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Identification of Two Cysteine Residues Forming a Pair of Vicinal Thiols in Glucosamine-6-phosphate Deaminase from *Escherichia coli* and a Study of Their Functional Role by Site-Directed Mutagenesis†

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ABSTRACT: The nucleotide sequence of the *nagB* gene in *Escherichia coli*, encoding glucosamine-6-phosphate deaminase, located four cysteinyl residues at positions 118, 219, 228, and 239. Chemical modification studies performed with the purified enzyme had shown that the sulfhydryl groups of two of these residues form a vicinal pair in the enzyme and are easily modified by thiol reagents. The allosteric transition to the more active conformer (R), produced by the binding of homotropic (D-glucosamine 6-phosphate or 2-deoxy-2-amino-D-glucitol 6-phosphate) or heterotropic (*N*-acetyl-D-glucosamine 6-phosphate) ligands, completely protected these thiols against chemical modification. Selective cyanylation of the vicinal thiols with 2-nitro-5-(thiocyanato)benzoate, followed by alkaline hydrolysis to produce chain cleavage at the modified cysteines, gave a pattern of polypeptides which allowed us to identify Cys118 and Cys239 as the residues forming the thiol pair. Subsequently, three mutated forms of the gene were constructed by oligonucleotide-directed mutagenesis, in which one or both of the cysteine codons were changed to serine. The mutant proteins were overexpressed and purified, and their kinetics were studied. The dithiol formed by Cys118 and Cys239 was necessary for maximum catalytic activity. The single replacements and the double mutation affected catalytic efficiency in a similar way, which was also identical to the effect of the chemical block of the thiol pair. However, only one of these cysteinyl residues, Cys239, had a significant role in the allosteric transition, and its substitution for serine reduced the allosteric interaction energy, due to a lower value of K_T.

Glucosamine-6-phosphate deaminase catalyzes the reversible conversion of D-glucosamine 6-phosphate (GlcN6P)[1] into D-fructose 6-phosphate and ammonia. It is allosterically activated by *N*-acetyl-D-glucosamine 6-phosphate (GlcNAc6P) (Comb & Roseman, 1958; Midelfort & Rose, 1977; Calcagno et al., 1984). The enzyme from *Escherichia coli* is an oligomeric protein composed of six identical polypeptide chains (Calcagno et al., 1984) whose primary structure is known from the DNA sequence of the gene *nagB* encoding this enzyme (Rogers et al., 1988). The gene, located at 15.5 min on the *E. coli* chromosome (White, 1968; Holmes & Russell, 1972),

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GlcN6P, D-glucosamine 6-phosphate; GlcNAc6P, *N*-acetyl-D-glucosamine 6-phosphate; NTCB, 2-nitro-5-(thiocyanato)benzoic acid; SDS, sodium dodecyl sulfate; TNB, 2-nitro-5-thiobenzoate.

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is part of the divergent *nagE–nagBACD* operons, which have been recently characterized (Rogers et al., 1988; Plumbridge, 1989; Vogler & Lengeler, 1989). The amino acid sequence of the enzyme (Rogers et al., 1988) shows four cysteinyl residues per enzyme subunit, corresponding to amino acids 118, 219, 228, and 239. Chemical modification studies on the purified deaminase from *E. coli* B had previously identified the presence of two reactive sulfhydryl groups per polypeptide chain in the native enzyme (Altamirano et al., 1987). They were shown to form a vicinal pair and to react with different thiol reagents, but only when the enzyme is in its less active allosteric conformation (Altamirano et al., 1989); upon allosteric activation by homotropic or heterotropic ligands, these thiols become completely protected (Altamirano et al., 1987, 1989). They are also involved in Zn^{2+} binding by the enzyme. When zinc-bound enzyme is allosterically activated, the Zn^{2+} ion is sequestered by the protein and is only released when it returns to its less-active conformation (Altamirano & Calcagno, 1990). This pair of sulfhydryls is essential for full activity of glucosamine-6-phosphate deaminase, affecting both the catalytic constant and allosteric activation. It has been shown that modification of the thiol pair by different reagents (Altamirano et al., 1989), or their participation in Zn^{2+} binding (Altamirano & Calcagno, 1990), produces a decrease of the molecular activity to half of the value for the native enzyme and a diminished cooperativity toward GlcN6P. This latter finding indicates that these thiols are playing some role in the allosteric transition and that the observed changes in their chemical reactivity are not a simple consequence of their passive displacement during the conformational transition. It is also possible that the region of the protein undergoing these structural changes involving vicinal thiols is located in the neighborhood of the active site, as suggested by the k_{cat} alteration in the SH-blocked enzyme.

