



HAL
open science

Functional domains and interdomain communication in *Candida albicans* glucosamine-6-phosphate synthase

Jaroslav Olchow, Iwona Gabriel, Slawomir Milewski

► **To cite this version:**

Jaroslav Olchow, Iwona Gabriel, Slawomir Milewski. Functional domains and interdomain communication in *Candida albicans* glucosamine-6-phosphate synthase. *Biochemical Journal*, 2007, 404 (1), pp.121-130. 10.1042/BJ20061502 . hal-00478677

HAL Id: hal-00478677

<https://hal.science/hal-00478677>

Submitted on 30 Apr 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Functional domains and interdomain communication in *Candida albicans* glucosamine-6-phosphate synthase

Jarosław OLCHOWY, Iwona GABRIEL and Sławomir MILEWSKI

Department of Pharmaceutical Technology and Biochemistry, Gdańsk University of Technology, 11/12 Narutowicza St., 80-952 Gdańsk, Poland

Corresponding author:

Sławomir Milewski

Department of Pharmaceutical Technology & Biochemistry

Gdańsk University of Technology

11/12 Narutowicza St.

80-952 Gdańsk, Poland

Phone: +48 58 347 21 07

Fax: +48 58 347 11 44

e-mail: milewski@chem.pg.gda.pl

Abbreviations used:

aa, amino acid; DON, 6-diazo-5-oxo-L-norleucine; DTT, dithiothreitol; Fru-6-P, D-fructose-6-phosphate; GAH, glutamine amide hydrolysing domain; GlcN-6-P, D-glucosamine-6-phosphate; GLUPA, γ -glutamyl-*p*-nitroanilide; IPTG, isopropyl- β -D-thiogalactopyranoside; ISOM, hexosephosphate-isomerising domain; UDP-GlcNAc, uridine 5'-diphospho-*N*-acetyl-D-glucosamine;

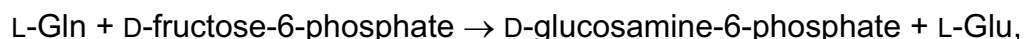
Running title: Domains of *C. albicans* GlcN-6-P synthase

Functional and structural properties of several truncated or mutated variants of *Candida albicans* glucosamine-6-phosphate synthase (Gfa1p) were compared to those of the wild-type enzyme. Fragments encompassing residues 1-345 and 346-712 of Gfa1p, expressed heterogenously in bacterial host as oligoHis fusions, were identified as functional domains: glutamine amide-hydrolysing (GAH) and hexose phosphate-isomerising (ISOM), respectively. It was found that native GAH is monomeric, while native ISOM forms tetramers, as does the whole enzyme. Spectrofluorimetric and kinetic studies of the isolated domains, the Δ 218-283Gfa1p mutant and the whole enzyme revealed that the binding site for the feedback inhibitor, UDP-GlcNAc, is located in the ISOM domain. Inhibitor binding affects amidohydrolysing activity of GAH and, as a consequence, the GlcN-6-P-synthetic activity of the whole enzyme. The 218-283 fragment is neither involved in ligand binding nor in protein oligomerisation. Comparison of the catalytic activities of V711FGfa1p, Δ 709-712Gfa1p, W97FGfa1p and W97GGfa1p to those of the native Gfa1p and the isolated domains provided evidence for an intramolecular channel connecting the GAH and ISOM domains of Gfa1p. The channel becomes leaky upon deletion of amino acids 709-712 and in W97F and W97G mutants. The W97 residue was found to function as a molecular gate, opening and closing the channel. The W97G and V711F substitutions resulted in an almost complete elimination of the GlcN-6-P-synthetic activity with the retention of the amidohydrolyase and sugar phosphate-isomerising activities.

Key words: domain, amidotransferase, glucosamine-6-phosphate synthase, site-directed mutagenesis, intramolecular channel.

INTRODUCTION

Glucosamine-6-phosphate /GlcN-6-P/ synthase, systemic name L-Glutamine:D-fructose-6-phosphate amidotransferase, EC 2.6.1.16, is an ubiquitous enzyme, catalysing the first committed step in the amino sugar biosynthetic pathway in prokaryotic and eukaryotic organisms. The enzyme catalyses a complex reaction:



that involves ammonia transfer and sugar phosphate isomerisation. GlcN-6-P synthase is an important point of metabolic control of amino sugar biosynthesis, the mammalian enzyme is proposed to be involved in the phenomenon of hexosamine-induced insulin resistance in diabetes [1] and the fungal GlcN-6-P synthase has been proposed as a potential target in antifungal chemotherapy [2]. For all these reasons, the enzyme, especially its eukaryotic type, is an interesting subject of study. The prokaryotic GlcN-6-P synthase has been relatively well characterised. The *Escherichia coli* enzyme has been purified to homogeneity [3], details of the molecular mechanism of catalysis have been deduced [4 and references cited therein] and its 3D structure has been determined [5]. Previous studies on *E. coli* GlcN-6-P synthase revealed that the enzyme is a homodimer. Each subunit is composed of two domains that can be released from the native protein upon limited proteolysis with chymotrypsin: the N-terminal glutamine amidohydrolase (GAH, residues 1-239) and the C-terminal ketose/aldose isomerase (ISOM, residues 240-608) [5]. The former binds L-Gln and hydrolyses it to glutamate and ammonia. The latter utilises the released ammonia for the conversion of D-fructose-6-phosphate to D-glucosamine-6-phosphate. Such a modular structure is characteristic for all amidotransferases composed of an amidohydrolase domain fused with an acceptor domain [6]. It is assumed that ammonia generated at the amidohydrolase domain is transferred to the second domain through the intramolecular channel. In most amidotransferases the channel is accessible for an exogenous ammonia, however the GlcN-6-P synthase seems to be unique in its apparent inability to use this substrate [6]. The presence of a solvent-inaccessible, relatively long channel composed of several hydrophobic amino acid residues, was demonstrated in the crystal structure of *E. coli* GlcN-6-P synthase [5]. However, some evidence was

presented for the close proximity of the domains' active sites [7] and, recently, for a hinged movement of the amidohydrolase domain towards the isomerase domain, induced upon substrate binding [8].

Only limited structural data are available for the eukaryotic GlcN-6-P synthase. There is little doubt that the essential active site amino acid residues are highly conserved among GlcN-6-P synthases. However, the eukaryotic enzyme differs from its prokaryotic counterpart in a number of features. The most important of these is the quaternary structure and regulation of the enzymatic activity. The eukaryotic GlcN-6-P synthase is undoubtedly homotetrameric, its activity is regulated by a feedback inhibition by UDP-GlcNAc, as well as by phosphorylation/dephosphorylation mediated by protein kinase A and protein phosphatase [9]. Moreover, the results of multiple sequence alignment analysis of the enzyme revealed the presence of a 30 – 80 aa fragment of unknown function at the C-terminal part of the eukaryotic GAH. No such fragment was observed in any known prokaryotic versions of the enzyme [9]. In this work we describe the results of our studies on GlcN-6-P synthase from the human pathogenic fungus *Candida albicans* (Gfa1p), involving structural and functional characterization of several truncated or mutated variants of this enzyme.

MATERIALS AND METHODS

Bacterial strains and media

The *Escherichia coli* XL1-Blue strain [*recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac* [F' , *proAB, lacI9, ZDM15, Tn10, (TetR)*]] from Stratagene was used in all cloning procedures. The *Escherichia coli* BL21(DE3) pLysS strain [F^- , *ompT, hsdS_B(r_B⁻, m_B⁻), gal, dcm, (DE3), pLysS*] from Novagen was used for the overexpression of both domains of *C. albicans* GlcN-6-P synthase. *Escherichia coli* strains were cultured at 37°C on LB solid medium (1.0% sodium chloride, 1.0% Tryptone, 0.5% Yeast Extract, 1.5% agar) and LB liquid medium (1.0% sodium chloride, 1.0% Tryptone, 0.5% Yeast Extract) supplemented with ampicillin (0.1 mg/ml) when necessary.

