Glucosamine-6-phosphate deaminase from *Escherichia coli* has a trimer of dimers structure with three intersubunit disulphides

Myriam M. ALTAMIRANO,* Jacqueline A. PLUMBRIDGE,† Hugo A. BARBA* and Mario L. CALCAGNO*‡

*Departamento de Bioquímica, Facultad de Medicina, Universidad Nacional Autónoma de México, P.O. Box 70-159, Ciudad Universitaria, 04510, Mexico City, Mexico, and †Institut de Biologie Physico-Chimique (CNRS, URA 1139), 75005, Paris, France

Glucosamine-6-phosphate deaminase is an oligomeric protein composed of six identical 29.7 kDa subunits. Each subunit has four cysteine residues located at positions 118, 219, 228 and 239. We have previously shown that Cys-118 and Cys-239 form a pair of vicinal thiols, the reactivity of which changes with the allosteric transition. The site-directed mutations Cys→Ser corresponding to the other two cysteine residues have been constructed, as well as some selected multiple mutations involving the four cysteines. Thiol and disulphide measurements on the wild-type and mutant enzymes indicate that thiols from Cys-219 are oxidized and form interchain disulphide bonds. The disulphide-linked dimer was

demonstrated by SDS/PAGE. This result is consistent with preliminary crystallographic data and thermal denaturation studies, and strongly suggests that glucosamine-6-phosphate deaminase is a trimer of disulphide-linked dimers. The mutant forms of the deaminase lacking the interchain disulphide bond or the thiol at Cys-228 are both stable hexamers showing the same sensitivity to urea denaturation as the wild-type protein. Furthermore, these Cys→Ser mutants display the same kinetics and allosteric properties as those already described for the wild-type enzyme.

INTRODUCTION

Glucosamine-6-phosphate deaminase [D-glucosamine-6-phosphate ketol-isomerase (deaminating) EC 5.3.1.10] is an oligomeric protein composed of six identical polypeptide chains (Calcagno et al., 1984) the primary structure of which is known from the DNA sequence of its gene, *nagB* (Rogers et al., 1988). The enzyme has been recently crystallized and preliminary crystallographic data are available from diffraction measurements at 0.21 nm (2.1 Å) resolution. It has been found that this protein has a point group symmetry of the type 32, centred on a threefold axis; crystallographic evidence also suggests that the hexamer subunits are arranged as dimers with three local twofold axes passing through the centre of the three dyads forming the hexamer (Horjales et al., 1992). This structure is also consistent with thermal denaturation data of the deaminase obtained by differential scanning calorimetry at neutral pH, which suggests that deaminase dissociates into dimers in two successive steps (Hernández-Arana et al., 1993).

The existence of disulphide bonds in the deaminase molecule can be presumed from the study of its four cysteine residues. Recently, we have identified Cys-118 and Cys-239 as the cysteines bearing two superficial vicinal thiols, the reactivity of which changes with allosteric transition. The other two cysteine residues are one that we have described as 'hidden', as it reacts with thiol-blocking reagents only under denaturing conditions, and one that does not react with thiol-blocking reagents at all, implying that it may be present in the disulphide form (Altamirano et al., 1992). As the enzyme has only four cysteines, these residues must be Cys-219 and Cys-228. In this work we report the identification of these two cysteines by the analysis of proteins carrying the site-directed mutations Cys-219→Ser and Cys-228→Ser. Other proteins carrying multiple Cys→Ser mutations were also made by combination with the mutations at

Cys-118 and Cys-239 constructed previously. These modified proteins were used to re-examine the question of the presence of a disulphide bond in the deaminase molecule and to assess its structural and functional role.

MATERIALS AND METHODS

Bacteria and enzyme

Escherichia coli wild-type glucosamine-6-phosphate deaminase was prepared from an overproducing strain, as previously described (Altamirano et al., 1991). Plasmids expressing the Cys-219 to Ser and Cys-228 to Ser mutant forms of the protein were constructed as described previously (Altamirano et al., 1992). The double mutation Cys-219, -228→Ser, the triple Cys-219, -228, -239→Ser and the quadruple mutation (all Cys residues changed to Ser) were also constructed. The host strain for the expression of these mutated proteins was IBPC546R, which is *nagB::kan,recA1*, a recombination-deficient strain carrying a null mutation in the chromosomal copy of the *nagB* gene (Plumbridge, 1991). All mutations were checked by DNA sequencing.

