-phosphate isomerase deaminase obtained from E. coli and to evaluate the role of these sulfhydryl groups in the catalysis and the allosteric control of this enzyme.

MATERIALS AND METHODS

ENZYME. Glucosamine-6-phosphate isomerase deaminase from E. coli B (strain ATCC 11303) was purified and stored essentially, as previously described (2). The protamine sulfate step was omitted in the purification scheme, without appreciable change in the characteristics of the enzyme obtained. Purity of each enzyme batch was always verified by denaturing polyacrylamide gel electrophoresis. The specific activity of the enzyme at pH 7.7 and 30°C was in the

range of 650-680 μmoles min^{-1} mg^{-1}. The same value was obtained in the presence of 5 mM dithiothreitol and after its removal by dialysis. Enzyme concentrations were calculated using the molar absorptivity $\varepsilon_{275} = 20.02 \times 10$ M^{-1} cm^{-1}, determinated by the gravimetric method of Kupke and Dorrier (5), weighing three enzyme samples of 0.5 mg each. The value obtained from the complete amino acid composition, using the absorptivity data published by Edelhoch (6), was somewhat lower (16.0×10^{-4} M^{-1} cm^{-1}). Thus, the value previously reported (2) was inaccurate.

Enzyme assays were run as described (2), in the direction of GlcN6P deamination. For each assay, substrate concentration was varied from 0.5 to 10 mM in the presence or absence of 2.5 mM GlcNAc6P. Data were fitted to the Michaelis-Henri equation according to Wilkinson (7). Homotropic cooperative kinetics was fitted to Hill equation by the Atkins procedure (8) to evaluate the maximal velocity and the Hill coefficient.

MATERIALS. GlcNAc6P was prepared from GlcN6P following the procedure of Leloir and Cardini (9); 2-deoxy-2-amino-glucitol-6-phosphate, (sorbitolamine-6-phosphate, SorN6P) was obtained by reduction of GlcN6P with sodium borohydride and purified by ion-exchange chromatography, essentially as described by Midelfort and Rose (10). Purity of both products was verified by thin layer chromatography on cellulose plates, using two different solvent systems (ethyl acetate: acetic acid: water: ammonia 6:2:2:1 and isopropanol: ammonia 4:1). Other biochemicals and DTNB were from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Methyl iodide was from J. T. Baker (U.S.A.) and was distilled before using. All other chemicals were reagent-grade products.

SULFHYDRYL GROUP ANALYSIS. Sulfhydryls were determined spectrophotometrically with varying amounts of DTNB at 25°C in Tris-HCl buffer with 5 mM EDTA in a final volume of 500 µl. The reactions were carried out at room temperature under pseudo-first-order conditions, i.e., with a high molar excess (600-1200 fold) of DTNB. Reactions were started by adding the enzyme, while recording the absorbance at 412 nm with a Pye Unicam SP-1800 double beam spectrophotometer. The number of sulfhydryl groups that reacted per mole of enzyme was determined from the absorbance at the end of the reaction, using the molar absorptivity $\varepsilon_{412} = 14140\ M^{-1}\ cm^{-1}$ (11). Kinetic measurements of sulfhydryl reactivity were carried out under the same conditions, both in absence or presence of ligands. Pseudo-first-order or second-order rate constants were determined by fitting the corresponding equations in their linear form by means of the least squares method.

Alkylation of sulfhydryl groups with methyl iodide was achieved in a 1.0 µM enzyme solution in 50 mM Tris-HCl buffer (pH 8.0) at room temperature in a closed tube. The reaction was started by adding the alkylating agent (2 µl per ml of reaction mixture) to a final concentration of 32 mM.

Treatment of the enzyme with m-periodate was performed in 0.1 M Tris-HCl buffer (pH 8.0) at 20°C, with a 5 µM enzyme solution and a 120 or 1200 times molar excess of sodium m-periodate. Samples of the reaction mixture were taken at different times and diluted into 100 volumes of a 0.1 M glycerol solution to destroy the excess of m-periodate anions and to prepare the enzyme for assay.