Plasmids, enzymes, and other materials

The plasmids used were: pET23b (Novagen), pUC19 (Fermentas), pUC19-CaGFA1 and pET23b-CaGFA1 [10]. Restriction and modification enzymes were purchased from Fermentas, Lithuania and New England Biolabs, USA. Purification of His-tagged proteins was performed on Ni²⁺-IDA agarose (his•Bind Resin, Novagen). DNA molecular weight markers were from DNA-Gdańsk II s.c., Poland. The GelCode™ Phosphoprotein Staining Kit was from Pierce, USA. Yeast Glc-6-P dehydrogenase and other reagents were from Sigma, USA.

DNA manipulations

Isolation of plasmid DNA was carried out according to the protocol of the Plasmid Mini kit (A&A Biotechnology, Poland). DNA fragments were isolated from agarose gels following the standard procedure of the DNA Gel-Out kit (A&A Biotechnology, Poland). DNA purification after enzyme treatment was performed according to the instructions in the DNA Clean-up kit (A&A Biotechnology, Poland). DNA digestion with restriction enzymes was carried out according to the enzyme supplier's instructions. DNA fragments were ligated and *Escherichia coli* cell were transformed according to the standard methods [11].

Construction of expression plasmids containing domain-coding sequences

The fragment of the *GFA1* gene encoding residues 1-345 of Gfa1p was amplified from the pUC19-CaGFA1 plasmid by PCR. The primers used in the amplification were: GLN1-KN (Table 1) and GLN2-HX, 5'-cct gcc aag ctt ctc gag aat ttc att caa ttc cat ttc taa agt ttg-3'. The artificial cloning sites: *KpnI*, *NdeI* (GLN1-KN) and *XhoI*, *HindIII* (GLN2-HX), at the 5' ends of primers (underlined) were introduced to facilitate the cloning procedure. The PCR product (1071 bp) purified from an agarose gel band was cloned directionally into *KpnI* and *HindIII* sites of the pUC19 vector and then re-cloned into the pET23b plasmid, giving a recombinant expression plasmid pET23b-GLN /4628 bp/. The identity of the plasmids was confirmed by restriction analysis and DNA sequencing. The obtained construct encoded the putative GAH domain of *C. albicans* GlcN-6-P synthase, containing an additional octapeptide (LEHHHHHH) at the C-terminus (GAHp-His₆).

Analogously, the respective *GFA1* gene fragment encoding residues of Gfa1p 356-712 was amplified from the pUC19-CaGFA1 plasmid by PCR using the following primers: Fru1-BNK, 5'- cct ggc gga tcc cat atg cac cac cac cac cac cac ggt acc atg aaa ggc ccc tat aaa cat ttt atg -3' and Fru2-Eco, 5'- cct gcc gaa ttc tta ctc aac agt aac tga ttt agc ca -3'. The artificial cloning sites: *Bam*HI and *Nde*I (Fru1-BNK) and *Eco*RI (Fru2-Eco), at the 5' ends of primers (underlined) were introduced to facilitate the cloning procedure. After cloning into pUC19 and subsequent re-cloning into pET23b, a recombinant expression plasmid pET23b-FRU /4755 bp/ was obtained. The construct encoded the putative ISOM domain of *C. albicans* GlcN-6-P synthase containing an additional nonapeptide (MHHHHHGT) at the N-terminus (His₆-ISOMp).

Construction of expression plasmids containing truncated versions of *GFA1*

Δ218-283*GFA1*. Two fragments were amplified from the pUC19-CaGFA1 template by PCR using the following primers: fragment 1 GLN1-KN (Table 1) and InGln-X, 5'- tcc agg ctc gag atc agt ttt aac acc aac caa taa tg-3', fragment 2 Infru-X, 5'-cct acc ctc gag ttc ttt tta tct tct gat cct gc-3' and Fru2-Eco (see above). Artificial restriction recognition sites for *Xho*I are underlined. The PCR products were purified and digested with *Bsu*15I and *Xho*I (GLN1-KN/ InGln-X product) and *Xho*I and *Eco*RI (Infru-X /Fru2-Eco product), to give 3 fragments of different size in each case (digests 1 and 2). A 413 bp fragment was isolated from digest 1 and a 1293 bp fragment from digest 2. The pET23b-CaGFA plasmid was digested with *Bsu*15I and *Eco*RI and a 3865 bp fragment was isolated from the digest. The 413 bp, 1293 bp and 3865 bp fragments were ligated, the *E. coli* XL1-Blue cells were transformed with the post-ligation mixture. The resulting plasmid construct pET23b-CaGFA1Δ218-283 was isolated and sequenced to confirm that it encodes the truncated Gfa1p lacking the 218-283 fragment.

Δ709-712*GFA1*. A *GFA1* fragment was amplified from the pUC19-CaGFA1 template by PCR using the following primers: Trpgly-5 (Table 1), GFA709X, 5'-atg acg ctc gag tta tga ttt agc caa gtt acg agg-3'. The PCR product was purified and digested with *Bsp*119I and *Xho*I to give 3 products, of which the 1052 bp one was isolated. The pET23b-CaGFA plasmid was digested with the same restriction enzymes and a 4666 bp fragment was isolated from the digest. The isolated

fragments were ligated, and *E. coli* XL1-Blue cells were transformed with the ligation product. The resulting plasmid construct pET23b-CaGFA1 Δ 709-712 was isolated and its identity was confirmed by sequencing.

Construction of expression plasmids containing site-directed mutant versions of GFA1

The site-directed mutants W97G and W97F of *GFA1* were constructed using the overlap extension method with pUC19-CaGFA1 as a template. Primers used for PCR are listed in Table 1. The V711F mutant was amplified from pUC19-CaGFA1 with Gln1-KN and Valphe primers. The PCR products were isolated, digested with *Mlu*I, *Kpn*I and *Bgl*II (W97F), *Kpn*I, *Kas*I and *Bgl*II (W97G), *Bsp*119I and *Xho*I (V711F) and ligated to the pET23b-CaGFA1 vector, digested with the appropriate restriction enzymes. *E. coli* XL1-Blue cells were transformed with the ligation products. The resulting plasmid constructs pET23b-CaGFA1W97G, pET23b-CaGFA1W97F and pET23b-CaGFA1V711F were isolated and their identity was confirmed by sequencing. Construction of the pET23b-CaGFA1S208A plasmid was described previously [12].

Protein expression and purification

Escherichia coli BL21(DE3)pLysS cells, transformed with the appropriate expression plasmid, were grown in LB medium at 37 °C. Expression was induced by the addition of IPTG, 0.5 mM, to the cultures growing to OD₆₀₀ = 0.6 – 0.8 and incubation was continued for another 5 h. Cells were harvested by centrifugation (5000 *g*, 10 min, 4 °C).

The His-tagged putative domains of Gfa1p were purified by metal affinity chromatography. The bacterial pellet was suspended in buffer A (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 5 mM imidazole, 0.1% Triton X-100, 0.5 mM PMSF) and the cells were disrupted by sonication on ice. The total lysate was centrifuged for 20 min at 16000 *g*, 4 °C. The supernatant (crude extract) was applied to a Ni²⁺-IDA agarose (his•Bind Resin, Novagen) column, bed volume 10 ml, equilibrated with four volumes of buffer A. Next, the column was washed with 20 ml of the same buffer, followed by washing with 40 mL of buffer W (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 80 mM imidazole, 0.1% Triton X-100, 0.5 mM PMSF). The His-tagged proteins were eluted

with two 5 ml portions of elution buffer E (20 mM Tris pH 7.9, 500 mM imidazole, 0.5 M NaCl, 0.1% Triton X-100, 0.5 mM PMSF). For further assays the eluates were concentrated by ultrafiltration using Vivaspin concentrators, 10 kDa cut-off limit (Viva Science Ltd, UK) at 7000 *g* and the buffer was exchanged for buffer B (25 mM potassium-phosphate, pH 7.5, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF) using the HiTrap™ Desalting Columns (Pharmacia LKB).

The wild-type Gfa1p, its truncated and site-directed mutant variants were purified by a multi-step procedure, essentially as described previously [10].