To better understand the role of these thiols in the mechanism of catalysis and control of the deaminase, we have undertaken their localization along the enzyme's polypeptide chain, and we have used this information to construct the corresponding Cys to Ser mutants. This approach makes it possible to characterize the functional contribution of each thiol group individually. As both sulfhydryls are equally reactive to chemical reagents, their chemical modification does not allow us to evaluate their separate contribution to catalysis and regulation.

Experimental Procedures

Bacteria and Enzyme. Wild-type *E. coli* glucosamine-6-phosphate deaminase was prepared from a strain carrying a plasmid which overproduced the protein from the *lac* promoter, as previously described (Altamirano et al., 1991). Similar plasmids expressing the three mutated forms of the protein (missing one or both of the vicinal cysteines; see below) were constructed. The host strain for expression of these mutated proteins was IBPC546R, where expression of the chromosomal copy of the *nagB* gene is eliminated by insertion of a kanamycin-resistance cassette in the gene (Plumbridge, 1991). This strain is in addition Δ*lac*, so that expression of the deaminase is constitutive. Expression of *nagB* gene was measured by enzyme assays and SDS–polyacrylamide gel electrophoresis in the discontinuous Laemmli system (Laemmli, 1970). Overproduction of both wild-type and mutated proteins was estimated to be in the range 20–25% as judged by Coomassie blue G-250 staining of electrophoresis gels. Bacteria were grown and mutant deaminases were prepared as described for the wild-type enzyme (Altamirano et al., 1991). The *N*-(ε-aminohexanoyl)-D-glucosamine 6-phosphate agarose affinity column, which binds deaminase by the allosteric site, also proved to be useful for the purification of the mutant enzymes. Purity of all enzyme preparations was verified electrophoretically in polyacrylamide gels, both in the denaturing Laemmli system or with the native protein, in 4–30% pore-gradient gels, as described (Calcagno et al., 1984). The concentration of wild-type or genetically modified enzymes was calculated from the absorbance at 278 nm in a solution of 25 mM Tris-HCl buffer, pH 7.8, using the known molar absorptivity for the wild-type protein (Altamirano et al., 1987). Mutations at the two reactive cysteine residues were not expected to change the absorption at 278 nm (Edelhoch, 1967). Enzyme assays and analysis of the kinetic data were performed as described previously (Altamirano et al., 1987, 1989). Unless otherwise specified, kinetic data were obtained at pH 7.7 and 30 °C, in the presence of 2.5 mM EDTA.

Specific Cyanylation of the Reactive Thiols. A 5 μM solution of wild-type deaminase was cyanylated under nondenaturing conditions with 2 mM 2-nitro-5-(thiocyanato)-benzoate (NTCB) in 50 mM potassium phosphate buffer, pH 7.50, containing 2.5 mM disodium EDTA (buffer A). The time course of the reaction was followed spectrophotometrically at 412 nm, with the release of TNB anion measured against an appropriate blank in a double-beam spectrophotometer.

Identification of the Cysteine Residues Reactive with NTCB in the Native Protein. Deaminase polypeptide chain, specifically cyanylated at the reactive thiols, was cleaved at these modified residues by alkaline hydrolysis according to Dégani and Patchornik (1974). Aliquots of 2–3 nmol of deaminase in 100-μL samples were cyanylated overnight under the nondenaturing conditions described above. The reaction mixtures were then extensively dialyzed against buffer A to remove NTCB and TNB and then treated with 50 mM *N*-ethylmaleimide (NEM) for 4 h in 3.5 M guanidinium thiocyanate. This step was introduced in an attempt to block a buried sulfhydryl group, which becomes highly reactive on denaturation (Altamirano et al., 1987) and which would produce undesirable side reactions with the thiocyanoalanine residues. Samples were then dialyzed against water; the precipitated protein was dissolved by the addition of solid guanidinium thiocyanate to give a final concentration of 3.5 M, and the pH was adjusted to 9.5 with 0.1 volume of 0.9 M potassium borate buffer, pH 9.5, and some 0.1 M KOH, if necessary. In other samples, 0.5% SDS was used instead of guanidinium thiocyanate. Samples were incubated at 37 °C for 12–48 h, and then they were dialyzed against 50 mM Tris-HCl buffer, pH 6.8, using a dialysis membrane with a cutoff of 2 kDa (benzoylated dialysis tubing, Sigma Chemical Co.) and stored frozen. Diluted samples were concentrated by lyophilization. Dialyses were performed in microchambers made by cutting 1.4-mL plastic Eppendorf tubes transversely 4 mm below the border and closing the small chamber under the lid with a piece of dialysis membrane. The compartment containing the sample became sealed when the lid was placed in the tube. The position of the membrane is the same used by Reinard and Jacobsen (1989) in their equilibrium dialysis microchambers. Dialysis occurs through the open end of the cut tube; the assembled microchambers were submerged in a flask containing the dialysis buffer and were vigorously stirred.