RESULTS

DETERMINATION OF SULFHYDRYL GROUPS OF GLUCOSAMINE-6-P DEAMINASE WITH DTNB. When the release of TNB anions in the presence of a high molar excess of DTNB was followed spectrophotometrically, simple pseudo-first-order kinetics were obtained (Fig. 1). In the presence of 8 M urea or 0.5 per cent SDS the reaction was almost instantaneous. From these experiments, 12 sulfhydryl groups per mole of native enzyme and 18 per mole of enzyme, denatured by urea or SDS (2 and 3 residues per subunit respectively), can be calculated (Table I). In another experiment, the enzyme was treated simultaneously with denaturing and reducing reagents. An enzyme sample (0.4 nmoles) was dialyzed against 500 volumes of a solution containing 8 M urea, 50 mM Tris-HCl buffer (pH 8.0, 20°C), and 10 mM dithiothreitol. The content of the dialysis sack was freed of the latter reagent by further dialysis against a deaerated urea-Tris solution of similar composition, and the sulfhydryls were determined with DTNB under the conditions described. The results (Table I) indicate that the number of cysteines in denatured and reduced enzyme is 28 per mole, i.e., 5 residues per chain (nearest integer). The table also shows that in absence of the denaturant, dithiothreitol does not produce any change in the number of sulfhydryls reacted.

In another experiment, a sample of native enzyme was treated with methyl iodide for 30 minutes under the conditions described in Methods, and dialyzed exhaustively against 50 mM Tris-HCl (pH 8.0); afterwards, sulfhydryls were determined by DTNB reaction. As shown in Table I, methylation blocks the two reactive sulfhydryls per subunit, whereas a third one remains unchanged and can be determined with the DTNB reaction in the presence of SDS.

EFFECT OF MODIFICATION OF REACTIVE SH GROUPS UPON ENZYME ACTIVITY. Native enzyme, fully reacted with DTNB and containing two TNB groups per polypeptide chain, was totally inactive. In other series of experiments, samples of the reaction mixture containing native enzyme and DTNB were taken at different time intervals and the fraction of Vmax with respect to zero time was determined for each one. The plot of the fraction of activity, as a function of the number of sulfhydryl groups reacting per mole of enzyme, is shown in Fig. 2.

To reactivate the enzyme, modified by the addition of TNB groups, a fully reacted sample (0.9 nmoles) was dialyzed against a volume excess of 10 mM dithiothreitol in 50 mM Tris-HCl buffer, pH 7.7, for 6 hours at 4°C. Under these conditions, 65 per cent of the original activity was recovered (average of two determinations).

The enzyme having the 12 reactive thiols modified by methylation remains practically unchanged in its kinetic behaviour. Homotropic cooperativity towards GlcN6P (Hill coefficient = 2.3), and activation by GlcNAc6P giving hyperbolic kinetics in its presence at saturating concentrations (Km for GlcN6P = 0.5 ± 0.04 mM), were essentially the same as in the untreated enzyme. Nevertheless, the specific activity of the modified enzyme was 463 μmoles min^{-1} mg^{-1} , 76 per cent of the expected value (average of two experiments). This methylated enzyme is insensitive to inhibition by DTNB.

m-PERIODATE TREATMENT OF THE ENZYME. The enzyme treated with m-periodate under the conditions described in Methods, retained its specific activity for 30 min; after this time, no modifications in thiol content were detected with DTNB. The enzyme kinetics and the activation by GlcNAc6P were the same

as in the untreated enzyme. Similar results were obtained using 120 or 1200 times molar excess of m-periodate.

DTNB REACTION AND EFFECT OF LIGANDS ON THE REACTIVITY OF THIOL GROUPS. The 12 exposed groups present in the native enzyme displayed pseudo-first-order kinetics when reacting with DTNB under the large molar excess of the reagent used (Fig. 1). Reaction order with respect to DTNB was also one (Fig. 3).