Determination of enzymatic activities and enzyme kinetic analysis

The amidohydrolysing activity was determined using L- γ -glutamyl-*p*-nitroanilide (GLUPA) as a substrate. Mixtures containing 1 mM GLUPA, 1 mM EDTA, 1 mM DTT and appropriately diluted enzyme preparation in 20 mM Hepes buffer, pH 7.5, were incubated at 25 °C. In some experiments, Fru-6-P (D-fructose-6-phosphate), 10 mM was also included as a component of the incubation mixture. The released *p*-nitroaniline was quantified spectrophotometrically at 420 nm, assuming $\epsilon_{420}(\textit{p}$ -nitroaniline) = 9 300 M⁻¹cm⁻¹. One unit of specific activity was defined as an amount of the enzyme that catalysed formation of 1 μ mol of *p*-nitroaniline/min/mg protein.

The rate of L-glutamine hydrolysis was determined by quantification of the released ammonia with the Nessler's reagent. Mixtures containing 10 mM L-Gln, 1 mM EDTA, 1 mM DTT and an appropriately diluted enzyme preparation in 20 mM Hepes buffer, pH 7.5, were incubated at 25 °C. Aliquots of 0.1 ml were withdrawn at 5 min intervals, diluted with 1.4 ml of water and 0.2 ml of a commercial solution of the Nessler's reagent was added. The mixtures were left for 10 min at room temperature and absorbance at 480 nm was measured. The released ammonia was quantified, assuming a molecular absorbance index for the yellow product of the Nessler reaction with a standard NH₄Cl solution, $\epsilon_{480} = 3 \times 10^8$ M⁻¹cm⁻¹.

The hexosephosphate-isomerising activity was determined using the Glc-6-P dehydrogenase-coupled assay, as described by Noltmann *et al.* [13]. Incubation mixtures containing 2 mM Fru-6-P, 0.5 mM NADP⁺, 1 mM EDTA, Glc-6-P dehydrogenase, 2.6 unit/ml and appropriately diluted GlcN-6-P synthase preparation in 50 mM Tris-HCl buffer, pH = 7.5, were incubated at 25 °C. The reduction of NADP⁺ to NADPH was monitored spectrophotometrically at 340 nm, assuming

$\epsilon_{340}(\text{NADPH}) = 6\,220\text{ M}^{-1}\text{cm}^{-1}$. One unit of specific activity was defined as an amount of the enzyme that catalysed formation of 1 μmol of Glc-6-P/ min/mg protein.

GlcN-6-P-synthetic activity was assayed as described previously [14].

For the determination of kinetic constants, the amidohydrolysing, Fru-6-P-isomerising or GlcN-6-P-synthetic activities were determined at variable (0 – 10 mM) initial concentrations of appropriate substrates. The data were plotted as Michaelis-Menten graphs. The kinetic data were subjected to the nonlinear regression analysis using the *Enzyme Kinetics Module 1.10 for Sigma Plot* software. The K_m and k_{cat} values were determined in triplicate, to give the mean values \pm S.D.

Phosphorylation of Gfa1p, Gfa1S208Ap and GAHp-His₆ *in vitro*

Gfa1p, Gfa1S208Ap or GAHp-His₆ (100 $\mu\text{g/ml}$) were incubated for 30 min at 25 °C with 10 μM cAMP, 1 mM ATP, 10 mM EDTA, 40 mM NaF and, if necessary, with the specific kinase A inhibitor H-89, 10 $\mu\text{g/ml}$. The reaction was initiated by the addition of the cAMP-dependent kinase from beef heart (30 units/ml). After 30 min, H-89 (10 $\mu\text{g/ml}$) was added to each sample to stop the kinase A activity. Samples were subjected to SDS-PAGE analysis and assayed for amidohydrolysing or GlcN-6-P-synthetic activity.

Detection of phosphorylated proteins in SDS-PAGE gels

The gels obtained after SDS-PAGE separation of protein mixtures incubated with kinase A, were stained with the GelCode™ Phosphoprotein Staining Kit, according to the protocol provided by the manufacturer. Gel images were documented, the gels were destained by several washings with demineralised water and stained with Coomassie Brilliant Blue R-250 to visualise all the protein bands.

Steady state fluorescence measurements

Fluorescence emission spectra were obtained using the Perkin Elmer LS55 spectrofluorimeter at room temperature. The excitation wavelength was set at 300 nm for selective excitation of tryptophan fluorescence. Incubation mixtures contained 0.3 μM of the respective domain, mutant or wild-type Gfa1p, 1 mM EDTA and/or a ligand at appropriate concentration in 25 mM phosphate buffer, pH 7.5. The incubation solution was stirred continuously in a 1.0 cm \times 1.0 cm cuvette. The

bandwidths for both the excitation and the emission monochromators were 4 nm. The spectra were corrected for background and Raman scattering by subtracting buffer spectra containing all ingredients except the protein.

Molecular mass determination of a native protein

Size-exclusion chromatography was performed on Superdex 200 HR 10/30 (Pharmacia LKB), eluted at 0.5 ml/min with 25 mM potassium-phosphate buffer, pH 7.0, containing 0.15 M NaCl, 1 mM EDTA and 1 mM DTT. Protein elution was monitored at 280 nm. The molecular mass standards were: thyroglobulin (669 kDa), apoferritin (443 kDa), α -amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa).

Other methods

The wild-type *C. albicans* GlcN-6-P synthase (Gfa1p) was overproduced in *E. coli* and purified to near homogeneity according to the multi-step procedure described by Sachadyn *et al.* [10]. Construction and purification of His₆-Gfa1p and Gfa1p-His₆ was described previously [15]. Protein was determined by the Bradford method [16]. Discontinuous SDS-PAGE was performed by the method of Laemmli [17] with 10% separating gel and 5% stacking gel.

RESULTS

Structural alignment of bacterial and fungal Gfa1p sequences

Amino acid sequences of *E. coli* and *C. albicans* GlcN-6-P synthases were aligned and the result is shown in Fig. 1. The sequences demonstrate 36% identity and 52% similarity, however several gaps, indicating regions which are absent from the shorter, bacterial variant of the enzyme, are clearly shown. Most of them are present in the N-terminal 1-239 part of the bacterial protein, constituting the GAH domain, with the largest one corresponding to the 218-283 region of the *C. albicans* enzyme.

Construction, expression, purification and characterization of the His₆-tagged domains of *C. albicans* Gfa1p

The two-domain structure of *E. coli* GlcN-6-P synthase is well documented, as well as a role played by each domain in the mechanism of catalysis. As shown in Fig. 2, the GAH domain (residues 1-239) is connected with the ISOM (residues 249-608) by a flexible nonapeptide linker. In the first step of our present studies we constructed two separate fragments of the *C. albicans* Gfa1p and characterised their functional properties. One of them comprised residues 1-345 and another one - residues 346-712. Both constructs contained oligoHis tags to facilitate their purification, however in the 1-345 construct, the tag was located at the C-terminus, while in the 356-712 construct it was at the N-terminus. Such localisations were an obvious consequence of the results of our previous studies on the construction and functional properties of the N- and C- His-tagged versions of the complete enzyme. We showed that the presence of an oligoHis tag at either end almost entirely inactivated the GlcN-6-P-synthesising activity of the enzyme. On the other hand, the N-tagged enzyme lost the ability to hydrolyse L-Gln but not to interconvert Glc-6-P and Fru-6-P, while the opposite was found for the C-tagged enzyme [15].

Expression plasmids pET23b-GLN and pET23b-FRU were constructed and their identities were confirmed by restriction analysis and gene sequencing. The fusion proteins, called GAHp-His₆ and His₆-ISOMp, respectively, were overproduced in *E. coli* BL21(DE3)pLysS cells that had been transformed with the respective overexpression plasmid. Densitometric analysis revealed that in each case the recombinant protein constituted 15-20% of the total cytoplasmic protein pool. GAHp-His₆ and His₆-ISOMp were purified by metal-affinity chromatography using the Ni²⁺ IDA-agarose resin. A single-step elution resulted in both cases in almost homogenous protein preparations (~98% homogeneity), isolated with 79 –81% yield. The exemplary results of the SDS-PAGE analysis of His₆-ISOMp purification are shown in Fig. 3.