Electrophoretic Separation of Cleavage Products. Deaminase samples cleaved at cysteinyl residues corresponding to vicinal thiols were fractionated by SDS–polyacrylamide gel electrophoresis using Laemmli discontinuous system and 0.75-mm-thick gel slabs. The stacking gel had 6.5% total

acrylamide concentration with 3.5% cross-linking. The separation gel was 21% with a cross-linkage of 0.5%. Low cross-linking was used to improve gel pliability; both gels contained 10% glycerol, to slow diffusion. These gels yield good separations in the range 30–3 kDa. Protein bands were detected with the silver-staining procedure of Wray et al. (1981). Cyanogen bromide fragments of sperm whale myoglobin, carbonic anhydrase, trypsinogen (phenylmethanesulfonyl fluoride treated), soybean trypsin inhibitor, and glucagon were used as molecular weight standards. Myoglobin BrCN fragments, which were prepared according to Gross and Witkop (1962), gave the five-band pattern described by Kratzin et al. (1989).

Construction of Mutated nagB Genes. The *nagB* gene, on a 1.4-kb *Eco*RI–*Hin*dIII fragment, was cloned from pUC-(*nagB*) (Altamirano et al., 1991) into M13mp19. Oligonucleotide mutagenesis was performed by the Kunkel method, as described (Sambrook et al., 1989). In one of the mutants, the Cys(TGC)118 was changed to Ser(AGC); in the other, Cys(TGC)239 was changed to Ser(AGC). Oligonucleotides of lengths of 21 and 23 bases, respectively, were used. They corresponded to the sense strand of the *nagB* gene and were centered around the base to be mutagenized. The double mutation was made by introducing the Cys239 change into the M13 carrying the Cys118 mutation. The mutations were checked by sequencing using oligonucleotides conveniently placed within the *nagB* coding sequence. The changes are to highly expressed serine codons and are not expected to change the expressivity of the protein. The mutagenized genes were excised as *Eco*RI–*Hin*dIII fragments and were recloned back into pUC18 and also pBR322. In the latter plasmid, there is no strong promoter positioned to express the *nagB* gene, which is just expressed at a low level from plasmid transcription. This expression is however sufficient to allow complementation of the classic *nagB2* mutation (Rogers et al., 1988) present in strain IBPC571R (*thi*-1, *argG6*, *argE3*, *his-4*, *mtl-1*, *xyl-5*, *tsx-29*, *rpsL*, Δ*lacX74*, *nagB2*, *zbf507*::Tn10). The three mutated alleles were maintained in *recA* strains to minimize their loss by recombination. For purification purposes, the pUC(*nagB*) mutant plasmids were introduced into IBPC546R (*thi*-1, *argG6*, *argE3*, *his-4*, *mtl-1*, *xyl-5*, *tsx-29*, *rpsL*, Δ*lacX74*, *nagB*::km, *recA1*, *srl*::Tn10), the recombination-deficient strain carrying a null mutation in the chromosomal copy of the *nagB* gene (Plumbridge, 1991).

Measurements of the Reactive and Total SH Groups in Deaminase Mutant Proteins. The number of reactive thiols corresponding to the vicinal pair was determined with 5-5′-dithiobis(2-nitrobenzoic) acid (DTNB), at pH 8.0, 20 °C, in the presence of 5 mM EDTA, by measuring the release of 2-nitro-5-thiobenzoate (TNB), as described previously (Altamirano et al., 1987). After completion of the reaction, 10% SDS was added to give a final concentration of 1%, and the increase in TNB was measured, to determine buried sulfhydryls. Wild-type enzyme was also measured as reference.

Results

Specific Cyanylation of Vicinal, Reactive SH Groups. Wild-type, nondenatured deaminase was cyanylated with NTCB as described under Experimental Procedures. The reaction followed pseudo-first-order kinetics, as expected for two equally reacting groups. The stoichiometry of the reaction, calculated from the amount of TNB released, was 2.07 sulfhydryls/enzyme subunit. A second-order rate constant of 2.1 × 10^2 M^{-1} s^{-1} for the reaction at pH 8.0 and 20 °C was calculated. The kinetic study of the S-cyanylated enzyme at the deaminating direction of the reaction showed a k_{cat} of 820 ± 42 s^{-1} and a K_m for GlcN6P of 1.20 ± 0.07 mM, when assayed in the allosteric activated form in the presence of 1 mM GlcNAc6P. In the absence of the activator the enzyme displayed positive cooperativity, with a maximal Hill coefficient of 1.60 (Figure 1).

