Candida albicans glucosamine-6-phosphate (GlcN-6-P) synthase was purified to apparent homogeneity with 52% yield from recombinant yeast YRSC-65 cells efficiently overexpressing the GFA1 gene. The pure enzyme exhibited $K_m$(Gln) = 1.56 mM and $K_m$(Fru-6-P) = 1.41 mM and catalyzed GlcN-6-P formation with $K_m$ = 1150 min$^{-1}$. The isoelectric point of 4.6 ± 0.05 was estimated from isoelectric chromatofocusing. Gel filtration, native polyacrylamide gel electrophoresis, subunit cross-linking, and SDS-polyacrylamide gel electrophoresis showed that the native enzyme was a homotetramer of 79.5-kDa subunits, with an apparent molecular mass of 330–340 kDa.

Results of chemical modification of the enzyme by group-specific reagents established an essential role of a cysteinyl residue at the glutamine-binding site and histidyl, lysyl, arginyl, and tyrosyl moieties at the Fru-6-P-binding site. GlcN-6-P synthase in crude extract was effectively inhibited by UDP-GlcNAc ($I_C = 0.67$ mM).

Purification of the enzyme markedly decreased the sensitivity to the inhibitor, but this could be restored by addition of another effector, glucose 6-phosphate. Binding of UDP-GlcNAc to the pure enzyme in the presence of GlcN-6-P showed strong negative cooperativity, with $n_H = 0.54$, whereas in the absence of this sugar phosphate no cooperative effect was observed. Pure enzyme was a substrate for cAMP-dependent protein kinase, the action of which led to the substantial increase of GlcN-6-P synthase activity and its sensitivity to phosphorylation. The maximal level of activity was observed for the enzyme molecules containing 1.21 cysteinyl residues at the glutamine-binding site and histidyl, lysyl, arginyl, and tyrosyl moieties at the Fru-6-P-binding site. GlcN-6-P synthase in crude extract was effectively inhibited by UDP-GlcNAc ($I_C = 0.67$ mM).

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L-glutamine+D-fructose-6-phosphate amidotransferase (hexose-isomerizing) EC 2.6.1.16, known under a trivial name of glucosamine-6-phosphate (GlcN-6-P)$^1$ synthase, catalyzes the complex reaction involving ammonia transfer and sugar phosphate isomerization: L-glutamine + D-fructose 6-phosphate → D-glucosamine 6-phosphate + L-glutamate. This reaction is the first committed step of the cytoplasmic biosynthetic pathway leading to the formation of uridine 5’-diphospho-N-acetylg glucosamine (UDP-GlcNAc). The final product of this pathway is an activated precursor of numerous macromolecules containing amino sugars, including chitin and mannoproteins in fungi, peptidoglycan and lipopolysaccharides in bacteria, and glycoproteins in mammals. GlcN-6-P synthase belongs to the class II amidotransferase family but is unique among other amidotransferases due to its apparent inability to use exogenous ammonia as a nitrogen donor (1). The enzyme is widely distributed in nature, and its activity has been detected in almost every organism and tissue; several genes coding for GlcN-6-P synthase have been cloned and sequenced (for review see Ref. 1). However, only prokaryotic GlcN-6-P synthases have been purified to apparent homogeneity from Escherichia coli and from the thermophilic bacteria Thermus thermophilus (3). The E. coli enzyme has been crystallized (4), and a structure of the glutamine-binding domain has been elucidated (5). Availability of the pure protein has facilitated extensive studies on its structure and molecular mechanism of the enzymatic reaction (6–8).

On the other hand, there is very little known of the molecular structure of eukaryotic GlcN-6-P synthases, and all the studies performed so far have been done on partially purified preparations. Several lines of evidence indicate that the eukaryotic enzyme could be different from its prokaryotic counterpart. Comparison of the available gene sequences has revealed a relatively large region (about 200 base pairs) that is lacking in the prokaryotic proteins (9). Eukaryotic but not prokaryotic GlcN-6-P synthases are the subject of feedback inhibition by UDP-GlcNAc (10). Sensitivity to this inhibitor in Blastocladia emersonii (11) and probably in Aspergillus nidulans (12) enzymes is modulated by reversible phosphorylation/depolymerization mediated by protein kinase(s) and phosphatase(s).

Fungal GlcN-6-P synthase is a subject of interest as a potential target in antifungal chemotherapy (13). Rationally designed oligopeptides containing an inhibitor of this enzyme showed promising chemotherapeutic effect in the murine model of disseminated candidiasis (14). Moreover, the fungal enzyme is a probable point of regulation of chitin biosynthesis.
Characterization of C. albicans GlcN-6-P Synthase

In the opportunistically pathogenic fungus Candida albicans, activity of this enzyme increases 4–5-fold during yeast-to-mycelium (Y → M) morphological transformation (15), correlating with a similar change in a chitin content in the cell wall (16). This transformation is considered to be a virulence factor during pathogenesis of human tissues (17).

The GFA1 gene encoding C. albicans GlcN-6-P synthase has been recently cloned and sequenced (9). In the present communication we describe the results of our further studies concerning characterization of properties of the gene product, aimed especially at regulation of its activity during morphological transformation of C. albicans cells.