The purified GAHp-His₆ and His₆-ISOMp were characterised in terms of their enzymatic activities and molecular mass/size and these properties were compared to those of the wild-type complete enzyme and its oligo-His-tagged fusions. The amidohydrolysing activity was tested using L- γ -glutamyl-*p*-nitroanilide (GLUPA) as the substrate. The hexosephosphate-isomerising activity was determined with Fru-6-P as the substrate, by continuous monitoring of the Glc-6-P formation in the Glc-6-P dehydrogenase-coupled assay. The GlcN-6-P formation was followed by the modified Elson-Morgan reaction [14]. The obtained results are summarised in Table

2. As expected, both GAHp-His₆ and His₆-ISOMp were completely devoid of the GlcN-6-P- synthetic activity. On the other hand, both fragments exhibited partial activities: GAHp-His₆ catalysed GLUPA hydrolysis and His₆-ISOMp was able to isomerise Fru-6-P to Glc-6-P. Kinetic parameters of both reactions were very similar to those found for the wild-type intact enzyme. Catalytic properties of GAHp-His₆ were also very similar to those of Gfa1p-His₆, while His₆-ISOMp demonstrated catalytic properties almost identical to those of His₆-Gfa1p. Therefore, there is little doubt that in *C. albicans* GlcN-6-P synthase, the 1-345 fragment constitutes the GAH domain and the remaining part is the ISOM domain.

Molecular mass of the native GAHp-His₆, determined under non-denaturing conditions (size-exclusion chromatography), was 42 ± 2 kDa and that of the denatured form of this protein (determined by SDS-PAGE) was 41 ± 0.5 kDa. The values found for His₆-ISOMp were: 159 ± 2 kDa and 39.5 ± 0.5 kDa, respectively. One may therefore conclude that GAHp-His₆ is monomeric in solution, while His₆-ISOMp is homotetrameric.

Our previous studies showed that *C. albicans* Gfa1p can be phosphorylated by a cAMP-dependent protein kinase and that the GlcN-6-P-synthetic activity of the enzyme increases upon protein kinase A-driven phosphorylation [14]. Moreover, we identified the S208 residue as an unique phosphorylation site [12]. In the present studies we checked whether the isolated GAH domain of Gfa1p, containing serine 208, was a substrate for a commercially available cAMP-dependent protein kinase from beef heart. GAHp-His₆ was incubated with the protein kinase under conditions optimal for protein phosphorylation and its amidohydrolysing activity was monitored. For comparison, the same activity was measured for Gfa1p and S208AGfa1p, together with the determination of possible changes in the GlcN-6-P-synthetic activity. The results of these experiments are shown in Fig. 4. It was found that incubation of GAHp-His₆ and Gfa1p with protein kinase A in the presence of ATP and cAMP, resulted in an almost two-fold increase of the amidohydrolysing activity of both proteins, closely correlated with a very similar enhancement of the synthetic activity of the whole enzyme. On the other hand, no change in both activities of S208AGfa1p, lacking the phosphorylation site, was noted. It seems therefore very likely that the enhanced GlcN-6-P-synthetic activity of Gfa1p phosphorylated at S208, is a direct consequence of the elevated amidohydrolysing activity of GAH.

In order to confirm protein phosphorylation and the identity of the phosphorylation site, SDS-PAGE analysis of the reaction mixtures, followed by specific gel staining, was performed. The results of this experiment are shown in Fig. 5. The gel shown in Fig 5A was stained with the GelCode™ kit, allowing a specific detection of the *O*-phosphorylated serine residues. The stained bands can be clearly seen in lanes 1, 4 and 5, thus confirming the presence of an *O*-phosphorylated serine in the kinase A-treated GAHp-His₆ and Gfa1p. Presence of Fru-6-P in the incubation mixture did not affect Gfa1p phosphorylation. A specific kinase A inhibitor, H-89, almost stopped phosphorylation of GAHp-His₆ (very faint band in lane 3). No band can be seen in lane 6, thus confirming that S208 is indeed the residue phosphorylated by kinase A in Gfa1p.

The hexosephosphate-isomerising activity of His₆-ISOMp and the amidohydrolysing activity GAHp-His₆ were virtually unaffected by UDP-GlcNAc, known to be a feedback inhibitor of eukaryotic GlcN-6-P synthase [9 and references cited therein]. In the presence of 2 mM UDP-GlcNAc, the K_m of His₆-ISOMp for Fru-6-P was 1.5 ± 0.1 mM and k_{cat} was 0.53 ± 0.04 min⁻¹, while the K_m of GAHp-His₆ for GLUPA was 0.29 ± 0.03 mM and k_{cat} was 0.48 ± 0.05 min⁻¹. On the other hand, UDP-GlcNAc lowered the GlcN-6-P-synthetic activity and amidohydrolysing activity of Gfa1p. For the former, the k_{cat} value determined in the presence of 2 mM UDP-GlcNAc was 285 ± 18 min⁻¹, compared to 720 ± 24 min⁻¹, determined for the inhibitor-free enzyme. In the case of the amidohydrolysing activity, the effect was much stronger when Fru-6-P was also present in the incubation mixture. The k_{cat} for GLUPA hydrolysis determined in the presence of 2 mM UDP-GlcNAc was 0.62 ± 0.06 min⁻¹ and in the presence of 2 mM UDP-GlcNAc + 10 mM Fru-6-P was 19.3 ± 1.1 min⁻¹, compared to 0.74 ± 0.05 min⁻¹ (- Fru-6-P) and 54.8 ± 2.8 min⁻¹ (+ Fru-6-P) determined in the absence of UDP-GlcNAc. The K_m values were not affected by UDP-GlcNAc.

Characterisation of Δ 218-283Gfa1p and the UDP-GlcNAc binding site

The polypeptide chain of GlcN-6-P synthase of eukaryotic origin is generally 60 - 100 aa longer than its prokaryotic counterpart. Most of the inserts responsible for this elongation are located in the GAH region, and one of them is especially long in all known sequences [9]. In the *C. albicans* Gfa1p, the long insert comprises the 218-283 region (see Fig. 1). In order to find any evidence for the possible role of the 218-

283 part of the enzyme, we constructed its truncated version, Δ 218-283Gfa1p. The protein was isolated and characterised. Molecular mass of the native Δ 218-283Gfa1p was found to be 290 ± 2 kDa, while that determined under denaturing condition was roughly four times lower (74 ± 1 kDa), thus indicating that the removal of the 218-283 fragment does not affect the quaternary structure of Gfa1p.

The catalytic properties of the truncated enzyme were also characterised and compared to those of the wild-type enzyme. The measured activities are summarised in Table 3. It is evident that the truncated Gfa1p is not able to form GlcN-6-P but retains amidohydrolysing and hexosephosphate isomerising activity of the intact enzyme.

Gfa1p contains three tryptophanyl residue, namely: W97, W388 and W690. As the first one is located in the GAH domain and the remaining two in the ISOM domain, we were able to use tryptophan fluorescence to study the interaction of GAHp-His₆, His₆-ISOMp, Δ 218-283Gfa1p and Gfa1p with UDP-GlcNAc, by monitoring conformational changes induced in these proteins upon ligand binding. Emission spectra, taken at the Trp-specific excitation wavelength, were obtained and evaluated. We observed that the presence of UDP-GlcNAc strongly diminished the tryptophan-derived fluorescence of Gfa1p, thus indicating changes in the protein conformation, upon which at least one tryptophanyl residue becomes more exposed to the bulk solvent. The same effect was observed in the His₆-ISOMp/UDP-GlcNAc spectrum, while the GAHp-His₆ fluorescence was not affected by the ligand (spectra not shown). On the other hand, changes observed in the fluorescence spectrum of Δ 218-283Gfa1p induced by UDP-GlcNAc (not shown) were very similar to those found for the intact enzyme. The decrease in Gfa1p, Δ 218-283Gfa1p and His₆-ISOMp fluorescence induced by UDP-GlcNAc was ligand concentration-dependent and some subtle changes were observed even at micromolar level (data not shown), corresponding to the level of the protein concentration, thus indicating a specific ligand binding.