Wild-type and genetically modified forms of glucosamine-6-phosphate deaminase were obtained and assayed using previously reported procedures including allosteric-site affinity chromatography (Altamirano et al., 1991, 1992).

Reagents

2-Nitro-5-thiosulphobenzoate (NTSB) was prepared as described by Thannhauser et al. (1987) and conserved in solution. Biochemicals, protein standards and carbohydrate derivatives were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Most chemicals were reagent-grade products.

Measurements of the reactive and total SH groups

The total thiol groups in the wild-type and mutant forms of the deaminase were determined with 5,5'-dithiobis-(2-nitrobenzoate) (DTNB) at pH 7.7 and 25 °C in the presence of 0.5% SDS or 3.5 M guanidinium thiocyanate and 5 mM EDTA. The product of the thiol-disulphide exchange reaction, 2-nitro-5-thiobenzoate (TNB), was measured spectrophotometrically with a double-beam instrument. Calculations were made using the known molar absorption coefficient for this compound (Means and Feeney, 1971) and for glucosamine-6-phosphate deaminase [ϵ_{278} $20 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Altamirano et al., 1987)].

Detection and measurement of disulphide bonds

Disulphide bonds were analysed by the method of Thannhauser et al. (1987). This procedure measures both thiols and disulphides. The reaction consists of a sulphytolysis of the S-S bonds by sulphite at pH 9.0, and the simultaneous reaction of pre-existing thiols and those formed by sulphytolysis, with NTSB in the presence of 3.5 M guanidinium thiocyanate as protein denaturant.

Electrophoretic procedures

Different deaminase samples were subjected to electrophoresis as described by Laemmli (1970). When necessary, sample buffer was prepared without any thiol-reducing reagent. Electrophoreses under non-denaturing conditions were performed in pore gradient gels (4–30% gels), as previously described (Calcagno et al., 1984). Electrophoreses in polyacrylamide gel slabs with transverse urea gradients, initially described by Creighton (1979), were by the method of Goldenberg (1989).

High-performance analytical gel filtration

Gel filtration was carried out on a TSK-G 3000 SW (LKB) column (7.5 mm $\times$ 600 mm) equilibrated with 100 mM Tris/HCl buffer, pH 7.5, containing 0.075 mM potassium acetate. The following molecular-mass standards were used: ferritin

(440 kDa), catalase (240 kDa), wild-type glucosamine-6-phosphate deaminase (178 kDa) and haemoglobin (66 kDa).

RESULTS AND DISCUSSION

Measurements of reduced and total cysteine residues

The reaction of wild-type glucosamine-6-phosphate deaminase with the NTSB/sulphite reagent (Thannhauser et al., 1987) produces 3.5 mol of TNB/mol of enzyme subunit. As the denatured enzyme has only three reactive thiols, and from the known stoichiometry of the Thannhauser et al. reaction, which releases one TNB anion/disulphide cleaved as well as one TNB for each free thiol, this result indicates the existence of one disulphide bridge for two deaminase subunits. Table 1 shows the results of thiol and disulphide measurements on wild-type and a set of Cys→Ser mutant enzymes. From these data it can be inferred that cysteine residues at position 219 form a disulphide bond between neighbouring polypeptide chains.

Electrophoretic isolation of the denatured dimer linked by a disulphide

In standard SDS/PAGE, with samples prepared in a buffer containing mercaptoethanol or other reducing reagents, all enzymes from Table 1 give a single electrophoretic band at 30 kDa (not shown). In the electrophoresis gels shown in Figure 1, samples were prepared in a buffer without reducing agent. Wild-type enzyme gives two bands, corresponding to 30 and 60 kDa (Figure 1, lanes 6 and 9). The mutant enzyme in which only Cys-219 has been replaced with serine produces a single band corresponding to the monomer (lanes 1 and 7). The same result is obtained in other multiple Cys→Ser mutations con-

Table 1 Titration of thiols and disulphides in wild-type and Cys→Ser mutations of *E. coli* glucosamine-6-phosphate deaminase

Results are means of three or four determinations. The number of thiol groups or disulphide links is expressed per chain of deaminase polypeptide. Thiols were measured with DTNB at pH 7.7, 30 °C. Thiols + disulphides were measured with the reagent NTSB-sulphite (Thannhauser et al., 1987) at pH 9.0 in the presence of 3.5 M guanidinium thiocyanate. SH titrations were carried out in the presence of 3.5 M guanidinium thiocyanate for the 'denatured' results. Similar results were obtained when the denaturant was 0.5% SDS.