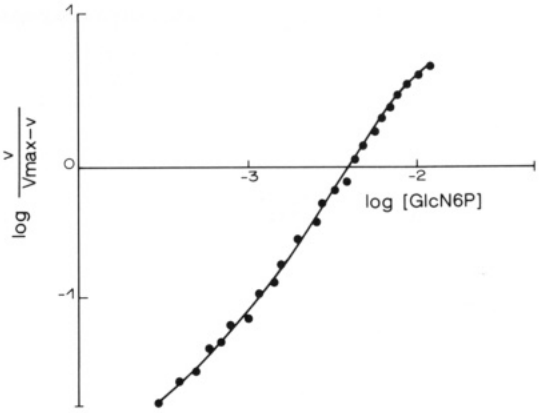


FIGURE 1: Kinetics of glucosamine-6-phosphate deaminase modified by cyanylation at the reactive pair of cysteinyl residues (Hill plot). Data plotted are averages from two independent experimental series. The maximum Hill coefficient for these data is 1.60.

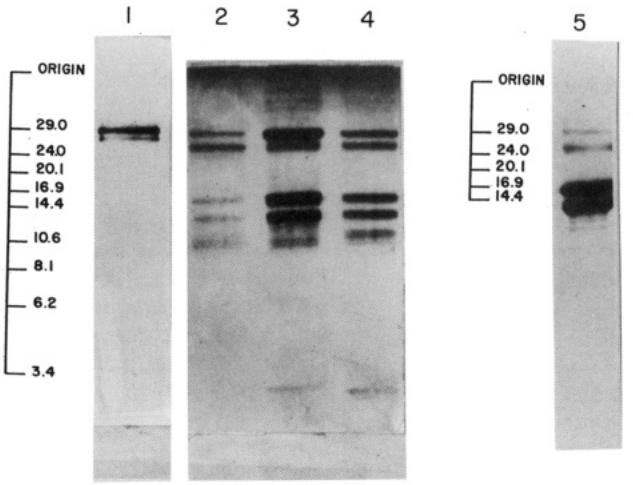
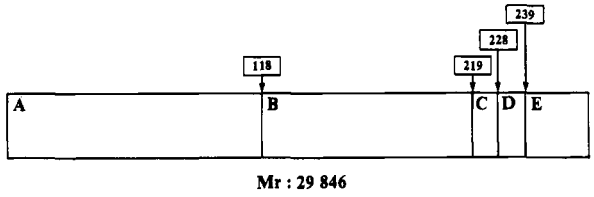


FIGURE 2: Electrophoretic separation of polypeptide chain fragments of *E. coli* glucosamine-6-phosphate deaminase produced by cleavage at the selectively cyanylated sulfhydryl groups. Lanes 2, 3, and 4: alkaline hydrolysis performed for 12, 24, and 48 h, respectively, in the presence of 0.5% SDS. Lane 5: alkaline hydrolysis performed in 3.5 M guanidinium thiocyanate for 24 h. Lane 1: control sample prepared with a noncyanylated enzyme, submitted to alkaline hydrolysis under the same conditions as the sample in lane 5. Total protein samples analyzed were in the range 0.5–2 mg, and the gel was silver stained. Molecular mass markers were carbonic anhydrase (29.0 kDa), trypsinogen (24 kDa), soybean trypsin inhibitor (20.1 kDa), myoglobin (16.9 kDa), myoglobin chain fragment 1–131 (14.4 kDa), myoglobin chain fragment 56–153 (10.6 kDa), myoglobin chain fragment 56–131 (8.2 kDa), myoglobin chain fragment 1–55 (6.2 kDa), and glucagon (3.5 kDa). Other experimental details are described in the text.