One may therefore conclude that in the complete Gfa1p the conformational change triggered in ISOM upon UDP-GlcNAc binding is subsequently transferred to GAH through some interdomain interactions to affect there the amidohydrolysing activity, whereas the 218-283 fragment of GAH does not seem to be involved in interactions with UDP-GlcNAc.

Interdomain communication and signalling

Structural studies on *E. coli* GlcN-6-P synthase revealed the presence of a 18 Å-long hydrophobic channel (see Fig. 2) between the active sites of the GAH and ISOM domains, constituted by the residues L601, A602, V605, V608 of ISOM and R26 and W74 of GAH, with the additional participation of K503 and H504 of the neighbouring protein subunit in the functional dimer. The channel allows ammonia transfer from GAH to ISOM and is gated by the indole ring of W74 [5, 8]. All the above mentioned residues are strongly conserved in the known GlcN-6-P synthase sequences [9] and are also present in *C. albicans* Gfa1p as: R32, W97, L705, A706, V709, V711, K606 and H607. In the present studies we have constructed and characterised a few recombinant versions of Gfa1p, truncated or mutated at sites supposed to be crucial for the functioning of the putative channel. Particularly, four C-terminal residues were removed to disturb the channel integrity, valine 711 was substituted by phenylalanine to block the channel and finally tryptophan 97 was exchanged for phenylalanine or glycine to reduce or remove a putative molecular gate responsible for channel opening and closure. All the constructed mutants were tested for catalytic activity. The results are presented in Table 3. It is clear that the removal of residues 709-712 results in the complete elimination of the GlcN-6-P-synthetic activity of Gfa1p, while its hexosephosphate-isomerising activity remains unaffected. The ability to synthesise GlcN-6-P was not restored when we used ammonium chloride (10 – 50 mM) and 10 mM Fru-6-P as substrates (detailed data not shown). On the other hand, substitution of W97 for phenylalanine or glycine affected the amidohydrolysing activity and GlcN-6-P formation but not the hexose phosphate isomerisation. The effect was much stronger in the case of the W97G mutant, which lost almost all synthetic activity and its ability to hydrolyse GLUPA was reduced to 10% of the original value. Interesting differences and changes in the amidohydrolysing activity, measured in the absence or presence of Fru-6-P were noted. We observed a 70 – 80-fold increase of the amidohydrolysing activity of Gfa1p, V711FGfa1p, W97FGfa1p and W97GGfa1p in the presence of Fru-6-P, while that of Δ 709-712Gfa1p and Δ 218-283Gfa1p was only slightly enhanced. These results indicate a possibility of an interdomain signalling in *C. albicans* Gfa1p. It should be noted that Mouilleron *et al.* suggested that in the *E. coli* GlcN-6-P synthase, interactions between residues constituting the C-tail of ISOM, especially T606, and C1 and N98 of GAH were responsible for a substantial

enhancement of the amidohydrolase activity of GAH upon Fru-6-P binding at ISOM [8]. Our studies thus provided evidence supporting that suggestion, since the C-tail: GAH interactions and, in consequence, the interdomain signalling, were apparently precluded upon $\Delta 709-712$ deletion. The negligible effect of Fru-6-P on amidohydrolysing activity of $\Delta 218-283$ Gfa1p suggests that the interdomain signalling is also lost upon the $\Delta 218-283$ deletion. Rather unexpectedly, the amidohydrolysing activity of $\Delta 709-712$ Gfa1p, measured in the absence of Fru-6-P, was nearly 25-fold higher than that of Gfa1p. This is surprising, since $\Delta 709-712$ Gfa1p lacks a part of the C-tail, including the T710 residue, corresponding to T606 of the bacterial GlcN-6-P synthase. Possibly some new interactions between the remaining part of the C-tail and GAH, enhancing amidohydrolysing potential of GAH but practically independent of Fru-6-P binding, were created upon the removal of residues 709-712. Obviously, such an explanation is only speculative, unless the Gfa1p and $\Delta 709-712$ Gfa1p structures are determined.

The data presented in Table 2 indicate that the K_m values for the substrate in the amidohydrolysing activity of GAH-His₆ and Gfa1p were not very different. This is in contrast to the previous observation by Isupov *et al.*, made on the bacterial GlcN-6-P synthase, where the K_m for the whole enzyme was more than two orders of magnitude lower, as compared to that of the isolated domain [18]. Teplyakov *et al.* suggested that the higher affinity of the *E. coli* whole enzyme for glutamine was due to the interdomain interactions involving the R539 residue of ISOM and the Q-loop (residues 73-80) of GAH [5]. Our results may thus indicate that such interactions affecting the glutamine binding do not exist in *C. albicans* Gfa1p, in spite of the fact that the above-mentioned residues are conserved in the fungal enzyme (see Fig. 1).

Differences in the amidohydrolysing activity of the obtained muteins were of special interest. However, it must be kept in mind that the activity was measured with an artificial substrate. In order to check whether the observed phenomena take place also in the case of the physiological substrate, we measured ammonia release from L-glutamine in the absence of Fru-6-P for muteins and the wild-type enzyme. The results are shown in Fig. 6. They confirm all the previous findings concerning the amidohydrolysing activity measured with GLUPA.

The V711F mutant exhibited extremely low but measurable synthetic activity, thus indicating that the introduced phenyl ring effectively blocks the interdomain

channel and thus virtually prevents ammonia transfer from GAH to ISOM. The channel blockage did not influence catalytic actions of both domains, since the measured respective activities were very much the same as those of the wild-type enzyme.

DISCUSSION

Results of our studies presented here indicate that the two-domain structure, demonstrated previously in the subunit of the bacterial GlcN-6-P synthase [5, 18-20], is also a feature of the fungal enzyme and the GAH functionality can be attributed to the 1-345 fragment of *C. albicans* Gfa1p, while the remaining part of the enzyme is catalytically active as ISOM. Our present results also show that formation of the previously suggested homotetrameric structure of *C. albicans* GlcN-6-P synthase [14,21] is mediated by interactions between the ISOM domains, while the GAH domains do not seem to be involved in the oligomerisation. Thus the quaternary structure of this enzyme is clearly different from that of its bacterial, homodimeric counterpart, as well as from that of the “sister” class II amidotransferase, namely L-glutamine: phosphoribosylpyrophosphate amidotransferase, for which it was previously found that the formation of the tetrameric structure was mediated by both domains [22].

Previous studies on fungal GlcN-6-P synthase provided kinetic evidence that the UDP-GlcNAc binding site is different from the L-Gln and D-Fru-6-P binding sites [14,23]. Our present results demonstrate that the inhibitor binding site is located at ISOM and the binding of UDP-GlcNAc induces conformational changes in this domain. Such location of the UDP-GlcNAc binding site(s) confirms suggestions made by Chou for the mammalian GlcN-6-P synthase, based on *in silico* homology modelling [24]. Our current results also show that despite the detected conformational changes, the UDP-GlcNAc binding to Gfa1p does not affect the hexosephosphate-isomerising activity of ISOM but lowers the amidohydrolysing activity of GAH and, in consequence, the GlcN-6-P-synthetic activity of the entire enzyme. A structural basis of this effect remains unclear until the exact location of the UDP-GlcNAc binding site in ISOM is known.

Regulation of the GlcN-6-P synthase activity by enzymatic phosphorylation and dephosphorylation was previously demonstrated for the mammalian and fungal

enzymes [14,25 - 27]. Our present results prove that an increase of GlcN-6-P synthetic activity of the *C. albicans* Gfa1p, observed upon phosphorylation of the S208 residue of this enzyme, is most likely due to the enhancement of the GAH amidohydrolysing activity. The molecular mechanism of this effect remains unknown, until a structure of the fungal enzyme is solved. However, it seems worth mentioning that in the 3D structure of the bacterial enzyme, the region corresponding to the kinase A recognition site in the eukaryotic GlcN-6-P synthase, is located close to the catalytic C1 residue. Particularly, the hydroxyl group of S176 (homologous to S208 of the *C. albicans* enzyme), is only 5.4 Å from the protonated α -amino group of C1 [5,8,18]. If the same is true for the fungal enzyme, one may assume that the phosphorylated S208 may help keep C1 in the active conformation. However, the fungal GAH contains several inserts in the 1-208 region, absent from the bacterial enzyme, which may also affect the relative positioning of C1 and S208.

