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PURIFICATION AND SOME PROPERTIES OF INDUCIBLE *N*-ACETYLGLUCOSAMINE KINASE FROM *CANDIDA ALBICANS*

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Summary

N-Acetylglucosamine kinase (ATP:2-acetamido-2-deoxy-D-glucose 6-phosphotransferase, EC 2.7.1.59) catalyzes the first reaction in the inducible *N*-acetylglucosamine catabolic pathway of *Candida albicans*, an obligatory aerobic yeast. As a part of continuing biochemical studies concerning the regulation of gene expression in a simple eukaryote, *N*-acetylglucosamine kinase has been purified and characterized biochemically. The enzyme has been purified about 300-fold from the crude extract and its molecular weight of 75 000 has been determined by Sephadex G-100 gel filtration. Isolation and analysis procedures are described.

The kinase reaction is optimal within a pH range of 7—8. The enzyme is strictly specific for GlcNAc as phosphate acceptor; ATP is the phosphoryl group donor for the kinase reaction and to a lesser extent dATP and CTP. K_m values for GlcNAc and ATP are 1.33 mM and 1.82 mM, respectively. The enzyme requires Mg^{2+}, which may be replaced by other bivalent metal ions such as Mn^{2+}, Ca^{2+}, Ba^{2+} and Co^{2+} for a lesser degree of effectiveness. The purified enzyme is extremely sensitive to thermal denaturation and becomes completely inactive by heating at 65°C for 2 min. The enzyme is also inactivated by sulphydryl reagents such as *p*-chloromercuribenzene sulfonic acid and *N*-ethylmaleimide.

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Abbreviations: GlcNAc, *N*-acetylglucosamine; ManNAc, *N*-acetylmannosamine.

Introduction

Recent studies in our laboratory indicate that only pathogenic yeasts (e.g. *Candida albicans*) are able to grow on GlcNAc medium and have the capacity to induce the enzyme involved in GlcNAc catabolic pathway [1—5]. We have been able to induce all these enzymes in *C. albicans* using a single inducer, GlcNAc. Furthermore, *N*-acetylglucosamine-3-epimerase, which is responsible for the interconversion of GlcNAc and ManNAc, is also induced by using either GlcNAc or ManNAc [6]. Our aim is to characterize this system thoroughly, both genetically and biochemically, since it appears to provide an exceptionally favourable opportunity to elucidate, at the molecular level, the mechanism of eukaryotic genetic regulation [7]. As a part of the programme to isolate mRNA specific to substrate inducible *N*-acetylglucosamine kinase (ATP:2-acetamido-2-deoxy-D-glucose 6-phosphotransferase; EC 2.7.1.59), we have purified this enzyme from *C. albicans* to raise antibodies against it, which in turn can be used to precipitate specifically the polysomes synthesizing nascent chains of the enzyme.

Materials and Methods

Materials. Agar, peptone and yeast extract were obtained from Difco. The following chemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.: *N*-acetylglucosamine, *N*-acetylgalactosamine, *N*-acetylmannosamine, glucosamine-HCl, UDP-*N*-acetylglucosamine, nucleotides, EDTA, Trizma base, 2-mercaptoethanol, NADH, *p*-chloromercuribenzene sulfonic acid, *p*-dimethylaminobezaldehyde, bovine serum albumin, pyruvate kinase and lactate dehydrogenase. Sephadex G-25 and G-100, molecular weight calibration kit containing ribonuclease A, chymotrypsinogen A, ovalbumin and Blue Dextran 2000 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. DEAE-cellulose (DE-52) was obtained from Whatman Inc., U.S.A. Calcium phosphate gel was prepared by the method of Keilin and Hartree [8]. All other reagents were of analytical grade.

Organism and growth conditions. Candida albicans 3100, a wild-type yeast strain obtained from the National Chemical Laboratory, Pune (India), was used throughout this study. The conditions for growth and enzyme induction have previously been described in detail [2].

Enzyme assays. N-Acetylglucosamine kinase was assayed either by the rate of disappearance of GlcNAc (Assay I) or formation of ADP (Assay II) according to the procedure of Datta [9]. The only modification was that the reaction mixture contained Tris-HCl (pH 7.6) instead of glycine-NaOH (pH 9.0). However, other components of the reaction mixtures were the same and the incubation was carried out at 30°C for 30 min unless otherwise stated. Assay I was used in the initial stages of enzyme purification (step 1—3) whereas Assay II was used in the final stages of enzyme purification (step 4—6).

Protein was estimated by the method of Lowry et al. [10] with bovine serum albumin as the standard.

Molecular weight determination. The molecular weight of *N*-acetylglucosamine kinase was determined by gel filtration on a Sephadex G-100 column

(2 × 73 cm) according to method of Determann and Michel [11]. The column was standardized with ribonuclease (13 700), chymotrypsinogen (25 000), ovalbumin (45 000) and bovine serum albumin (67 000).