The second order rate constant at pH 8.0, 20°C was of about 2.0 - 2.5 M^{-1} s^{-1} . In comparison, the second order rate constant for the reaction between reduced glutathione and DTNB, at the same pH and temperature, was four orders of magnitude larger (4×10^4 M^{-1} s^{-1}).

The reactivity of exposed SH groups towards DTNB at pH 8.0 in the presence of several enzyme ligands is compared in Table II. None of the ligands tested influenced the kinetic pattern of thiol reactivity or the number of groups that reacted. When pseudo-first-order rate constants are compared, some inhibition of reactivity was observed in the presence of 20 mM GlcN6P or Fru6P; no effect was produced by ammonia or its dead-end analog methylammonium. The allosteric activator, GlcNAc6P, markedly diminished the availability of thiols to DTNB. The same effect is obtained with the competitive inhibitor, SorN6P, (Table II).

DISCUSSION

Sulfhydryl group analyses of glucosamine-6-phosphate isomerase deaminase reveal three of these groups per polypeptide chain, two exposed and reactive and a third unreactive one, that is either buried in the enzyme structure or involved in some kind of side-chain interactions which make it

unavailable for the reagent. The simple pseudo-first-order kinetics of the reaction implies also that the exposed groups react at the same rate, indicating a similar local environment. A true first-order reaction (zero order for DTNB)was not found, as would be expected if there were a rate-limiting first-order step, such as the unfolding of protein structure. On the other hand, second order kinetics is expected for groups exposed on the protein surface. Nevertheless, reactivity of these exposed groups towards DTNB is considerably lower than glutathione sulfhydryl, taken as a model of an interaction-free group. This finding shows that significant interactions also exist in which these thiols participate.

The reduction experiment of the urea-denatured enzyme with dithiothreitol reveals two additional sulfhydryl groups per enzyme subunit. This observation could be explained as the reduction of a preexisting disulfide linkage in each subunit. This result accounts for the five cysteic acid residues per oxidized polypeptide chain reported to be present (2), although, disulfide-containing proteins are not generally produced by E. coli (12) due to its high intracellular reducing potential. We can not exclude the oxidation of the enzyme during preparation or storage, or even the presence of the two thiols being blocked in some other way. Further experiments are necessary to explain this finding.

The exposed sulfhydryl groups were not modified by treatment of the enzyme with m-periodate, under the same conditions that according to Rippa et al. (1) produce oxidation of vicinal thiols in different proteins. This result reveals a noticeable structural difference with enzymes studied by these authors.

Although the reaction of the 12 exposed sulfhydryls with DTNB completely inactivates the enzyme (Fig. 2), its alkylation with methyl iodide, a smaller and charge-free reagent, does not produce significant kinetic changes, except for a decrease in the turnover number. This could be due to side reactions of methyl iodide with other amino acid side chains. Alkylation experiments directly prove that SH groups are not involved in the catalysis or the allosteric control of the enzyme. Inhibition by DTNB or mercuri - als (3) can be a consequence of conformational distortion of protein structure by the introduction of bulky charged groups. This change can be only partially reversed by dithiothreitol.

Enzyme ligands decrease the reactivity towards DTNB of the 12 exposed sulfhydryl groups. The allosteric activator: GlcNAc6P, and the competitive inhibitor: SorN6P, markedly reduce the reactivity of these SH groups, and protect them entirely at high concentrations. The change of the rate constant produced by the allosteric effector, parallels the effect of this ligand to reduce positive cooperativity for the reaction of GlcN6P deamination, [(2), Fig. 1]. SorN6P, an analog of the open-chain form of the substrate (10) and able to occupy the entire active site, produces the same effect. The inhibited enzyme displays hyperbolic kinetics, as expected for an allosteric K-system (Hill coefficient is near 1 between 20 and 30 μM SorN6P).