A possible role of the 218-283 fragment in *C. albicans* Gfa1p remains one of the particularly intriguing issues. Similar inserts, smaller or slightly larger, are present in all known eukaryotic sequences, although they demonstrate very little, if any, sequence homology [9], that could suggest that the respective regions may not have any particular function. Our present results indicate that the 218-283 fragment is neither involved in UDP-GlcNAc binding nor in the formation of the quaternary structure. The fact that the Δ 218-283Gfa1p lacks the GlcN-6-P-synthetic activity can be, in our opinion, interpreted as a consequence of a possible improper relative orientation of GAH and ISOM that prevents the assembly of the interdomain channel and thus precludes the ammonia transfer from GAH to ISOM. This suggestion seems to be additionally supported by the observed disturbance of the interdomain signalling.

The loss of the GlcN-6-P-synthetic activity of Gfa1p upon removal of the four C-terminal residues, indicates that the integrity of the interdomain channel is crucial for the ammonia transfer from GAH to ISOM. Therefore, it is very likely that the channel in Δ 709-712Gfa1p is leaky and ammonia molecules, generated from L-glutamine at the GAH active site, do not reach the ISOM active site but are released to the bulk solvent. On the other hand, the escape route created by the 709-712 deletion seems to be unidirectional, since the truncated enzyme was still unable to use exogenous ammonia as the substrate for GlcN-6-P formation. However, one

must keep in mind that GlcN-6-P synthase, unlike some other amidotransferases [6], does not possess any separate binding site for ammonia. Therefore, the NH₃ molecules from the bulk solvent, randomly interacting with enzyme molecules, may have little chance to enter the ISOM active site from a proper direction.

Finally our present results provide evidence for the crucial role of the W97 residue which is a counterpart of W74 of the *E. coli* enzyme. In our W97F and W97G mutants, this putative molecular gate was modified: reduced in the former or virtually eliminated in the latter. These modifications have been shown to substantially decrease the GlcN-6-P-synthetic activity of the enzyme, by an order and more than two orders of magnitude, respectively. In our opinion this phenomenon may be well explained in light of the previous observations of Mouilleron *et al.*, who found that the indole ring of W74 in *E. coli* enzyme, after rotation induced upon glutamine binding, opened the channel and at same time sealed its side wall [8]. We assume therefore, that the channel in the W97F W97G mutants of Gfa1p is leaky to some extent, resulting in lower efficiency of ammonia transfer from GAH to ISOM. Interestingly enough, both substitutions resulted also in a slight decrease of the amidohydrolysing activity. This effect might be in turn caused by the fact that W74 in *E. coli* GlcN-6-P synthase, and possibly its counterparts in other enzyme variants, are parts of the Q-loop (residues 73-80 in the *E. coli* enzyme; 98-103 in the *C. albicans* homologue), functioning as a lid that closes the entrance to the GAH active site when L-glutamine is bound [5,8]. The W97F and W97G substitutions may distort the lid structure, thus making it less efficient as a “substrate keeper”.

The V711F substitution appeared to create an additional molecular gate in the interdomain channel. This effect was in fact expected, as comparison of the structures of channel segments in V711F and the wild-type enzyme, made by molecular homology modelling (Fig. 7), revealed that the phenyl ring should provide a steric hindrance to the channel. A similar phenomenon had been observed in the G359Y and G359F mutants of *E. coli* carbamoyl phosphate synthetase (CPS) [28, 29], in the L109A mutant of *E. coli* CTP synthase [30] and in the L415A mutant of glutamine phosphoribosylpyrophosphate amidotransferase [31]. In all those cases the partial reactions of glutamine hydrolysis and the final product formation became uncoupled, but both enzymes remained able to use exogenous ammonia as the substrate. It was postulated that the molecular channel in bacterial CPS becomes leaky upon Gly 359 substitution with Tyr or Phe [29], while the channel in the CTP

synthase was constricted upon the L109A exchange [30]. Our V711F mutant did not gain any ability to use ammonia for GlcN-6-P formation, but the partial activities were not affected. It seems therefore that in the V711F mutant, L-glutamine or L- γ -glutamyl-*p*-nitroanilide is hydrolysed at GAH and the respective product, namely ammonia or *p*-nitroanilide, is released to the bulk solvent.

In conclusion, we were able to identify the separately expressed 1-345 and 346-712 fragments of *C. albicans* Gfa1p as the glutamine amide-hydrolysing domain (GAH) and the hexose phosphate-isomerising domain (ISOM), respectively. The interactions between the ISOM domains are responsible for the formation of a homotetrameric structure of the native Gfa1p, while GAH is not involved in protein oligomerisation. The binding site for the feedback inhibitor, UDP-GlcNAc, is located in the ISOM domain. Binding of UDP-GlcNAc at ISOM or phosphorylation of the S208 residue affects the amidohydrolysing activity of GAH and, in consequence, the GlcN-6-P synthetic activity of the whole enzyme. No specific function can be attributed to the 218-283 fragment of GAH, however its deletion strongly disrupts the interdomain communication and signalling. A similar effect is observed upon the removal of four C-terminal residues. The intramolecular channel connecting GAH and ISOM is constituted by several hydrophobic residues and one of them, namely W97, functions as a molecular gate. The channel becomes leaky upon the deletion of amino acids 709-712, as well as upon the reduction of the molecular gate size. On the other hand, the channel can be blocked upon the V711F substitution.

Acknowledgement

The authors acknowledge the financial support of these studies from the Polish Ministry of Science and Higher Education (grant No P06A 036 25) and in part from the Faculty of Chemistry, Gdańsk University of Technology. Thanks are given to Dr. Marek Wojciechowski for his help in molecular modelling.

REFERENCES

- 1 Hebert, L.F., Daniels, M.C., Zhou, J., Crook, E.D., Turner, R.L., Simmons, S.T., Neidigh, J.L., Zhu, J.S., Baron, A.D. and McClain D.A. (1996) Overexpression of glutamine:fructose-6-phosphate amidotransferase in transgenic mice leads to insulin resistance. *J. Clin. Invest.* **98**, 930-936.
- 2 Borowski E. (2000) Novel approaches in the rational design of antifungal agents of low toxicity. *Farmacologia* **55**, 206-208.
- 3 Badet, B., Vermoote, P., Haumont, P.Y., Lederer, F. and LeGoffic, F. (1987) Glucosamine synthetase from *Escherichia coli*: purification, properties and glutamine-utilizing site location. *Biochemistry* **26**, 1940-1948.
- 4 Teplyakov, A., Leriche, C., Obmolova, G., Badet, B. and Badet-Denisot, M.-A. (2002) From Lobry de Bruyn to enzyme-catalyzed ammonia channelling: molecular studies of D-glucosamine-6P synthase. *Nat. Prod. Rep.* **19**, 60-69.
- 5 Teplyakov, A., Obmolova, G., Badet, B. and Badet-Denisot, M.-A. (2001) Channeling of ammonia in glucosamine-6-phosphate synthase. *J. Mol. Biol.* **313**, 1093-1102.
- 6 Massière, F. and Badet-Denisot, M.-A. (1998) The mechanism of glutamine-dependent amidotransferases. *CMLS. Cell. Mol. Life Sci.* **54**, 205-222.
- 7 Leriche, C., Badet-Denisot, M.-A. and Badet, B. (1997) Affinity labeling of *Escherichia coli* glucosamine-6-phosphate synthase with a fructose 6-phosphate analog. Evidence for proximity between the N-terminal cysteine and the fructose-6-phosphate-binding site. *Eur. J. Biochem.* **245**, 418-422.
- 8 Mouilleron, S., Badet-Denisot, M.-A. and Golinelli-Pimpaneau, B. (2006) Glutamine binding opens the ammonia channel and activates glucosamine-6P synthase. *J. Biol. Chem.* **281**, 4404-4412
- 9 Milewski, S. (2002) Glucosamine-6-phosphate synthase – the multifacets enzyme, *Biochim. Biophys. Acta.* **1597**, 173-192.
- 10 Sachadyn, P., Jędrzejczak, R., Milewski, S., Kur, J. and Borowski, E. (2000) Purification to homogeneity of *Candida albicans* glucosamine-6-phosphate synthase overexpressed in *Escherichia coli*. *Prot. Expr. Purif.* **19**, 343-349.
- 11 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