Cleavage at Vicinal Sulfhydryls. Wild-type glucosamine-6-phosphate deaminase, stoichiometrically cyanylated at the reactive vicinal thiols, was cleaved at these residues according to Dégani and Patchornik (1974). Cleavage fragments were separated by SDS–polyacrylamide gel electrophoresis and identified by their molecular masses (Figure 2, lanes 2–4). A sample of noncyanylated enzyme, submitted to all subsequent steps described under Experimental Procedures, was also prepared and run as a control for nonspecific cleavage (Figure 2, lane 1). The three main fragments observed were found to correspond to molecular masses of 27, 17, and 13 kDa. A



A :	13 210
B :	11 021
C :	1 090
D :	1 250
E :	3 272

Cleavage fragments Theoretical Mr		Fragments found (kDa)
A	13 210	13.0
B C D E	16 636	17.0
A B C D	26 574	27.0
B C D	13 364	13.0
E	3 272	3.3

FIGURE 3: Schematic representation of the polypeptide chain of *E. coli* glucosamine-6-phosphate deaminase. The position of cysteinyl residues is indicated, and the fragments between them are designated with letters from A to E, in order along the protein sequence. Molecular masses of fragments found are the estimated values from electrophoresis gels.

faint band at 3.3 kDa was also observed; probably most of this component is lost in the fixing and staining steps. A considerable amount of enzyme remained uncleaved when the denaturant was 0.5% SDS; the 29.8-kDa band corresponding to the intact protein was more conspicuous in samples hydrolyzed for 12 h (lane 2) than in samples hydrolyzed for 24 and 36 h (lanes 3 and 4). After incubation for 24 h, hydrolysis was more complete when guanidinium thiocyanate was used instead of SDS (lanes 5 and 3, respectively). Under the conditions of incomplete cleavage, 27-, 17-, and 13-kDa fragments were apparent, suggesting that they correspond to the splitting at one or the other of the two possible cleavage sites. When hydrolysis was closer to completion, as judged by the amount of unhydrolyzed enzyme present (Figure 2, lanes 4 and 5), the 27-kDa component diminished and the 13-kDa band increased. The possible cleavage sites, at the four cysteinyl residues of glucosamine-6-phosphate deaminase, are depicted in Figure 3. In this figure, the segments between cysteinyl residues are designated with letters from A to E, and their calculated molecular masses are given. We have found a fragment of 17 kDa, which most likely corresponds to BCDE, with a calculated molecular mass of 16 636 Da. There was, in addition, the appearance of an equivalent amount of a 13-kDa polypeptide which could correspond to fragment A (molecular mass 13 210 Da). The simultaneous appearance of these two bands suggests that Cys118 is a cleavage site. The existence of a 27-kDa peptide is consistent with the other cleavage site being located near the C-terminal end of the chain, where the other three cysteine residues are found. Taking into account the observation of a band at 3.3 kDa, which can only correspond to fragment E (molecular mass 3272 Da), the 27-kDa fragment can be reasonably assigned to fragment ABCD (molecular mass 26 574 Da). Due to this, the other cleavage site corresponds to Cys239. According to this interpretation, the intense 13-kDa band corresponds to the superimposed peptides A (13 210 Da) and BCD (13 364 Da). If cyanylation and hydrolysis occurred at Cys219, fragments of 24 and 5.5 kDa should be seen, while cleavage at Cys228 would generate fragments of 25 and 4.5 kDa. We have not observed peptides corresponding to these molecular masses.

Mutated Forms of Glucosamine-6-phosphate Deaminase at Cys118 and Cys239. After identification of Cys118 and Cys239 as the cysteinyl residues bearing the vicinal thiols, the corresponding mutant genes were constructed, where the cysteine codons were converted to serine. The modified enzymes carrying the mutations Cys118 to Ser, Cys239 to Ser, and the corresponding double mutant involving both residues were overproduced and purified as described under Experimental Procedures. Our initial sequence assignment for the two reactive cysteinyls was confirmed by direct thiol titration with the three genetically modified enzymes under native and denaturing conditions. As shown in Table I, single-mutant enzymes lack one reactive sulfhydryl, and both thiols are absent in the double mutant. Data from the wild-type enzyme are also included for comparison.

Table I: Number of Sulfhydryl Groups Reacting with DTNB, per Deaminase Subunit, in Wild Type and Cys to Ser Mutants[a]

	nondenatured	1% SDS
wild type	2.04 ± 0.09	3.10 ± 0.11
Cys118 to Ser	0.96 ± 0.08	2.02 ± 0.05
Cys239 to Ser	1.050 ± 0.13	1.89 ● 0.07
Cys118 to Ser, Cys239 to Ser	0.11 ± 0.04	0.96 ± 0.06

[a] Experimental conditions are described in the text. Data are averages ± standard errors, from at least five determinations.