Fru6P and GlcN6P, both allosteric substrates (2), also produce some inhibition of SH reactivity at the concentrations tested. It should be taken into account that, under these conditions, saturation kinetics is not

attained yet, because of the low apparent affinity of the deaminase for the sugar-phosphates in the absence of the activator [(2), Fig. 2]. Ammonium, a substrate, and the inhibitor methylammonium, do not modify the reactivity of the exposed thiols. Since the kinetic mechanism of the enzyme is still to be determined, this result may be explained in terms of an ordered sequential addition of substrates, but it is also interesting to note that ammonium is not an allosteric substrate for this enzyme (2).

The results presented, taken as a whole, rule out the possible participation of sulfhydryl groups in the active or allosteric site of the enzyme or their participation in interactions essential for the allosteric conformational change. A similar finding, the inhibition by DTNB and iodoacetamide, and the protection against these inhibitors provided by methylating the reactive surface sulfhydryls, has been described also for pig muscle-3-phosphoglycerate kinase by Dékány and Vas (13).

Reactivity of exposed sulfhydryls is notably modified by ligands that induce the allosteric conformational transition. This correlation suggests that the reactivity changes found are a simple consequence of a conformational change in which the thiols do not play an essential role.

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FOOTNOTES

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2 To whom all correspondence should be addressed.

3 The following abbreviations are used: GlcN6P, D-glucosamine-6-phosphate; Fru6P, D-fructose-6-phosphate; GlcNAc6P, N-acetyl-D-glucosamine-6-phosphate; SorN6P, 2-amino-2-deoxy D-glucitol-6-phosphate, or sorbitolamine-6-phosphate; DTNB, 5'5'-dithiobis-(2-nitrobenzoic acid); TNB, 5'-thio-2-nitrobenzoic acid; SDS, sodium dodecyl sulfate.

LEGENDS FOR THE FIGURES

Fig. 1. DTNB titration of SH groups of native glucosamine-6-phosphate isomerase deaminase. In a 500 µl microcell, 0.65 µM enzyme and 0.5 mM DTNB were mixed under the conditions described in Methods. Absorbance data at 412 nm, were read against a blank without enzyme. The total number of SH groups per mole of enzyme (12) was calculated from absorbance at the end of the reaction, and used to determine the unreacted groups for each time.

Fig. 2 Effect of modification of reactive SH groups with DTNB on the activity of glucosamine-6-phosphate isomerase deaminase. Enzyme (0.65 µM) was reacted with DTNB (2.0 mM) under the conditions described in Methods. At appropriate times during the reaction, 15 µl samples of the reaction mixture, were taken and the reaction stopped by 20 times dilution with water. Enzyme was assayed immediately as described, and fraction of Vmax was plotted against the number of SH groups reacting with DTNB per mole of enzyme at the time when the reaction was brought to a halt.

Fig. 3 Reaction order with respect to DTNB.
Pseudo-first-order rate constants, represented in the vertical axis, were obtained under the same experimental conditions described for Fig. 2, except that the reagent concentration was varied.

TABLE I.

Sulfhydryl groups reacting with DTNB per mole of enzyme (a)

Reaction conditions	Thiol groups per mole enzyme
Unmodified	12.2 ± 0.3
8 M urea (b)	17.3 ± 0.9
0.5 % SDS (b)	18.4 ± 1.5
10 mM dithiothreitol (c,d)	11.9
10 mM dithiothreitol + 8 M urea (c,d)	28.0
Methylated enzyme (c,d)	0
Methylated enzyme, 0.5 % SDS (c,d)	6.1

(a) All the reactions were performed at 20°C, with 0.7 μM enzyme, 0.5 mM DTNB, 5 mM EDTA and 0.1 M Tris-HCl buffer (pH 8.0), in a final volume of 500 μl (data from 6 determinations).

(b) Reaction made in the presence of the denaturing agent

(c) See details in the text.

(d) Average of two independent experiments.