Results

Purification of N-acetylglucosamine kinase

Unless otherwise indicated, all the operations were performed at 0—4° C and all potassium phosphate buffers contained 1 mM EDTA and 10 mM 2-mercaptoethanol.

Step 1. Crude extract. C. albicans cells at mid-log phase were harvested by centrifugation, washed twice with 15 mM KH_2PO_4 and resuspended in induction medium containing 15 mM KH_2PO_4 and 22 mM *N*-acetylglucosamine [2]. After 3 h, cells were collected by centrifugation, washed free of the sugar and stored frozen. The cells can be stored in the deepfreeze at —18° C for several days, before kinase extraction, without much loss of activity. Frozen cells (30 g) in 30 ml of potassium phosphate buffer (50 mM, pH 7.6) were ground for 10 min in a mortar and pestle with acid washed sand. After centrifugation at 3000 × *g* for 5 min, the supernatant fraction was collected and the residue was reextracted with the same buffer. The supernatants were then combined, centrifuged at 18 000 × *g* for 30 min and the supernatant (crude extract) collected.

Step 2. Ammonium sulphate precipitation. The crude extract (350 ml) was brought upto 30% saturation with solid $(NH_4)_2SO_4$. After standing for 15 min, the inactive residue was discarded by centrifugation at 16 000 × *g* for 20 min. The resulting supernatant was brought upto 65% saturation by further addition of solid $(NH_4)_2SO_4$ and the resulting precipitate was collected by centrifugation. The pellet was gently resuspended in 14 ml of buffer A (20 mM potassium phosphate, pH 7.6/1 mM EDTA/10 mM 2-mercaptoethanol) and passed through a Sephadex G-25 column (2.5 × 30 cm) previously equilibrated with buffer A to remove $(NH_4)_2SO_4$.

Step 3. DEAE-cellulose column chromatography. The pooled enzyme fractions from step 2 were applied to a DEAE-cellulose column (Whatman DE-52, 3.5 × 20 cm) previously equilibrated with buffer A. The column was washed with buffer A and then with buffer A containing 150 mM KCl until no absorption of the effluent at 280 nm could be registered. The enzyme was then eluted with 78 ml of buffer A containing 300 mM KCl.

Step 4. Sephadex G-100 column chromatography. The enzyme obtained from step 3 was concentrated to 10 ml in an Amicon ultrafiltration cell using Diaflo membrane PM-10 and was chromatographed on a Sephadex G-100 column (2 × 73 cm) previously equilibrated with buffer A. The flow rate of the column was 11.7 ml/h collecting 2-ml fractions. Before loading the sample, Blue Dextran was used for the determination of the void volume. The active enzyme fractions (44—62) were combined.

Step 5. Calcium phosphate gel adsorption. Calcium phosphate gel (38 ml; dry wt. 36 mg/ml) was sedimented by centrifugation. The sediment was resuspended in 38 ml of enzyme fraction from step 4 by gentle stirring with a glass rod. After standing for 10 min, the enzyme in the supernatant was

collected by centrifugation at 5000 × *g* for 5 min.

Step 6. DEAE-cellulose column chromatography. The enzyme solution obtained in step 5 was applied to a DEAE-cellulose column (1.5 × 15 cm) previously equilibrated with buffer A. The column was then washed with buffer A containing 100 mM KCl until no absorption of the effluent at 280 nm could be registered. The enzyme was then eluted with a 400 ml linear gradient of 0.1—0.4 M KCl in buffer A, at a flow rate of approx. 25 ml/h collecting 4-ml fractions. *N*-Acetylglucosamine kinase activity and protein content of the eluted fractions are shown in Fig. 1. The active enzyme fractions (54—76) were pooled, concentrated by $(NH_4)_2SO_4$ precipitation and stored at 0—2° C.

The procedure resulted in a purification of approx. 300-fold with an overall recovery of about 10%. A summary of the purification procedure is presented in Table I.

Properties of N-acetylglucosamine kinase

Molecular weight. The molecular weight of the enzyme was determined by chromatography on a Sephadex G-100 column with proteins of known molecular weight. From the observed K_{av} of *N*-acetylglucosamine kinase, the molecular weight of the enzyme was estimated to be 75 000.

Stability. The enzyme stored at 0—2° C in buffer A was stable for at least a week. The enzyme was unstable to freezing and thawing and labile to heat and low pH. Almost 100% activity was lost on heating at 65° C for 2 min. The enzyme was stable to dialysis.

Substrate specificity. The enzyme displayed a high degree of specificity for GlcNAc, and did not act on a variety of other sugars including *N*-acetylmannosamine, *N*-acetylgalactosamine, glucosamine, UDP-*N*-acetylglucosamine, glucose and fructose. The phosphoryl donor ATP could not be replaced by UTP, GTP, TTP, dGTP or dCTP. Only CTP and dATP had some donating capacity (22 and 71% of ATP, respectively). This is in contrast to the kinase from hog spleen where GTP had some (30% of ATP) donating capacity [12].