EXPERIMENTAL PROCEDURES

Yeast Strains and Culture Conditions

Saccharomyces cerevisiae BJ1991 (MATa pep4-3 phr1 ura3 leu2 trp1) was provided by I. Purvis (Glaxo Group Research, Greenwood, UK). C. albicans ATCC 10261 was a gift from M. Payton (GIMB, Geneva, Switzerland). C. albicans and yeast cells were grown in YPD medium (2% glucose, 2% bacterial peptone, 1% yeast extract) at 28 °C with shaking at 200 rpm.

Morphological Transformation

C. albicans cells grown overnight in YPD were harvested, washed with saline, and starved overnight in saline at 4 °C. Starved cells were used to inoculate either the YCB/BSA medium containing 1.17% yeast carbon base, 1% bacteriological peptone, and 0.2% bactepeptone serum albumin or the Lee’s medium (18). Yeast form cells grew efficiently in both media at pH 4.5, 28 °C, and Y → M transformation was performed at pH 6.5, 37 °C. Efficiency of the morphological transformation was assessed by cell counting in a Burker chamber.

Bacterial Strains and Plasmids

E. coli DH5α (Life Technologies, Inc.) was used for plasmid selection and amplification. Plasmid YEpGW42 (8.7 kb) carrying the S. cerevisiae GFA1 gene on a 3.5-kb SacI fragment inserted into YEp352 (19) was a gift from W. Tanner (Regensburg, Germany). YEpMA91 was a yeast shuttle vector carrying the LEU2 marker and the promoter and terminator from PKK1 separated by a BgIII site (20).

DNA Isolation and Manipulations

Standard procedures were used for the isolation and subcloning of plasmid DNA fragments (21). Methods for Southern and Northern analyses were the same as cited previously (9). PCR amplification was for 30 cycles (1 min at 94 °C, 2 min at 50 °C, and 3 min at 72 °C) followed by 8 min at 72 °C then cooling to 40 °C. The reaction mix used standard concentrations recommended by Perkin-Elmer. The primers were designed to incorporate BamHI sites at either end of the structural gene, while maintaining an optimum environment around the start codon (22): 5′-oligo-5′-GAG AAA AAT ggA Tcc TAT TAA Aaa ATG TGG TGT GG-3′; 3′-oligo-5′-CAG ACA gga TcC ATT TTC ATT ACT CAA CAG-3′. The start codon and stop anti-codon are underlined. Mismatches are in lowercase.

Yeast Transformation

S. cerevisiae deletion strains YRSu3-21 and YRSu3-31 were propagated in YPD containing t-glucosamine, 5 mg ml⁻¹. The cells were transformed by the lithium acetate method (23). Selection for transformants was for LEU¹ on YNB minimal agar plates. YRS-C65 and YRS-C53 transformants were propagated in defined YNB media containing 1% glucose, 0.65% YNB, and appropriate supplements at 50 μg/ml and then transferred to YPD medium.

Purification of the Enzyme

Preparation of Crude Extract—YRSC-65 cells (10 g wet weight) from the overnight culture on YPD were harvested by centrifugation (5,000 × g, 4 °C, 10 min) and washed with cold buffer A (25 mM potassium phosphate buffer, pH 6.8, 1 mM EDTA). Cell paste was mixed with 20 g of alumina and frozen. The mixture was carefully thawed and cells were disrupted by grinding in a mortar. Buffer B (25 mM potassium phosphate buffer, pH 6.8, 1 mM EDTA, 1 mM DTT) was added in small portions until the cell paste became sticky, and grinding was continued. Cell debris and alumina were spun down (10,000 × g, 4 °C, 5 min). Supernatant was saved, and the solid residue was extracted again with buffer B, followed by centrifugation. Both supernatants were combined and centrifuged (35,000 × g, 4 °C, 45 min). Precipitate was discarded, and supernatant was saved as a crude extract.

Protein Treatment—Solution containing 1% pepsinamine sulfate in buffer B was added to the crude extract (1 ml per 70 mg of protein present in the crude extract), stirred moderately at 4 °C for 1 h. The precipitated solid was harvested. Supernatant was discarded, and precipitate was washed with buffer B and then combined with 6 ml of buffer C (0.1 mM pyrophosphate buffer, pH 6.8, 1 mM EDTA, 1 mM DTT, 10 mM Fru-6-P). This suspension was stirred for 30 min at 4 °C and centrifuged (10,000 × g, 4 °C, 10 min). Precipitate was discarded, and supernatant was saved as a pyrophosphate extract.

FPLC on Mono Q—Pyrophosphate extract containing GlcN-6-P synthase activity was filtered through the 0.22-μm Millipore membrane filter, diluted 1:2 with buffer D (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT), and loaded on Mono Q HR 5/5 FPLC column equilibrated with buffer D. The column was washed with 5 ml of buffer D, and elution was performed with a linear 0–0.5 mM NaCl gradient in buffer D at 1.0 ml min⁻¹. Active fractions were pooled and concentrated by ultrafiltration with Centricon 10 device.

FPLC on Superdex 200—The concentrated active fraction from Mono Q was loaded on Superdex 200 HR 10/30 column equilibrated with buffer B containing 0.15 mM NaCl. Proteins were eluted with the same buffer at the flow rate of 0.5 ml min⁻¹. Active fractions were pooled.