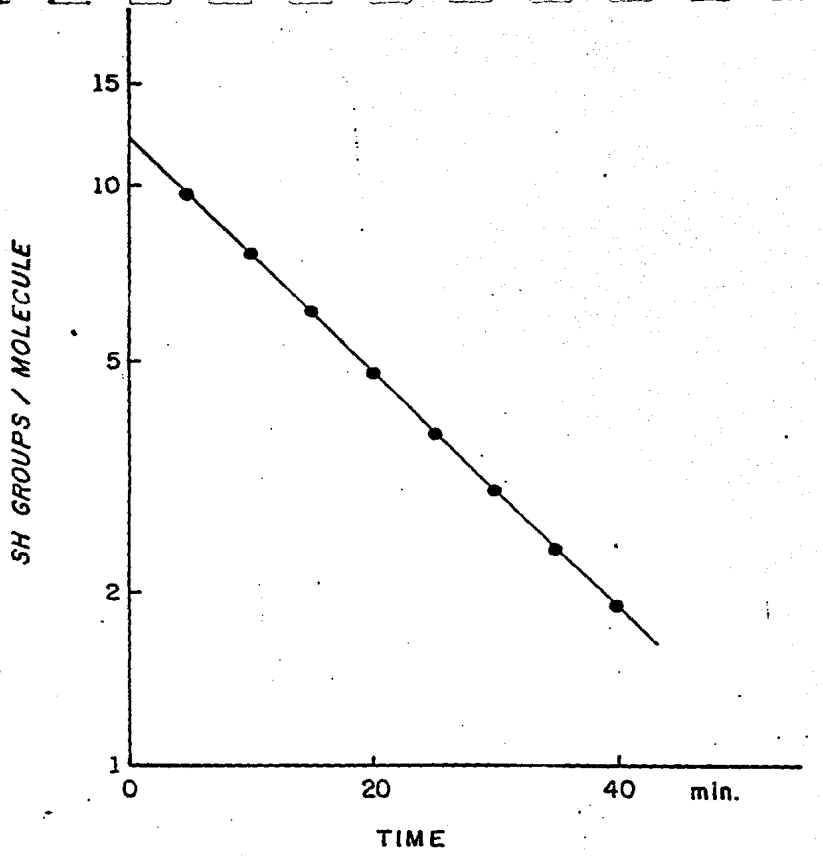
TABLE II.

Effect of ligands on the reactivity of thiol groups of Glucosamine - 6-P isomerase deaminase (a).

Ligand	concentration mM	2nd order rate constant $\times 10^{-3}$ M^{-1} s^{-1}	Reactivity (b)
None		2.43	1
GlcNAc6P	0.75	1.08	0.44
	1.50	0.64	0.26
	2.5	0.18	0.07
SorN6P	0.012	1.60	0.66
	0.030	0.21	0.09
	20	0	0
GlcN6P	15	2.07	0.85
Fru6P	15	1.80	0.74

(a) Conditions were the same as in Table I, except that DTNB was 2 mM. From the spectrophotometric determination of the reaction, the pseudo-first-order rate constant was fitted by linear regression of the semilogarithmic form of the first-order equation; the corresponding second order constant was obtained dividing this result by the DTNB concentration.