	Thiols		Thiols + disulphides
	Native	Denatured	
Wild-type	2.06	2.95	3.58
Cys-219→Ser	2.10	3.07	3.11
Cys-228→Ser	2.00	1.97	2.55
Cys-219, 228→Ser	1.94	2.12	2.10
Cys-118, -228, -239→Ser	0	0	0.47
Cys-118, -219, -228, -239→Ser	0	0	0

Figure 1 Comparative analysis of wild-type and some Cys→Ser mutations of glucosamine-6-phosphate deaminase

SDS/PAGE was carried out for wild-type glucosamine-6-phosphate deaminase and some site-directed Cys→Ser mutations. The gels (total acrylamide = 11.2%, cross-linker = 2.7%) were prepared as described by Laemmli (1970), and electrophoresis was carried out at 20 mA and 10 °C. Sample buffer was prepared without thiol-reducing agents. Lanes 1 and 7, Cys-219→219→Ser; lane 2, Cys-219, -228→Ser; lane 3, Cys-228→Ser; lane 4, Cys-118, -219, -228, -239→Ser (i.e. all four Cys residues changed to Ser); lane 5, Cys-118, -228, -239→Ser (this sample was dissolved at room temperature); lanes 6 and 9, wild-type; lane 8, Cys-118, -228, -239→Ser (this last sample was boiled for 1 min). The gel was stained with Coomassie Brilliant Blue R-250. Standards: BSA (66 kDa); *E. coli* N-acetyl-D-glucosamine-6-phosphate deacetylase (41 kDa); carbonic anhydrase (30 kDa).

Figure 2 Transverse urea gradient PAGE of wild-type and Cys-219, -228→Ser double mutant of glucosamine-6-phosphate deaminase

Deaminase samples were subjected to electrophoresis at pH 8.5 in a gel with a urea transversal concentration gradient from 0 to 8 M prepared as described by Goldenberg (1989). The gel (total 12%, cross-linker 2.4%) was loaded with 35 μ g of protein, run at 4 °C at 20 mA for 2.5 h and stained with Coomassie Brilliant Blue R-250. The urea concentration gradient is from left to right. (a) Wild-type glucosamine-6-phosphate deaminase; (b) Cys-219, -228→Ser mutant enzyme.

taining the change of Cys-219 to Ser (lanes 2 and 4). The triple Cys-118, -228, -239→Ser mutation produces a band corresponding to the dimer, accompanied by a small amount of monomer (lane 5). The sample in this lane was prepared at room temperature. Another sample treated by heating for 1 min in a boiling water-bath gives a larger amount of monomer (nearly 25% by densitometry). These results and those obtained with the other Cys to Ser mutations studied (Figure 1, lanes 4–6), confirm that Cys-219 is the only cysteine residue involved in formation of the interchain disulphides. The cysteine residue at position 228 is identified as having the 'hidden' thiol, reacting only in the denatured protein (Table 1). In wild-type enzyme, the interchain disulphide is unstable and the lighter component predominates in non-reducing SDS/polyacrylamide gels, probably because of the reducing effect of the other three thiols present in the polypeptide chain. It would be expected that the triple mutation Cys-118, -228, 239→Ser would give a pure 60 kDa band; the variable amounts of monomer always present suggest that the denatured disulphide-linked dimers are partially unstable under the conditions of electrophoresis. The dimeric form after elution from the polyacrylamide gel and re-electrophoresis on a second gel again also contains a small and variable proportion of monomer. The amounts of dimer and monomer were determined by extraction of Coomassie Blue from the stained protein bands excised from polyacrylamide gels, as described by Ball (1986). The dimer/monomer ratio obtained with the mutant Cys-118, -228, -239→Ser was 4:1–6:1 for the first electrophoresis and 14:1 for the re-electrophoresis.