Determination of GlcN-6-P Synthase Activity

Colorimetric Method—A standard incubation mixture consisted of 10 mM Fru-6-P, 10 mM L-glutamine, 1 mM EDTA, 1 mM DTT, 50 mM potassium phosphate buffer, pH 6.8, and appropriately diluted enzyme preparation and inhibitors when necessary. Final concentration of the pure GlcN-6-P synthase was 0.5–1.0 μM ml⁻¹. The reaction was started by adding the enzyme, incubated at 37 °C for 30 min, and terminated by heating at 100 °C for 1 min. The concentration of GlcN-6-P formed by the enzyme, determined by the modified Elson-Morgan procedure (24), increased linearly for at least 60 min. One unit of specific activity was defined as an amount of enzyme that catalyzed the formation of 1 μmol of GlcN-6-P min⁻¹ mg protein⁻¹.

Spectrophotometric Assay—L-Glutamate formed by GlcN-6-P synthase was determined by coupling with glutamate dehydrogenase, essentially as described by Badet et al. (2). This method was used to confirm the results obtained from kinetic experiments performed at low concentrations of the substrates.

Determination of GlcN-6-P Synthase Activity in Situ—C. albicans cells grown in Lee’s medium were harvested and suspended in 8.5 ml portions of 0.1 mM imidazole/HCl buffer, pH 7.0, containing 0.2 mM KCl and 1 mM MgCl₂, at cell density 1.2–1.6 × 10⁶ cells ml⁻¹. Aliquots (1.5 ml) composed of toluene/ethanol/Triton X-100, 5:20:2, were added to the cell suspensions, and the mixtures were vortexed for 5 min at room temperature. The cells were washed three times with 50 mM potassium phosphate buffer, pH 6.8, containing 1 mM EDTA and 1 mM DTT and suspended in the same buffer at 10⁶ cells ml⁻¹. L-Glutamine, 10 mM, and Fru-6-P, 10 mM, and inhibitors when necessary were added, and the suspensions were incubated for 30 min at 37 °C. Cells were removed by centrifugation, and GlcN-6-P concentration was assayed in the supernatant, as described above.

Inactivation of GlcN-6-P Synthase with Glutamine Analogues

Incubation mixtures containing 5 μg of GlcN-6-P synthase, 50 mM potassium phosphate buffer, pH 6.8, 1 mM EDTA, 15 mM Fru-6-P, and inactivators at various concentrations in a total volume of 1 ml were incubated at 25 °C. To follow an inactivation of the enzyme, 200-μl aliquots were withdrawn from the mixture and applied at the top of 1-ml columns packed with Sephadex G-25 (equilibrated previously with 50 mM potassium phosphate buffer, pH 6.8), and centrifuged (500 × g, 1 min, 4 °C). Under these conditions the unbound inhibitor was separated from the enzyme, and protein was recovered in clean test tubes. Appropriate effluent aliquots were used for the determination of the residual enzyme activity.

Chemical Modification of the Enzyme

GlcN-6-P synthase, 5 μg, was incubated with group-specific reagents under following conditions: (a) with NTGB, IAA, phenylmethylsulfonyl fluoride, and NAI in 50 mM potassium phosphate buffer, pH 6.8, containing 1 mM EDTA; (b) with DEP in 50 mM potassium phosphate buffer, pH 6.0, containing 1 mM EDTA; (c) with BD in 50 mM bicarbonate buffer, pH 8.5; (d) with CMC in 50 mM MES buffer, pH 6.0; (e) with

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Characterization of C. albicans GlcN-6-P Synthase

Inc.). The backbone of the deletion cassette was obtained by band purification of an 8.5-kb fragment after digestion with XhoI and BglII. A 1.1-kb HindIII fragment encoding a functional URA3 gene was obtained by digestion of pSPUR1, band-purified, and ligated into the polylinker of pGem7Zf(-) (3,000 base pairs, Promega). Transformants that gave white colonies on isopropyl β-D-thiogalactoside, 5-bromo-4-chloro-3-indolyl beta-D-galactopyranoside plates were isolated. The 1.1-kb fragment was then excised by digestion with XhoI and BamHI, thus generating URA3 fragment containing ends compatible with the deletion cassette backbone. Ligation yielded plasmid pRS where residues -31 to 971 of GFA1 had been replaced with the URA3 gene. Yeast strain BJ1991 was transformed with the mixture of the restriction fragments excised with SspI and URA3 colonies were selected. Four were picked and shown to require glucosamine for growth on YPD plates. YRSu3-21 and YRSu-31 were isogenic deletion strains except for the orientation of insertion of the URA3 gene. Northern analysis confirmed that these strains did not produce any mRNA hybridizing to the GFA1 gene (Fig. 1), and no GlcN-6-P synthase activity could be detected.

The C. albicans GFA1 gene (9) was amplified by PCR using primers that incorporated BamHI sites 5′ and 3′ to the coding sequence. The PCR 2.1-kb product was digested with BamHI, band-purified, and ligated into the BglII site of pMA91. Restriction mapping was used to determine that obtained plasmids YEpRSC-65 and YEpRSC-53 contained the GFA1 gene in the correct orientation relative to the PGK1 promoter. These plasmids were transformed into strains YRSu3-21 and YRSu3-31. They complemented the glucosamine auxotrophy, restored GlcN-6-P synthase activity, and a GFA1 transcript of the correct size was detected by Northern analysis (Fig. 1).