(b) Calculated from each second-order rate constant relative to its value in absence of ligands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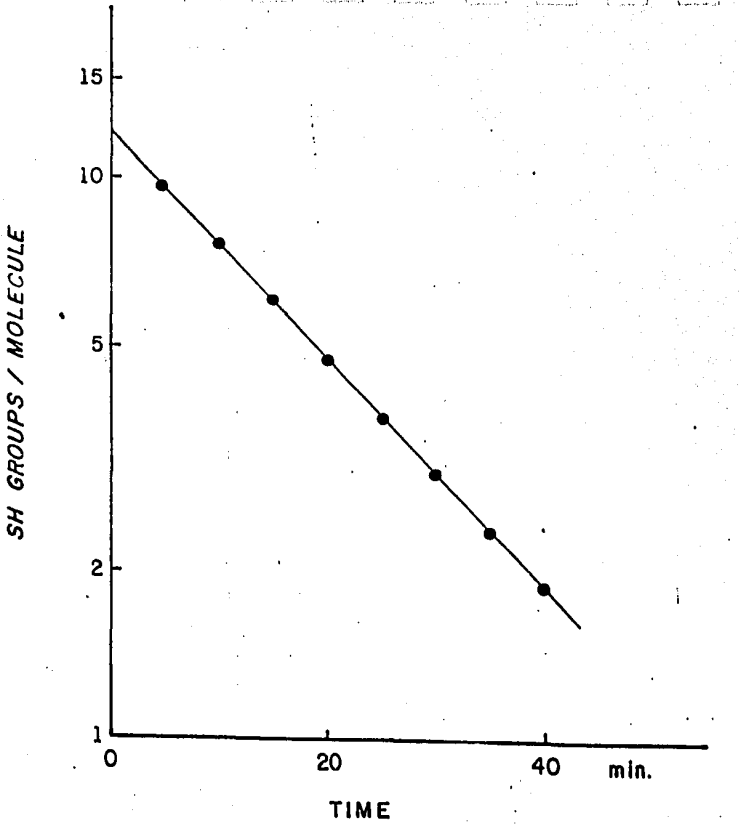

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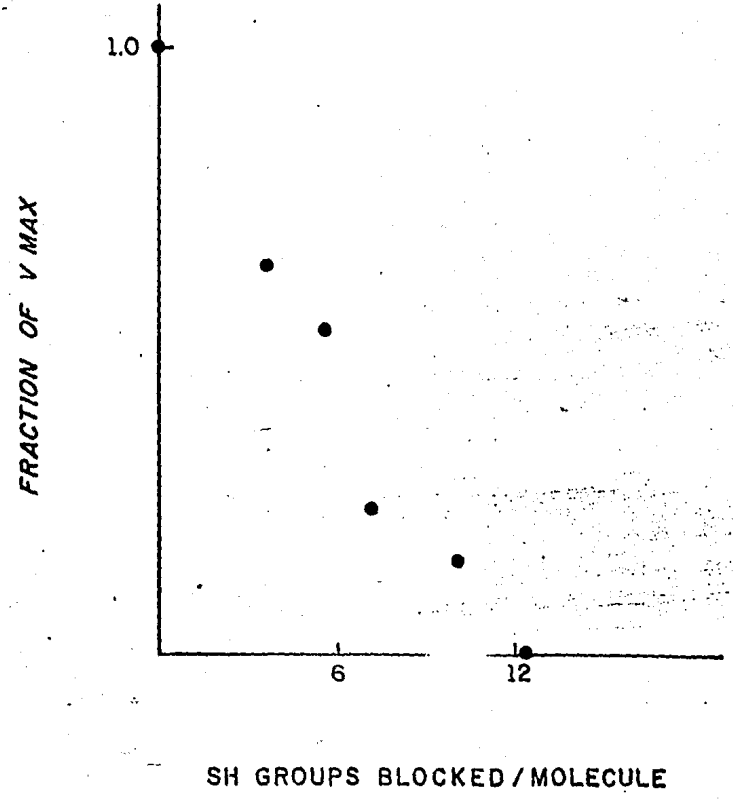