Table II: Kinetic Parameters of Wild Type and Cys to Ser Mutants of Glucosamine-6-phosphate Deaminase[a,b]

	K_m (mM)	k_{cat} (s^{-1})	h_{max}
wild type	1.89 ± 0.16	1810 ± 40	3.09 ± 0.07
Cys118 to Ser	0.84 ± 0.07	898 ± 14	3.03 ± 0.08
Cys239 to Ser	0.77 ± 0.03	842 ± 58	1.60 ± 0.10
Cys118 to Ser, Cys239 to Ser	0.81 ± 0.08	887 ± 44	1.45 ± 0.13

[a] Experimental conditions are described in the text. Data are averages ± standard errors, from at least four experiments.

Kinetics of Genetically Modified Glucosamine-6-phosphate Deaminases. The kinetic behavior of the three mutant forms of glucosamine-6-phosphate deaminase, the two single mutants, and the double mutant is summarized in Table II. Data from the wild-type enzyme were also included for comparison. The three mutant enzymes present similar changes in the apparent catalytic constant, $V_{max}/[E_t]$, i.e., the microscopic k_{cat} multiplied by the number of catalytical sites. This value corresponds to half of the value for the native enzyme. The replacement of one or both of the thiols also produced a modification in K_m for GlcN6P, which changed from 2.0 to 0.8 mM. Thus, the catalytic efficiency or specificity constant (k_{cat}/K_m) (Fersht, 1985) remains effectively constant for the wild-type and most mutants (about 10^6 M^{-1} s^{-1}). Since glucosamine-6-phosphate deaminase is an allosteric enzyme of the K-type (Calcagno et al., 1984), these data were obtained in the presence of a saturating concentration of the allosteric activator, GlcNAc6P, to produce hyperbolic kinetics. In the absence of this ligand, the enzyme from the double mutant displays positive cooperativity, but less than the wild-type enzyme, with a maximum Hill coefficient (h_{max}) of nearly 1.5. The enzyme having the mutation Cys118 to Ser displays an unmodified cooperative behavior, similar to that of the wild-type protein. On the other hand, the replacement Cys239 to Ser produces a deaminase distinctly less cooperative, with nearly the same Hill coefficient as that observed for the double-mutant enzyme (Figure 4 and Table II).

Effect of Zn^{2+} on Cys to Ser Glucosamine-6-phosphate Deaminase Mutants. The three genetically modified enzymes prepared were preincubated with zinc acetate, under conditions which result in saturation of the zinc-binding site (Altamirano & Calcagno, 1990); the enzyme was then assayed in the presence and in the absence of the allosteric activator. The kinetic parameters, K_m for GlcN6P and k_{cat} and h_{max} for the

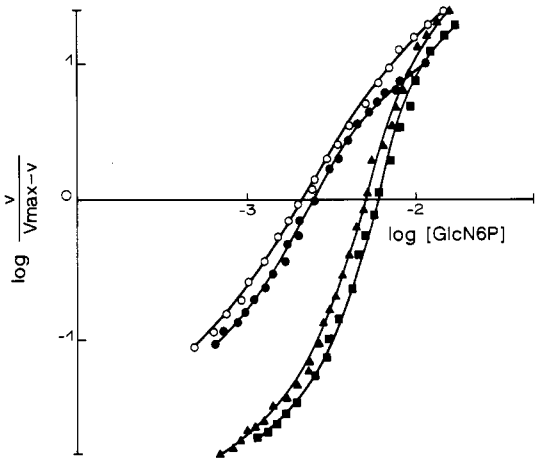


FIGURE 4: Kinetics of the wild type and Cys to Ser mutants of glucosamine-6-phosphate deaminase. Data of initial velocities as a function of GlcN6P concentration are presented as Hill plots. Details are described under Experimental Procedures. The data plotted are averages from two separate experimental series. Squares: wild-type enzyme. Triangles: Cys118 to Ser mutant. Open circles: Cys239 to Ser mutant. Filled circles: Double mutant Cys118 to Ser and Cys239 to Ser. The kinetic parameters derived from these curves are given in Table II.