Several isolated YRC-65 and YRS-53 transformants when grown in YPD medium reproducibly produced C. albicans GlcN-6-P synthase constituting 5–7% of total cytoplasmic proteins, as revealed by densitometric SDS-PAGE analysis. 

Enzyme Purification and Kinetic Properties—The C. albicans GlcN-6-P synthase, overproduced by YRS-65 cells, was purified to at least 97% homogeneity with 52% yield using a four-step procedure involving protamine sulfate precipitation, ion-exchange chromatography, and gel filtration, as summarized in Table I. Two enzymes that could affect the kinetic measurements, namely phosphoglucose isomerase and glutaminase, were precipitated by protamine sulfate. Glucose 6-phosphate dehydrogenase activity was assayed according to the procedure of Stein (27) and glutaminase activity by the method of Holcenberg (28). Glucose 6-phosphate activity was assayed as described by Lowry and Passoneau (29). Phosphoglucose isomerase activity was assayed by the method of Steiner (26). Glutaminase activity was assayed by the method of C. albicans GlcN-6-P synthase constituting 5–7% of total cytoplasmic proteins, as revealed by densitometric SDS-PAGE analysis. 

FIG. 1. Northern analysis of GFA1 gene expression. Total mRNA isolated from deletion strains YRsus-21, YRsus-31 and transformants YRS-65, YRS-53 was subjected to quantitative Northern analysis. Samples were probed with the BamHI fragment (0.8 kb) of the YEpGW42 plasmid, containing the S. cerevisiae GFA1 gene.
Characterization of C. albicans GlcN-6-P Synthase

TABLE I

| Purification of C. albicans GlcN-6-P synthase from YRSC-65 cells |
|------------------|-----------------|----------|------------------|--|________|
|                   | Volume ml       | Total protein mg | Total activity units | Specific activity units mg⁻¹ | Recovery % | Purification factor | fold |
| Crude extract     | 10              | 175         | 40.8            | 0.327             | 100        | 1                   |      |
| Pyrophosphate     | 8               | 29          | 34.0            | 1.18              | 83         | 3.6                 |      |
| Mono Q            | 4               | 7.4         | 21.7            | 2.93              | 53         | 8.95                |      |
| Superdex 200      | 4               | 4.4         | 21.2            | 4.83              | 52         | 14.8                |      |

FIG. 2. Determination of the molecular weight of native GlcN-6-P by native PAGE run under variety of polyacrylamide concentrations. GlcN-6-P synthase and the following marker proteins: 1, bovine serum albumin (68 kDa); 2, alcohol dehydrogenase (150 kDa); 3, β-amylase (200 kDa); 4, xanthine oxidase (280 kDa); 5, apoferitin (443 kDa); urease (483 kDa) were applied to 4, 5, 6, 7, 5, and 9% polyacrylamide gels and Coomassie Brilliant Blue-stained. For each protein a graph of a logarithmic function of the relative mobility versus acrylamide concentration was constructed (inset shown the graph obtained for GlcN-6-P synthase). The slopes of the graphs were plotted against molecular masses. Position of GlcN-6-P synthase is indicated by an arrow.

Month. More purified preparations were stable at -20 °C for weeks when stored in 50% glycerol. The presence of 1 mM DTT and 10 mM Fru-6-P was essential for the enzyme stability. The pure enzyme exhibited pH optimum 6.8 ± 0.05 in Tris, BisTris, HEPES, Mops, and phosphate buffers. \( K_m \) for l-Gln was 1.56 mM, \( K_m \) for GlcN-6-P was 1.41 mM, and \( k_{cat} \) was 1150 min⁻¹. The \( K_m \) values were also determined for the enzyme present in crude extracts prepared from C. albicans ATCC 10281 Y and M cells. The former exhibited \( K_m \) for l-Gln was 1.52 mM and \( K_m \) for Fru-6-P was 1.45 mM, whereas the latter had the same \( K_m \) for Fru-6-P, but the \( K_m \) for l-Gln was 0.82 mM, and an inhibition of the enzyme activity by an excess of this substrate was observed (details not shown). Substrate inhibition was not detected for the pure enzyme and for the enzyme present in the crude extract from C. albicans Y cells.

Isoelectric Point—Pure GlcN-6-P synthase was chromatographed using a 6 to 4 pH gradient on MonoP HR 5/5 FPLC column. The enzyme was partially denatured during column development, but an activity profile (not shown) enabled an estimation of an isoelectric point of 4.6 ± 0.05.

Studies on the Enzyme Structure—Molecular weight of the C. albicans GlcN-6-P synthase subunit was determined by SDS-PAGE. From the plot of \( \lg(M_r) \) versus migration distance, \( M_r = 79,500 \), which obtained is in a good agreement with the value 79,482, deduced from the gene sequence. The molecular weight of the native protein was determined by gel filtration and native PAGE run under variety of acrylamide concentrations. The first method gave reproducibly \( M_r = 340,000 \) and the latter \( M_r = 330,000 \) (Fig. 2). In both methods only single bands were detected.