- 12 Gabriel. I., Olchowoy, J., Stanisławska-Sachadyn, A., Mio, T., Kur, J. and Milewski, S. (2004) Phosphorylation of glucosamine-6-phosphate synthase is important but not essential for germination and mycelial growth of *Candida albicans*. FEMS Microbiol. Lett. **235**, 73-80.
- 13 Noltman, E. A. (1964) Isolation of crystalline phosphoglucose isomerase from rabbit muscle. J. Biol. Chem. **239**, 1545-1550.
- 14 Milewski, S., Kuszczak, D., Jędrzejczak, R., Smith, R.J., Brown, A.J. and Gooday, G.W. (1999) Oligomeric structure and regulation of *Candida albicans* glucosamine-6-phosphate synthase. J. Biol. Chem. **274**, 4000-4008.
- 15 Olchowoy, J., Kur, K., Sachadyn, P. and Milewski, S. (2006) Construction, purification and functional characterisation of His-tagged *Candida albicans* glucosamine-6-phosphate synthase expressed in *Escherichia coli*. Protein Expr. Purif. **46**, 309-315.
- 16 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248-254.
- 17 Laemmli, U. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**, 680-685.
- 18 Isupov, M. N., Obmolova, G., Butterworth, S., Badet-Denisot, M.-A., Badet, B., Polikarpov, I., Littlechild, J. A. and Teplyakov, A. (1996) Substrate binding is required for assembly of the active conformation of the catalytic site in Ntn amidotransferases: evidence from the 1.8 Å structure of the glutaminase domain of glucosamine-6-phosphate synthase. Structure, **4**, 801-810.
- 19 Denisot, M.-A., LeGoffic, F. and Badet, B. (1991) Glucosamine-6-phosphate synthase from *Escherichia coli* yields two proteins upon limited proteolysis: identification of the glutamine amidohydrolase and 2R ketose/aldose isomerase-bearing domains based on their biochemical properties. Arch. Biochem. Biophys. **288**, 225-230.
- 20 Teplyakov, A., Obmolova, G., Badet-Denisot, M.-A., Badet, B. and Polikarpov, I. (1998) Involvement of the C-terminus in intramolecular channeling in glucosamine-6-phosphate synthase: evidence from a 1.6 Å structure of the isomerase domain. Structure, **6**, 1047-1055.
- 21 Olchowoy, J., Jędrzejczak, R., Milewski, S. and Rypniewski, W. (2005) Crystallization and preliminary X-ray analysis of the isomerase domain of

- glucosamine-6-phosphate synthase from *Candida albicans*. *Acta Cryst.* **F61**, 994-996.
- 22 Muchmore C. R. A., Krahn, J. M., Kim, J. H., Zalkin, H. and Smith, J. L. (1998) Crystal structure of glutamine phosphoribosylpyrophosphate amidotransferase from *Escherichia coli*. *Protein Sci.* **1**, 39-51.
- 23 Endo, A., Kakiki, K. and Misato, T. (1970) Feedback inhibition of L-glutamine D-fructose 6-phosphate amidotransferase by uridine diphosphate N-acetylglucosamine in *Neurospora crassa*. *J. Bacteriol.* **103**, 588-594.
- 24 Chou, K.-C. (2004) Molecular therapeutic target for type-2 diabetes. *J. Proteome Res.* **3**, 1284-1288.
- 25 Chang, Q., Su, K., Baker, J. R., Yang, X., Paterson, A. J. and Kudlow, J. E. (2000) Phosphorylation of human glutamine-fructose-6-phosphate amidotransferase by cAMP-dependent protein kinase at serine 205 blocks the enzyme activity. *J. Biol. Chem.* **275**, 21981-21987.
- 26 Hu, Y., Riesland, L., Paterson, A. J. and Kudlow, J. E. (2004) Phosphorylation of mouse glutamine-fructose-6-phosphate amidotransferase 2 (GFAT2) by cAMP-dependent protein kinase increases the enzyme activity. *J. Biol. Chem.* **279**, 29988-29993.
- 27 Etchebehere, L. C. and Maia, J. C. (1989) Phosphorylation-dependent regulation of amidotransferase during the development of *Blastocladiella emersonii*. *Arch. Biochem. Biophys.* **272**, 301-310.
- 28 Thoden, J. B., Huang, X., Raushel, F. M., Holden, H. M. (2002) Carbamoyl-phosphate synthetase. Creation of an escape route for ammonia. *J. Biol. Chem.* **277**, 39722-39727.
- 29 Huang, X. and Raushel, F. M. (2000) An engineered blockage within the ammonia tunnel of carbamoyl phosphate synthase prevents the use of glutamine as a substrate but not ammonia. *Biochemistry* **39**, 3240-3247.
- 30 Lunn, F. A. and Bearne, S. L. (2004) Alternative substrates for wild-type and L109A *E. coli* CTP synthases. Kinetic evidence for a constricted ammonia tunnel. *Eur. J. Biochem.* **271**, 4204-4212.
- 31 Bera, A. K., Smith, J. L. and Zalkin, H. (2000) Dual role for the glutamine phosphoribosylpyrophosphate amidotransferase ammonia channel. Interdomain signaling and intermediate channeling. *J. Biol. Chem.* **275**, 7975-7979.

Table 1. PCR primers used for the construction of site-directed mutants of *GFA1*.

Restriction recognition sites are underlined. Capital letters indicate mismatches with the original *GFA1* sequence (bold – introducing mutation; normal – introducing restriction recognition site).

N- and C-terminal flanking oligonucleotides	
Gln1-KN:	5'-cctggcggtagcccatatgtgtggtatgtttggttacg-3'
cgfa-bam:	5'-cacgggatccttactcaacagtaactgatttagcca-3'
Mutation-introducing oligonucleotides	
Mutant	Primer designation and sequence
W97G	Trpgly-3: 5'-catgtgt <u>GgCgc</u> Ctctagtagcaataacc-3'
	Trpgly-5: 5'-cactactaga <u>GgcGcC</u> acacatggtcaacc-3'
W97F	Trpphe-3: 5'-agc GAa <u>AcGCg</u> tatgagcaataccaacatg-3'
	Trpphe-5: 5'-ctc <u>atacGCgTtTC</u> gctacacatggtcaac-3'
V711F	Valphe: 5'-cctgcc <u>ctcgagt</u> tactcaa A agtaactgatttagccaag-3'

Table 2. Kinetic parameters of reactions catalysed by Gfa1p, its oligoHis-tagged variants and oligoHis-tagged domains.

Data are the means of three independent determinations \pm S.D. ND – not determined

Enzymatic protein	Amidohydrolysing activity		Hexosephosphate isomerising activity		GlcN-6-P-synthetic activity		
	$K_{M(\text{GLUPA})}$ (mM)	k_{cat} (min^{-1})	$K_{M(\text{Fru-6-P})}$ (mM)	k_{cat} (min^{-1})	$K_{M(\text{Gln})}$ (mM)	$K_{M(\text{Fru-6-P})}$ (mM)	k_{cat} (min^{-1})
Gfa1p	0.67 \pm 0.04	0.74 \pm 0.05	1.20 \pm 0.08	1.28 \pm 0.09	0.34 \pm 0.01	1.41 \pm 0.05	720 \pm 24
His ₆ -Gfa1p	12.2 \pm 0.9	0.055 \pm 0.004	1.08 \pm 0.07	1.27 \pm 0.06	ND	ND	ND
Gfa1p-His ₆	0.64 \pm 0.08	0.80 \pm 0.07	6.50 \pm 0.31	0.24 \pm 0.03	ND	ND	12.6 \pm 0.4
GAHp-His ₆	0.31 \pm 0.02	0.45 \pm 0.03	ND	ND	ND	ND	ND
His ₆ -ISOMp	ND	ND	1.40 \pm 0.11	0.55 \pm 0.03	ND	ND	ND

Table 3. Enzymatic activities of Gfa1p, its truncated and mutated versions.