Table III: Kinetic Changes Produced by Zinc Ion on Cys to Ser Mutants of Glucosamine-6-phosphate Deaminase[a,b]

	K_m (mM)	k_{cat} (s^{-1})	h_{max}
wild type	0.95 ± 0.12	890 ± 65	1.60 ± 0.08
Cys118 to Ser	1.24 ± 0.06	1 030 ± 41	1.57 ± 0.09
Cys239 to Ser	1.04 ± 0.04	1086 ± 50	1.39 ± 0.10
Cys118 to Ser, Cys239 to Ser	2.20 ± 0.06	954 ± 32	1.80 ± 0.10

[a] A 40 nM enzyme solution in 50 mM Tris–MES buffer (pH 8.0) was incubated for 2 h with 1 μM zinc acetate. This incubation time is 10 times the half-life of the Zn^{2+}-binding reaction for the wild-type enzyme (Altamirano & Calcagno, 1990). The enzyme was assayed in the presence of 0.5 mM GlcNAc6P to determine K_m and K_{cat}, and also without activator to calculate h_{max}, as described in the test. Data are averages ± standard errors from at least four experiments. Reference values for the untreated wild-type enzyme are given in the first row of Table II.

three Cys to Ser mutants, are shown in Table III. Results corresponding to the wild-type enzyme are also included. We note that the Zn^{2+} ion did not induce any additional kinetic change on either mutant involving Cys239. On the other hand, the treatment with zinc on the Cys118 to Ser mutant enzyme, produced a change in cooperativity similar to the effect already described for the wild-type protein.

Discussion

Localization of the Reactive, Vicinal Cysteines along the Deaminase Sequence. The two vicinal cysteinyl residues of glucosamine-6-phosphate deaminase, which play an important role in catalysis and allosteric regulation of deaminase activity, have been located in the polypeptide chain of the enzyme. In the absence of ligands, they provide the unique thiols reactive in the native protein. In the presence of either homotropic or heterotropic ligands which shift the allosteric equilibrium to the R conformer, they become unreactive. When this property was taken advantage of, both residues were specifically cyanylated and the polypeptide chain was cleaved at these positions (Dégani & Patchornik, 1974; Jacobson et al., 1973). From the molecular mass of the hydrolysis fragments and the known amino acid sequence of the enzyme, we identified these cysteines as residues 118 and 239. The kinetic study of the deaminase stoichiometrically cyanylated at these cysteines showed changes which are similar to those produced by other chemical reactions involving the same groups (Altamirano et al., 1989, Altamirano & Calcagno, 1990).

Cys to Ser Mutants at Residues 118 and 239. The two single Cys to Ser mutants and the corresponding double mutant involving both residues were constructed, and the modified enzymes were overexpressed and purified. In these variant proteins, serine was chosen to replace cysteine, to introduce a structurally conservative mutation (Gosh et al., 1986). The spectrophotometric titration of thiol groups in these mutant proteins confirmed the sequence assignments made by chemical cleavage (Table I). These data also show the presence of a third sulfhydryl, reactive only under denaturing conditions. Glucosamine-6-phosphate deaminase from *E. coli* K12 has four cysteinyl residues per chain (Rogers et al., 1988), but only three are titrable in the denatured wild-type form, two in both single Cys to Ser mutants, and only one in the double mutant (Table I). This indicates that one of the remaining cysteinyl residues, Cys219 or Cys228, has its thiol group blocked and probably forms a disulfide.

Our previous data and the evidence presented here on the chemical reactivity of Cys118 and Cys239 show that they are specifically and stoichiometrically modified with many sulfhydryl reagents. This is an indication that they are located at the enzyme surface. Indeed, the predicted secondary structure of the enzyme (Altamirano et al., 1991) locates Cys118 at a single hydrophilic α-helical segment, also predicted as a flexibility peak. On the other hand, Cys239 appears in a predicted β-turn with high scores of flexibility and surface probability. The remaining two cysteinyl residues of the enzyme, Cys219 and Cys228, appear in segments predicted to be hydrophobic and are expected to be buried inside the deaminase structure.

Role of Cys118 and Cys239 in Allosteric Transition. From chemical modification experiments, we know that in the three-dimensional structure of the deaminase Cys118 and Cys239 have their thiols in a vicinal position (Altamirano et al., 1989). They are both equally reactive toward a set of sulfhydryl reagents (Altamirano et al., 1987, 1989; Altamirano & Calcagno, 1990), including NTCB (see Results); the chemical blockage of this thiol pair produces a deaminase with modified catalytic and allosteric properties. The present study allowed us to evaluate separately the role of each cysteinyl residue in the function of this enzyme. Data from Table II and Figure 4 show that the role ascribed to the vicinal thiols in the stabilization of the T allosteric conformer (Altamirano et al., 1989) is played only by Cys239. The mutant Cys118 to Ser presented the same cooperativity and has the same ability to be activated by GlcNAc6P as that of the wild-type protein. On the other hand, the single mutant Cys239 to Ser and the double mutant gave similar Hill plots which look like the data reported previously for deaminases having the thiol pair chemically blocked. As the two thiols from the vicinal pair are equally reactive, this was an unexpected result. We must assume that both thiols are involved in thermodynamically equivalent interactions, but with different segments of the polypeptide chain, and that only one of them, Cys239, participates in some kind of bonding having an important role in allosteric transition. Mutants involving Cys239 have a lower interaction energy due to a change in K_T and behaved as if they were in an intermediate position between R and T conformations. Upon saturation with the allosteric activator, the K_m for GlcN6P obtained with all Cys to Ser mutants studied was lower than the value of the wild-type enzyme (Table II); these K_m values can be considered identical to K_R. From Hill