The native enzyme was treated with glutaraldehyde for 8 h. SDS treatment of such a preparation and subsequent separation of its components by SDS-PAGE led to the appearance of bands corresponding to \( M_r = 80,000, 178,000, \) and 325,000 (not shown). It may be assumed that the bands corresponded to the monomeric, dimeric, and tetrameric forms of the enzyme, respectively. No band corresponding to the possible trimeric form was found.

Pure native C. albicans GlcN-6-P synthase was incubated with several chemical reagents under conditions ensuring selective modification of particular amino acid residues. Samples drawn from the reaction mixtures at time intervals were subjected to enzyme gel filtration, and activity of GlcN-6-P synthase was determined in effluent aliquots. Incubation with Cys-directed NTCB and IAA, Arg-directed BD, His-directed DEP, Lys-directed PLP, Asp/Glu-directed CMC, and Tyr-directed NAI led to a time- and concentration-dependent irreversible modification of the enzyme, whereas Ser-directed phenylmethylsulfonyl fluoride had no effect. In each case the inactivation was complete at the appropriate reagent concentration. The pattern of plots of apparent inactivation velocity constants \( (k_{app}) \) versus inactivator concentration derived from these experiments were the straight lines originating at zero point (not shown), thus indicating single-step reactions. Kinetic analysis of the results afforded the apparent second-order rate constants \( k_1 \), summarized in Table II. These values were rather low, except that found for DEP. Reaction orders determined from the plots of \( \lg(k_{app}) = n \lg([I]) \) were found to be in the 0.87–1.18 range. The residues modified by the group-specific reagents were not unequivocally identified, but the protective effect of enzyme substrates was studied. Presence of l-Gln in incubation mixtures protected the enzyme against inactivation caused by NTGB and IAA, whereas Fru-6-P prevented inactivation caused by DEP, BD, PLP, and NAI. Fru-6-P afforded only partial protection against CMC.

Inhibition of the Enzyme Activity—The pure GlcN-6-P synthase was inhibited by a number of glutamine analogs, competitively in respect to l-Gln and uncompetitively in respect to Fru-6-P. Inhibitory constants for these compounds are summarized in Table III. \( N^3-(4\text{-Methoxyfumaroyl})-1,2,3\text{-diaminopropionic acid} \) and \( N^2-p\text{-trans}-2,3\text{-epoxy}

Molecular mass (kDa)
Characterization of C. albicans GlcN-6-P Synthase

TABLE II
Chemical modification of C. albicans GlcN-6-P synthase

| Reagent (residue modified) | Reaction order | Protective effect* |
|---------------------------|----------------|-------------------|
|                          |                | K<sub>i</sub> s<sup>-1</sup> | K<sub>Inact</sub> s<sup>-1</sup> |
| NTCB (Cys)               | 4.33           | 1.0                | +                  |
| IAA (Cys)                | 3.1            | 0.94               | +                  |
| PLP (Lys)                | 0.93           | 1.0                | NT<sup>a</sup>     |
| DEP (His)                | 512            | 0.97               | –                  |
| BD (Arg)                 | 1.48           | 1.18               | –                  |
| CMC (AspGlu)             | 0.18           | 0.87               | NT                 |
| NAI (Tyr)                | 0.42           | 0.92               | NT                 |
| PMSF (Ser)               | NI<sup>b</sup> | NT                 | NT                 |

*The protective effect was considered positive (+) when k<sub>app</sub>/k<sub>app</sub><sub>i</sub> < 0.1 and negative (−) when k<sub>app</sub>/k<sub>app</sub><sub>i</sub> > 0.9.

<sup>a</sup> NT, not tested.

<sup>b</sup> NI, no inactivation.

TABLE III
Inhibition and inactivation of GlcN-6-P synthase by glutamine analogs

For inhibition, residual activity of pure GlcN-6-P synthase was assayed as described under “Experimental Procedures” in the presence of different concentrations of glutamine analogs, except the L-Gln concentrations were variable. Data were analyzed using Lineweaver-Burk plots. Inhibitory constants K<sub>i</sub> were determined from the secondary plots of k<sub>app</sub> versus inhibitor concentration, derived from the Lineweaver-Burk plots. For inhibition, pure GlcN-6-P synthase was incubated with FMDP or EADP in the presence of 10 mM Fru-6-P. An excess of inactivating agent was removed by enforced gel filtration, and a residual enzyme activity was determined. Limiting inactivation rate constants k<sub>i</sub> and inactivation constants K<sub>Inact</sub> were determined from the plots of inactivation half-times t<sub>1/2</sub> versus reciprocal of inactivator concentration.

| Reaction | Inhibition | Inactivation |
|----------|------------|-------------|
|          | K<sub>i</sub> × 10<sup>s</sup> | K<sub>Inact</sub> × 10<sup>s</sup> | k<sub>i</sub> | k<sub>2</sub>/K<sub>Inact</sub> (s<sup>-1</sup>) |
| FMDP     | 0.7        | 2.1         | 0.233       | 1850          |
| EADP     | 14.3       | 83          | 0.577       | 115           |
| γ-Glutamyl hydroxamate | 160        | NI<sup>a</sup> | NI | NI |
| Methionine sulfoximine  | 1500       | NI<sup>a</sup> | NI | NI |

<sup>a</sup> NI, no inactivation.

sugar nucleotide. The data presented in Fig. 4 clearly show that among the compounds tested, only glucose 6-phosphate enhanced the enzyme sensitivity to UDP-GlcNAc. This effect was Glc-6-P concentration-dependent and a relatively high level of this sugar phosphate, >10 mM, was necessary to reach the full enzyme sensitivity to UDP-GlcNAc. All the tested sugar phosphates alone, at concentrations <20 mM, had no direct effect on GlcN-6-P synthase activity.