Fig. 2

Fig. 3 Calculation of

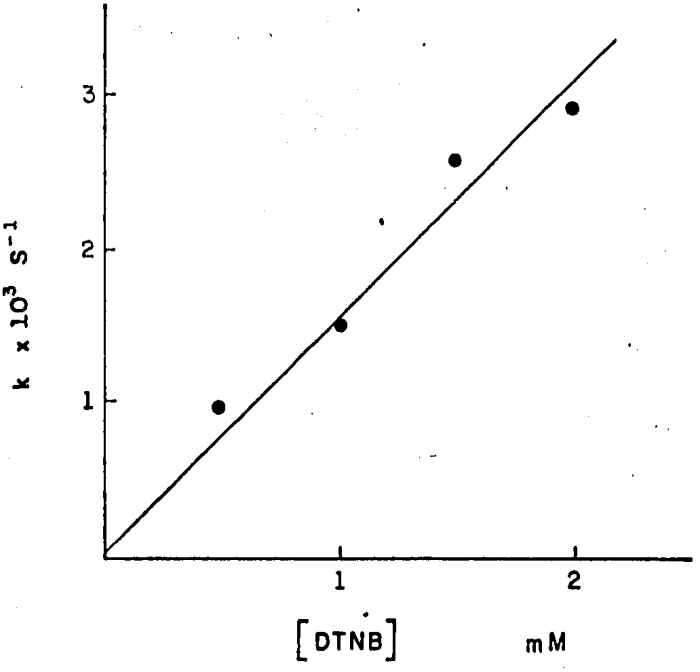

Fig. 3

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Title: Sulfhydryl groups of glucosamine-6-phosphate isomerase-deaminase from escherichia coli

Comments: (Use additional pages if necessary, on ordinary paper.)

This paper is clearly written and presents well-executed data on the chemical modification of sulfhydryl residues in glucosamine-6-phosphate isomerase. Although the data show clearly that the reactive residues are not critical for catalysis or allosteric behavior, the finding that they become less reactive in the presence of substrates does give some information about ligand-induced conformational changes in the enzyme.

My main reservation about the paper is that E. coli normally do not contain or produce sulfide-containing proteins (Wright and Schuster (1966) Proc. Natl. Acad. Sci. USA 63, 1043-1047). Ferredoxin, thioredoxin reductase, and ribonucleotide reductase (Holmgren (1985) Ann. Rev. Biochem. 54, 237). It is thought that this is because bacteria have a low intracellular redox potential. It is also possible that two of the sulfhydryl residues in this enzyme have been blocked or oxidized in some other way. I think that the authors should check their results and the literature with my reservation in mind and make some relevant comment on their findings.

November 6, 1986

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México

Dear Dr. Calcagno:

Your manuscript entitled "Sulfhydryl Groups of Glucosamine-6-Phosphate Isomerase-Deaminase from *E. coli*" has been thoroughly evaluated with the assistance of reviewers who are knowledgeable in the field. We consider this to be an interesting and well executed study. However, two of the reviewers have pointed out how unusual it is to find proteins of *E. coli* which have disulfides and suggest that you re-check this observation and discuss this result with reference to the available literature. In addition, suggestions have been made to improve the presentation of the paper. The detailed comments are enclosed, and corrections have also been made on two of the three copies of the manuscript which are being returned to you.

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Discusión.

En este trabajo se presentó el estudio de algunas características cinéticas y estructurales de la glucosamina 6-fosfato isomerasa (desaminasa) de *Escherichia coli*.

Los patrones de velocidad inicial, intersecantes en el eje de la abscisa (resultados del primer artículo), sugieren la formación de un complejo central E-Fru6P-NH_4^+ → E-GlcN6P (1), y también que las constantes de Michaelis y las de inhibición de los sustratos, cuando se estudia su papel como productos inhibidores, sean iguales si el mecanismo cinético fuera ordenado en estado estacionario. Si este fuera el mecanismo cinético, los patrones de inhibición por GlcN6P esperados son: competitivo, con el primer sustrato en unirse a la enzima, y no competitivo intersecante en el eje de las abscisas, con el segundo sustrato (2). La inhibición que se presenta en el segundo artículo indicaría que la Fru6P es el primer sustrato que se une y se esperaría que la $K_{M\ GlcN6P}$ sea igual a la $K_{I\ GlcN6P}$; sin embargo las constantes no son iguales. La $K_{M\ GlcN6P}$ es de 2.2 mM (ver resultados del primer artículo), y la $K_{I\ GlcN6P}$ aparente obtenida variando Fru6P con 50 mM de NH_4^+ y 2.5 mM de GlcNAc6P es de 0.17 mM (ver resultados del segundo artículo). Aunque para establecer el mecanismo cinético hace falta completar el estudio de inhibición por productos en ambos sentidos de la reacción, la no coincidencia de las constantes cinéticas para la GlcN6P es una evidencia de que no se trata de un mecanismo ordenado en estado estacionario.