plots, we can estimate a K_T of 38 mM for the wild-type or Cys118 to Ser enzyme. Mutants replacing Cys239 gave lower K_T values, near 9 mM. This indicates that the main allosteric change produced by the Cys239 to Ser mutation is a change in the difference of $K_T - K_R$ and, consequently, in Wyman's apparent interaction energy (Wyman, 1964), mainly at the expense of the K_T.

Catalytic Constants of Cys to Ser Mutants. The replacement of either vicinal cysteinyl residue (Cys118 or Cys239) on the deaminase catalytic constant has the same effect: the apparent k_{cat} for the fully activated enzyme is reduced to nearly half of the value for the wild-type enzyme. This contribution to the catalytic power of the enzyme requires both residues of the thiol pair, and neither Cys118 nor Cys239 alone is sufficient to restore the full catalytic activity. The same 2-fold activity reduction is observed whether the dithiol is suppressed by mutation or by chemical modification. The precise role of this pair in catalysis remains to be established. We can take into account that they retain their vicinal condition in the R conformer (Altamirano et al., 1989) and that their proximity to the active site would modify the proton dissociation constant of a neighboring group involved in the catalytic mechanism. A thorough kinetic study at different pH values of wild-type and the Cys to Ser mutant deaminases may throw some light on this point.

It is interesting to observe that the ratio k_{cat}/K_m (specificity constant) has almost the same value for the wild-type and the mutant enzymes, nearly 10^6 M^{-1} s^{-1}. According to Fersht (1974), a compensatory change in the affinity of the deaminases for GlcN6P is to be expected, if the binding of the transition state of GlcN6P to the protein is not affected by the Cys to Ser mutations.

Concluding Remarks. Modified deaminases in which both cysteines were changed to serine residues (Table II) behaved similarly to the wild-type enzyme in which both thiols have been blocked with different small-sized substituents (Altamirano et al., 1989). This result demonstrates that the kinetic changes described by chemical modification procedures are due to the lack of the free thiol pair and do not depend on steric factors caused by the presence of the chemical groups introduced. The kinetic changes observed in the Cys to Ser mutants point out the different roles of Cys118 and Cys239, in R and T conformers. We have previously discussed that changes in cooperativity and k_{cat} produced by chemical suppression of the dithiol could be accounted for by assuming half-of-the-sites kinetics (Altamirano et al., 1989). The results described here show that both effects are unreleated; only Cys239 is necessary for full cooperative behavior, but both cysteinyls must be present for maximum catalytic activity.

In a previous paper (Altamirano & Calcagno, 1990), we have reported the presence of one zinc-binding site per deaminase subunit. The vicinal thiols, now identified as Cys118 and Cys239, seemed to play an essential role in the Zn^{2+}-binding site. The results, summarized in Table III, show that, as expected, zinc did not produce additional kinetic changes in deaminases lacking the Cys239 thiol. Mutant deaminase lacking only Cys118, which displays an intense cooperativity similar to that of the wild-type enzyme (Table II), was modified by low zinc concentrations, giving the same kinetic pattern as that of the Cys239-lacking enzyme. This observation confirms the important role of Cys239 in the allosteric transition. It also proves that the Zn-binding site is still functional when the Cys118 sulfhydryl is replaced by a hydroxyl group in the Cys to Ser mutation. This demonstrates that Cys118 and Cys239 have different roles in the formation of the Zn-binding site.

Cooperative behavior of glucosamine-6-phosphate deaminase is strongly dependent on pH (Calcagno et al., 1984); it is maximal in the range 7.8–8.2. In the light of the present results, it may be interesting to study the role of sulfhydryl proton dissociation from Cys239 as one of the possible protonic equilibria involved in this pH effect. Ligands involving this group, as Zn^{2+} or H^+, may then play an important role in deaminase regulation.

Registry No. GlcNAc6P, 1746-32-3; GlcN6P, 3616-42-0; GlcN6P deaminase, 9013-10-9; Cys, 52-90-4; Zn, 7440-66-6; Ser, 56-45-1.

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