Inhibition of pure GlcN-6-P synthase by UDP-GlcNAc in the presence of Glc-6-P was non-competitive, both in respect to Fru-6-P (Fig. 5) and to L-Gln (not shown). The Hill coefficient and the inhibitory constant, determined from the log (v<sub>i</sub> − v<sub>i</sub>) plot (Fig. 5, inset), were n<sub>H</sub> = 0.91 and K<sub>i</sub> = 0.52 mM, respectively. The non-competitive mode of inhibition was not changed in the absence of Glc-6-P, but the n<sub>H</sub> value dropped to 0.54, whereas the K<sub>i</sub> increased to 3.7 mM.

Phosphorylation of the C. albicans GlcN-6-P Synthase in Vitro—The pure enzyme was tested as a possible substrate for commercially available cAMP-dependent protein kinase from beef heart. The GlcN-6-P synthase was subjected to the kinase action under conditions optimal for protein phosphorylation. GlcN-6-P synthase activity and sensitivity to UDP-GlcNAc were monitored. The 30-min incubation with protein kinase in the presence of ATP and cAMP resulted in a more than 100% increase of GlcN-6-P synthase-specific activity, whereas the sensitivity to the physiological feedback inhibitor was only slightly affected (Fig. 6). cAMP alone and a combination of cAMP and ATP had no effect on GlcN-6-P synthase, whereas the presence of the kinase inhibitor from rabbit muscle abolished the kinase effect, thus demonstrating that the observed enhancement of GlcN-6-P synthase activity is a consequence of protein phosphorylation. The enhancement of GlcN-6-P synthase activity upon the action of cAMP-dependent protein kinase was time-dependent and showed saturation kinetics with

FIG. 3. Inhibition of GlcN-6-P synthase by UDP-GlcNAc. Residual activity of pure and crude GlcN-6-P synthase was assayed as described under “Experimental Procedures.” Crude extract from YRSC-65 (●), crude extract from C. albicans ATCC 10261, Y forms (○), crude extract from C. albicans, M forms (▼), pure GlcN-6-P synthase (▲).
the maximum value observed after 90 min (Fig. 6, inset). Results of another experiment, shown in Fig. 7, in which GlcN-6-P synthase was phosphorylated with [γ-32P]ATP as a substrate, demonstrated correlation between GlcN-6-P synthase activity and an extent of its phosphorylation. The stoichiometry of the phosphorylation determined under optimal conditions was 1.21 ± 0.08 mol of P/mol of GlcN-6-P synthase.

Changes in the GlcN-6-P Synthase Properties during Y → M Transformation of C. albicans Cells—GlcN-6-P synthase activity and sensitivity to UDP-GlcNAc were measured in situ in C. albicans cells stimulated in defined media to Y → M transformation by temperature and pH shift. Data summarized in Table IV show about 5-fold increase in the enzyme activity measured in cells incubated in the Lee’s amino acid medium, pH 6.5, at 37 °C and this enhancement was correlated with the progress of morphological transition. On the other hand, the enzyme activity was practically constant when the cells were incubated in the Lee’s medium, pH 4.5, at 30 °C, i.e. under conditions ensuring the yeast-like morphology (detailed data not shown). Induction of Y → M transformation resulted also in an immediate desensitization of GlcN-6-P synthase to UDP-GlcNAc. Similar changes in the GlcN-6-P synthase activity and sensitivity to inhibition caused by UDP-GlcNAc were observed when morphological transformation was induced in YCB/BSA medium (not shown).

The Glc-6-P concentration in cytosol of C. albicans cells, measured after overnight starvation, fell to 0.85 ± 0.05 mol mg dry weight\(^{-1}\) from the value of 2.9 ± 0.15 nmol mg dry weight\(^{-1}\) found in Y cells harvested from the rich YPD medium. Incubation of the starved cells in Lee’s or YCB/BSA medium, pH 4.5, at 30 °C, i.e. under conditions ensuring the yeast morphology, caused an increase in Glc-6-P concentration to 3.2 ± 0.2 nmol mg dry weight\(^{-1}\) after 1 h, followed by constant, slow decrease. On the other hand, the maximal level of Glc-6-P detected in the cells induced to Y → M transformation in Lee’s or YCB/BSA, pH 4.5, at 37 °C, was as low as 1.3 ± 0.3 mg dry
The C. albicans GFA1 gene was overexpressed in S. cerevisiae, thus allowing the purification of its product, i.e. C. albicans GlcN-6-P synthase. It should be mentioned that the efficient purification of C. albicans gene of the yeast host was knocked out, thus allowing an minor component of the cells. It can be estimated that GlcN-6-P synthase is a very high, is comparable to that achieved previously for the E. coli GlcN-6-P synthase (34) and quite sufficient for quick and effective purification of the enzyme.