Otro mecanismo cinético congruente con los patrones de velocidad inicial intersecantes que se presentan, es al azar en equilibrio rápido cuando la unión de cualquiera de los sustratos, en el sentido birreactante, a la enzima libre no tienen efecto en la unión del siguiente sustrato. En el estudio de inhibición por productos en el mismo sentido; al disminuir el sutrato fijo la K_I aparente del producto inhibidor, disminuye aproximandose al valor de la constante de disociación (3); por el valor del K_I obtenido para la GlcN6P (0.17 mM) se

esperarla una $K_{s\ GlcN6P}$ por lo menos un orden de magnitud menor que la obtenida (2.2 mM). Sin embargo, para descartar de forma inequivoca este mecanismo, al igual que con el anterior, hace falta completar el estudio de inhibición por productos.

Otros dos posibles mecanismos son: al azar en estado estacionario y el ordenado en equilibrio rápido. Con el primero se obtienen los mismos patrones de velocidad inicial y de inhibición por productos que el de azar en equilibrio rápido (1), distinguiendose únicamente de éste por que las K_M son diferentes de las constantes de disociación (4). Este mecanismo podria ser apoyado por los valores diferentes de K_M y de K_I para la GlcN6P; sin embargo, para confirmarlo es necesario completar el estudio de inhibición por productos y medir las constantes de disociación de los sustratos en experimentos de equilibrio. Para el segundo los patrones de velocidad inicial esperados no son simétricos; para este mecanismo variando el primer sustrato con varias concentraciones fijas del segundo se espera un aumento en la velocidad máxima y una disminución del K_M aparente, en el otro patrón siempre se obtiene la misma velocidad máxima (5); por lo que queda descartado de acuerdo a los patrones de velocidad inicial presentados.

Con respecto a las propiedades alostéricas de la enzima, descritas en base al modelo de Monod (6); se menciona que el modulador positivo, GlcNAc6P, transforma la cinética sigmoide en hiperbólica. Este resultado se interpreta como fijación exclusiva del ligando (discusión del primer articulo). Sin embargo también se puede obtener una michaelización total en el caso general de este modelo, fijación no exclusiva de activador y sustrato, para varias combinaciones de parámetros y constantes; por ejemplo, con n mayor a 10, un coeficiente de fijación no exclusiva para el activador de 0.005, para el sustrato de 0.05, constantes alostéricas entre 1×10^4 y 1×10^6 y una concentración especifica de activador tan baja como 2.5. La cinética hiperbólica obtenida con el modulador

positivo de esta enzima se puede explicar por cualquiera de estas dos causas. Para decidir a cual de los dos casos se ajusta el comportamiento de esta enzima, es necesario cuantificar los sitios activos y alostèricos por mètodos directos, por ejmplo diàlisis en equilibrio.

Los resultados del primer articulo muestran que la enzima homogènea es un hexàmero que tiene 5 Cys por subunidad. Dos de ellos detectables en condiciones desnaturalizantes en presencia de ditiotreitol (resultados del tercer articulo), suguiriendo la existencia de puentes disulfuro; ya sea, uno intrasubunidad o que las subunidades podrlan estar unidas por puentes disulfuro, dos entre cada subunidad. El caracter homopolimèrico de la enzima sugerido en la discusiòn del primer articulo, es cuestionable. El electroenfoque en el que se apoya esta conclusiòn no se realizò en condiciones reductoras de puentes disulfuro. Aunque no se puede descartar que estos residuos de Cys se hayan oxidado durante el proceso de purificaciòn de la enzima o el almacenamiento; este no serla el primer caso de una enzima de *E. coli* en el que las subunidades estèn unidas por puentes disulfuro; se ha reportado que la triptofanasa de esta bacteria tiene un puente disulfuro por subunidad (7).

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