Data are the means of three independent determinations \pm S.D.

Enzyme version	Amidohydrolysing activity		Hexosephosphate isomerising activity	GlcN-6-P-synthetic activity
	[mU/mg]			
	- Fru-6-P	+ Fru-6-P		
Gfa1p (wild type)	12 \pm 0.6	890 \pm 24	63.1 \pm 1.9	12 \pm 0.5
Δ 218-283Gfa1p	11.6 \pm 0.2	12.5 \pm 0.7	61 \pm 2.2	<0.01
V711F	13 \pm 0.5	940 \pm 18	62.6 \pm 1.3	0.021 \pm 0.003
Δ 709-712	290 \pm 6	357 \pm 11	60.7 \pm 1.4	<0.01
W97F	4.3 \pm 0.3	256 \pm 8	62.1 \pm 1.2	1.20 \pm 0.06
W97G	1.3 \pm 0.1	95 \pm 2	63.2 \pm 1.4	0.05 \pm 0.01

Figure legends

Figure 1 The alignment of amino acid sequences of *C. albicans* and *E. coli* GlcN-6-P synthases.

The linker between the GAH and ISOM domains of *E. coli* GlcN-6-P synthase and residues substituted in the recombinant versions of the *C. albicans* enzyme, constructed for the purpose of this work, are indicated. *C. albicans* numbering is shown.

Figure 2 Stereo view of the 3D structure of *E. coli* GlcN-6-P synthase.

Functional domains, some relevant fragments and the intramolecular channel (small spheres) are indicated. The image has been generated from 1JXA [5].

Figure 3 SDS-PAGE analysis of the purification of *N*-oligoHis-tagged 346-712 fragment of Gfa1p overproduced in *E. coli*.

Lane M, molecular mass markers; lane 1, total lysate of *E. coli* BL21(DE3)pLysS pET23b-FRU cells; lane 2, total lysate of control *E. coli* BL21(DE3)pLysS pET23b cells; lanes 3-4, wash fractions with buffer W; lane 5, fraction eluted with 0.5 M imidazole

Figure 4 Changes in enzymatic activities of GAH-His₆, Gfa1p and S208AGfa1p upon phosphorylation with protein kinase A.

Samples of Gfa1p, S208AGfa1p or GAH-His₆ were incubated for 30 min in the presence of indicated compounds. A specific kinase A inhibitor H-89 (10 µg/ml) was then added and the samples were assayed for amidohydrolysing and/or GlcN-6-P-synthetic activity.

Figure 5 SDS-PAGE analysis of GAHp-His₆, Gfa1p and S208AGfa1p phosphorylation by kinase A.

Mixtures obtained after 30 min incubation of a given protein with indicated components were separated by SDS-PAGE. (A) Gel stained with the GelCode™ kit; (B) The same gel stained with Coomassie Brilliant Blue.

Lane 1, GAHp-His₆ + kinase A; lane 2, GAHp-His₆; lane 3, GAHp-His₆ + kinase A + H-89; lane 4, Gfa1p + kinase A; lane 5, Gfa1p + kinase A + 10 mM Fru-6-P; lane 6,

S208AGfa1p + kinase A; lane 7, Gfa1p. Each mixture contained 10 μ M cAMP, 1 mM ATP, 10 mM EDTA and 40 mM NaF. Protein load: 10 μ g (lanes 1-3) and 3 μ g (lanes 4-7). M – molecular weight markers: 120 kDa, 100 kDa, 85 kDa, 70 kDa, 60 kDa, 50 kDa, 40 kDa.

Figure 6 The time course of ammonia release from L-glutamine, catalysed by Gfa1p and its mutated versions.

Enzyme samples were incubated with 10 mM L-Gln and the released ammonia was quantified with the Nessler's reagent. Experiments were done in triplicate. Bars represent S.D.

Figure 7 The blockage of the interdomain channel in Gfa1p upon V711F substitution.

(A) Computed structure created by homology modelling using *E. coli* GlcN-6-P synthase (1JXA) [5] as a template. (B) Putative result of V711F substitution.

Residues marked with an asterisk are from the neighbouring subunit. The channel is indicated by black spheres.

	1	10	20	30	40	50	60	70	80	90
Ca	CGIFGYVNFLVDKSRGEIIDNLEGLQRLEYRGYDSAGI									
Ec	CGIVGAI.....AQRDVAEILLEGLRRLEYRGYDSAGLAVVDA.....EGHMTRL..RRLGKVQMLAQAAEEHPLHGGT.....									
		W97								
	100	110	120	130	140	150	160	170	180	
Ca	GIAHTRWATHGQPKTENCHPHKSDPKGEFIVVHNGIITNYAALRKYL LSKGHVFESETDTECIAKLFKHFDLNVKAGVFPDLNELTKQV									
Ec	GIAHTRWATHGEPSEVNAHPHVSE...HIVVVHNGIIEHHEPLREELKARGYTFVSETDTEVIA....HLVNWELKQG..GTLREAVLRA									
			S208							
	190	200	210	220	230	240	250	260	270	
Ca	LHELEGSYGLLVKSYHYPGEVCGTRKGSPLLVGVKTDKKLKVDFVDVEFEAQOQHRPQQPQINHGATSAAELGFIPVAPGEQNLRTSQS									
Ec	IPQLRGAYGTVIMDSRHPDTLLAARSGSPLVIGLGMG.....									
	280	290	300	310	320	330	340	350	360	
Ca	RAFLSEDDLMPVVEFFLSSDPASVVQHTKKVLFLEDDIAHIYDGEIIRHRASTKSAGESTVRPIQOTLEMELNEIMKGPYKHFMOKEIFE									
EcENFIASDQLALLPVTRRFIFLEEGDIAEITRRSVNIF...DKTGAEVKRODIES.NLOYDAGDKGIYRHYMOKEIYE									
								linker		
	370	380	390	400	410	420	430	440	450	
Ca	QPDSAFNTMRGRIDFENCVVTLGGLKSWLSTIRRCRRIIMIACGTSYHSCLATRSIFEELTEIPVSVELASDFLDRRSPVFRDDTCVSVS									
Ec	QPNAIKNTLTGRISHGQVDLSELGPNAD.ELLSKVEHIQILACGTSYNSGMVSRYWFEFLAGIPCDVEIASEFRYRKSAVRRNSLMITLS									
	460	470	480	490	500	510	520	530		
Ca	OSGETADSILALQYCLER GAL.TVGIVNSVGSMSRQTHCGVHINAGPEIGVASTKAYTSQYIALVMFALSLSNDSISRKGRHEEIIKGL									
Ec	OSGETADTLAGLRLSKELGYLGLSLAICNVPGSSLVRESDLALMTNAGTEIGVASTKAFTTQTLTVLLMLVAKLSRLKGLDASIEHDI VHGL									
	540	550	560	570	580	590	600	610	620	
Ca	QKIPEQIKQVVKLENKIKDL CNSLNDQKSLLLLGRGYQFATALEGALKIKEISYMHSEGVLAGELKHGILALVDEDLPIIAFATRDSL F									
Ec	QALPSRIEQMLSQDKRIEALAE.DFSKHHALFLGRGDQYPIALEGALKLKEISYIHA EAYAAGELKHGPLALIDADMPVIVVAPNNE LL									
	630	640	650	660	670	680	690	700	710	V711
Ca	PKVMSAIEQVTARDGRPIVICNEGDAIISNDKVHTTLEVPETVDCLQGLLNVIPLQLISYWLAVNRGIDVDFPRNLAKSVTVE									
Ec	EKLKSNIEEVRARGGQLYVFADQDAGFVSSDNMHI.IEMPHVEEVIAPIFYTVPLQLLAYHVALIKGTDVDQPRNLAKSVTVE									