A relatively simple four-step procedure was elaborated which allowed isolation of the electrophoretically pure enzyme from recombinant cells. The overproduced C. albicans GlcN-6-P synthase could be almost quantitatively precipitated from crude extract with protamine sulfate and recovered with good yield by elution from the precipitate with a pyrophosphate buffer. This step allowed separation of the enzyme from bulk amount of accompanying proteins. The pyrophosphate extract, containing about 25% pure GlcN-6-P synthase but not phosphoglucone isomerase and glutaminase, could be used for most kinetic measurements, since the results were not different from those obtained for the pure protein. The Mono Q chromatography, albeit carried out as quickly as possible, always caused a substantial loss of the enzyme activity. However, a relative instability of GlcN-6-P synthases from other sources during an ion exchange chromatography was previously reported (35). The described method can be easily scaled up if a Mono Q column of higher capacity is used.

The properties of the pure C. albicans GlcN-6-P synthase could be compared with those reported for the pure bacterial enzyme or partially purified preparations of eukaryotic GlcN-6-P synthase. The Km for l-glutamine is 2–3 times higher than most of the values determined so far, but Km for Fru-6-P and the pH optima are similar to those reported previously. We...
could confirm a previous observation of Chiew et al. (15) on a substantial increase of the enzyme affinity to L-glutamine occurring during Y → M transformation of C. albicans cells. An isoelectric point of the enzyme is 0.3 pH units lower than that found previously for its bacterial counterpart (2) but similar to the value of 4.5 found for the rat hepatoma enzyme (36). The acidic character of the protein explains its high affinity to protamine.

The bacterial GlcN-6-P synthase was previously unequivocally found to be a dimer of 70-kDa subunits (2). Our results clearly demonstrate that the fungal enzyme is a homotetramer of 79.5-kDa subunits. Such a quaternary structure seems to be a general feature of eukaryotic GlcN-6-P synthase, since molecular masses ranging from 300 to 380 kDa were previously reported for partially purified enzymes from eukaryotic sources (10, 36–38).

Chemical modification studies provided preliminary information on amino acid residues essential for the enzyme activity. Two such residues, Cys-1, the only catalytic residue located at the glutamine-binding domain, and Lys-608, participating in sugar phosphate isomerization at the Fru-6-P-binding domain, were previously unequivocally identified in E. coli GlcN-6-P synthase (2, 39). Since the C. albicans enzyme was effectively protected by glutamine against inactivation caused by cysteine-directed reagents, NTCB and IAA, and by Fru-6-P against lysine-directed PLP and, on the other hand, respective Cys-1 and Lys-708 residues are present in the highly conserved regions of the fungal protein (9), there is little doubt that these residues were actually modified in our experiments and play the same role as in the bacterial enzyme. The C. albicans GlcN-6-P synthase was effectively inactivated by a histidine-directed reagent DEP under conditions preventing interaction with the reactive cysteine residue. A protective effect of Fru-6-P suggests a location of this essential residue at the Fru-6-P-binding domain, where it could probably act as a general acid-base catalyst in isomerization of fructose imine phosphate. The previous investigation of the bacterial enzyme, irreversibly modified by DEP, provided evidence for the presence of an essential histidine at the glutamine-binding domain (40). The authors were not able to identify this residue, due to the instability of the DEP-derivatized histidyl residues. However, the later x-ray studies of the glutamine-binding domain of E. coli GlcN-6-P synthase did not reveal any histidyl residue participating in substrate binding or catalysis at this domain (5), thus denying a previously formulated conception of Cys-His-Asp catalytic triad (41). In our opinion the “essential” histidyl residue modified upon the action of DEP on the bacterial enzyme could be a moiety situated next to any really essential site. The most likely candidate seems to be His-97, since Asn-98 and Gly-99 were reported to be involved in stabilization of a tetrahedral intermediate (5). The respective His-123 residue of the C. albicans enzyme, adjacent to Asn-124 and Gly-125, does not seem to be accessible for chemical modification, since we did not observe any protective effect of L-glutamine against chemical modification of this protein caused by DEP.

In contrast to the glutamine-binding domain, amino acids participating in catalysis and substrate binding at the Fru-6-P-binding domain are not known, except the lysyl residue mentioned above. Our present studies provided preliminary evidence suggesting the presence of essential histidyl, arginyl, and tyrosyl residues at this site. The essential arginyl residue could be involved in phosphate binding. Such a role was previously suggested for the essential arginyl residue present at the active site of phosphoglucone isomerase, an enzyme catalyzing a reaction closely resembling sugar phosphate isomerization taking place at the Fru-6-P-binding site of GlcN-6-P synthase (42). On the other hand, an essential tyrosyl residue should be most likely involved in formation of hydrogen bonds stabilizing transition state intermediates. A precise location of a putative essential Glu/Asp moiety is not clear. The identity and actual roles of putative essential histidyl, arginyl, Glu/Asp, and tyrosyl residues remain to be revealed, especially by site-directed mutagenesis. This work is in progress in our laboratory.