Molecular mass and stability of Cys-219→Ser deaminase

The mutant form of the deaminase lacking the interchain disulphide bond is a hexamer, as demonstrated by pore gradient gel electrophoresis and gel-filtration h.p.l.c. of the native proteins (not shown); in all cases only the hexameric molecule was detected. The same results were obtained with the Cys-228→Ser mutation and the Cys-219, -228→Ser double mutant. To investigate the stability of the wild-type hexamer and our Cys→Ser enzyme mutants, we used the technique of transverse urea gradient gel electrophoresis (Creighton, 1979; Goldenberg, 1989). Wild-type enzyme (Figure 2a), both Cys→Ser single mutants and the Cys-219, -228→Ser double mutant (Figure 2b) behaved essentially the same; under these experimental conditions, discontinuities caused by oligomer dissociation are not apparent.

Kinetics of the deaminases carrying the site-directed mutations Cys-219→Ser and Cys-228→Ser

The kinetics and allosteric properties of the mutant deaminases in which serine replaces Cys-219 or -228 were the same as those already described for the wild-type enzyme (Altamirano et al., 1992). The same result was obtained with the double mutation.

Concluding remarks

We have demonstrated the presence of three interchain disulphide bonds in the deaminase molecule, formed between cysteine residues at position 219. According to the structural information available, the three S-S bonds in the deaminase molecule should be located on each of the three twofold symmetry axes (Horjales et al., 1992). Surprisingly, this disulphide is not essential for the stability of the oligomer under physiological conditions and does not modify the profile of deaminase on electrophoresis in transverse urea gradients (Figure 2). Nevertheless, quantitative denaturation studies by differential scanning calorimetry, for instance, may be of value in the characterization of the possible structural role of the thiol group from Cys-219. Moreover the elimination of the 'hidden' thiol from Cys-228 or the interchain disulphide formed by Cys-219 does not produce any significant change in glucosamine-6-phosphate deaminase function.

This research was supported by grants from the Third World Academy of Sciences (grant BC 91-028), Dirección General Asuntos del Personal Académico (DGAPA) (UNAM, Mexico), CNRS (France) and The European Economic Community (contact C11*-CT2-0038). M. M. A. holds a doctoral fellowship, from DGAPA, UNAM, México.

REFERENCES

- Altamirano, M. M., Mulliert, G. and Calcagno, M. (1987) *Arch. Biochem. Biophys.* **258**, 95–100
- Altamirano, M. M., Hernández-Arana, A., Plumbridge, J. A. and Calcagno, M. (1991) *Biochim. Biophys. Acta* **1076**, 266–272
- Altamirano, M. M., Plumbridge, J. A. and Calcagno, M. (1992) *Biochemistry* **31**, 1153–1158
- Ball, E. H. (1986) *Anal. Biochem.* **155**, 23–27
- Calcagno, M., Campos, P. J., Mulliert, G. and Suástegui, J. (1984) *Biochim. Biophys. Acta* **787**, 165–173
- Creighton, T. E. (1979) *J. Mol. Biol.* **129**, 235–264
- Goldenberg, D. P. (1989) in *Protein Structure: A Practical Approach* (Creighton, T. E., ed.), pp. 225–250, IRL Press, Oxford
- Hernández-Arana, A., Rojo-Domínguez, A., Altamirano, M. M. and Calcagno, M. L. (1993) *Biochemistry* **32**, 2644–2648

- Horjales, E., Altamirano, M. M., Calcagno, M. L., Dauter, Z., Wilson, K., Garratt, R. C. and Oliva, G. (1992) *J. Mol. Biol.* **226**, 1283–1286
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Means, G. E. and Feeney, R. E. (1971) *Chemical Modification of Proteins*, Holden Day Inc., San Francisco
- Plumbridge, J. A. (1991) *Mol. Microbiol.* **5**, 2053–2062
- Rogers, N. J., Ohgi, T., Plumbridge, J. and Söll, D. (1988) *Gene*, **62**, 197–207
- Thannhauser, R., Konishi, Y. and Scheraga, H. A. (1987) *Methods Enzymol.* **143**, 115–119

Received 1 April 1993/14 June 1993; accepted 23 June 1993