In our previous work we reported that C. albicans GlcN-6-P synthase, like its other eukaryotic counterparts, is inhibited by UDP-GlcNAc (9). However, the IC50 value is in the millimolar range, much higher than that found previously for the rat liver (10), Neurospora crassa (43), human liver (44), or jack bean (45) enzymes but is comparable to the yeast protein (19). The noncompetitive character of inhibition in respect to both substrates indicates that the UDP-GlcNAc-binding site is different from the active site. A similar situation was found previously for the N. crassa enzyme (43). However, it should be noted that in the case of other eukaryotic GlcN-6-P synthases, strikingly different modes of inhibition were reported, especially in respect to Fru-6-P. It was competitive for rat liver and plant enzymes (10, 45) or uncompetitive in A. nidulans protein (12). It is not clear if these results reflect the real differences in enzyme structure and properties or were a consequence of working with complex protein mixtures instead of the pure enzyme.

We found that the sensitivity of C. albicans GlcN-6-P synthase to UDP-GlcNAc was dependent on the presence of another specific effector, Glc-6-P. The previous reports suggesting that this compound could affect the properties of eukaryotic GlcN-6-P synthases were rather confusing (46). Having the pure fungal enzyme we were able to demonstrate unequivocally that Glc-6-P actually modulates the sensitivity of C. albicans GlcN-6-P synthase in a concentration-dependent manner. In the absence of Glc-6-P an enzyme affinity to UDP-GlcNAc is low, and the binding of this inhibitor is regulated by the strong negative cooperativity. This effect allows only partial, less than 50%, inhibition even at very high concentrations of UDP-GlcNAc. The presence of Glc-6-P enhances the enzyme affinity to the inhibitor and prevents the allosteric interactions.

Frisa and Sonnenborn (47) and Etchebehere et al. (48) provided evidence for the involvement of cAMP-dependent protein kinase and protein phosphatases 2A and 2C in reversible sensitization and desensitization of Blastocladiella emersonii GlcN-6-P synthase to inhibition caused by UDP-GlcNAc (47, 48). Our results show that phosphorylation and dephosphorylation are involved in the regulation of the C. albicans enzyme activity but not sensitivity to the feedback inhibitor. The latter seems to be entirely dependent on the intracellular Glc-6-P level. However, it is known that the biosynthesis of cAMP, being a part of one of the signal transduction systems, is often conjugated with changes in the intracellular concentration of Glc-6-P (49). Several authors showed that different signal transduction systems involving cAMP-dependent and Ca2+-dependent protein kinases operate during Y → M transformation (50, 51). A diffusable cAMP analog, dibutylryl cAMP, effectively triggers germ tube formation (52), whereas specific inhibitors of cAMP-dependent kinase inhibit germination under in vivo conditions (53). On the other hand, although several different environmental factors were reported to promote the morphological shift, it is believed that the low glucose level in the growth medium, neutral pH resulting in a slow glucose uptake and/ or serum proteins, protein amino acids or N-acetyl-D-glucosamine as the main carbon source, alternative to glucose, are the most important (54, 55). In this respect it is noteworthy that the cAMP cascade in C. albicans can be activated by glucagon (56), a human hormone produced by pancreas in response to low glucose level in the blood.
Characterization of C. albicans GlcN-6-P Synthase

It was previously demonstrated that the GFA1 mRNA level substantially increased at the beginning of yeast-to-mycelia shift but then fell down, despite further progress in morphological transformation (9). On the other hand, enhancement of GlcN-6-P synthase activity is correlated with extent of germination of C. albicans cells (Ref. 15 and this work), and immediate desensitization of the enzyme to UDP-GlcNAc is observed at the onset of Y → M transition (this work). These observations rather exclude a possibility of transcriptional regulation of GlcN-6-P synthase production in response to factors stimulating germination. Therefore, it seems possible to put forward a working hypothesis on the mechanism of post-transcriptional regulation of GlcN-6-P synthase activity in C. albicans. In Y cells containing less than 1% of chitin in their cell walls (16), grown in a rich medium, this enzyme remains in the semi-inhibited state, since the the intracellular concentration of its feedback inhibitor, UDP-GlcNAc, reported to be about 1 mM (57), exceeds the IC₅₀ value of the enzyme. Induction of germ tube formation lowers the intracellular level of GlcN-6-P and thus desensitizes the GlcN-6-P synthase to UDP-GlcNAc and, on the other hand, triggers cAMP formation. cAMP-activated protein kinase phosphorylates GlcN-6-P synthase thus enhancing its activity. The modified molecules of GlcN-6-P synthase can satisfy a requirement for the amino sugar, substantially enhanced in mycelial cells, containing 4–5% chitin in their walls (15). We are fully aware of the fact that the actual situation is probably much more complex, and our model may be valid only under specific conditions of germination used by us.

Further studies, especially aimed at identification of endogenous cAMP-dependent kinase involved in phosphorylation of GlcN-6-P synthase and its phosphorylation site, are necessary to confirm the above hypothesis and understand the complexity of regulation of this enzyme in C. albicans. A recent discovery, purification and characterization of the sole cAMP-dependent protein kinase from C. albicans (53), should obviously facilitate this study.

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Oligomeric Structure and Regulation of *Candida albicans* Glucosamine-6-phosphate Synthase

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