The kinase required Mg^{2+} for activity. Other bivalent metal ions, for instance Mn^{2+} and Ca^{2+}, could replace the Mg^{2+} requirement effectively. Some other

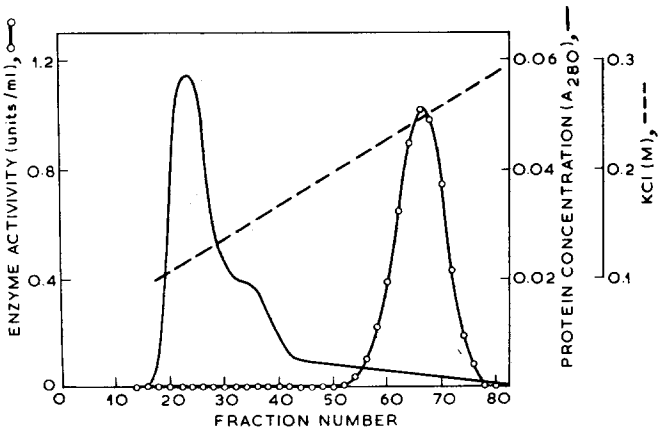

Fig. 1. DEAE-cellulose chromatography of *N*-acetylglucosamine kinase.

TABLE I

PURIFICATION OF *N*-ACETYLGLUCOSAMINE KINASE FROM *CANDIDA ALBICANS*

Enzyme activity in step 1—3 was measured by Assay I, whereas Assay II was used for step 4—6. A unit of enzyme activity was defined as the amount of enzyme producing 1 μmol of GlcNAc-6-*P* per 30 min under the standard assay conditions.

Purification step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification	
					Factor (-fold)	Yield %
1. Crude extract	350	655	610	0.93	1.0	100
2. Ammonium sulphate (30—65%) and Sephadex G-25	43	126	474	3.76	4.0	77.7
3. DEAE-cellulose	78	30	315	10.50	11.3	51.6
4. Sephadex G-100	38.5	14	251	17.93	19.3	41.1
5. Calcium phosphate gel	38.5	6.5	240	36.92	39.7	39.3
6. DEAE-cellulose	91.0	0.21	60	285.71	307.2	9.8

bivalent cations, e.g. Ba^{2+} and Co^{2+}, were also partly effective (Table II), while monovalent cations, e.g. Na^{+} and K^{+}, and bivalent cations like Fe^{2+} were completely ineffective.

Effect of pH, enzyme concentration and incubation time. The reaction rate was proportional to enzyme concentration and was linear with respect to time upto 40 min of incubation. The optimum pH was found to be 7.6.

Effect of substrate concentration. The K_m values, obtained by Lineweaver-Burk plots were 1.33 mM for GlcNAc measured with a fixed concentration of ATP (4 mM) and 1.82 mM for ATP when the GlcNAc concentration (4 mM) in the incubation mixture was fixed.

Effect of sulphydryl reagents on the activity of N-acetylglucosamine kinase. Sulphydryl reagents such as *N*-ethylmaleimide and *p*-chloromercuribenzene sulfonic acid markedly inhibited the enzyme activity. The kinase was pre-incubated at 30° C for 3 min with either *p*-chloromercuribenzene sulfonic acid or *N*-ethylmaleimide and the treated fractions were immediately diluted with

TABLE II

THE ACTIVITY OF *N*-ACETYLGLYCOSAMINE KINASE WITH VARIOUS IONS

The step 5 protein (15 μg) in the typical reaction mixture was incubated with 50 mM of the given ions at 30°C for 30 min.

Ion	*N*-Acetylglucosamine utilized (μmol)	Relative activity
Mg^{2+}	0.55	100
Mn^{2+}	0.47	86
Ca^{2+}	0.46	84
Ba^{2+}	0.29	53
Co^{2+}	0.28	51
Cd^{2+}	0.14	26
Sn^{2+}	0.10	18
Cu^{2+}	0.08	15
Ni^{2+}	0.07	13

the assay medium. For a protein concentration of 15 μg/ml, both the reagents at a concentration of 0.4 mM inactivated the kinase by about 60—65%.

Products of the reaction. A typical incubation mixture which contained 10 μmol each of GlcNAc, ATP and 25 μmol Mg^{2+} was incubated with *N*-acetylglucosamine kinase (step 6, 20 μg protein) in 1 ml of 0.05 M Tris-HCl (pH 7.6). After 30 min the reaction was terminated by the addition of 1 ml of 0.1 M acetic acid and the products were analysed as described previously for hog spleen kinase by Datta [12]. The position of the phosphate group of the product was established by periodiate oxidation in acetate buffer by the method of Jeanloz and Forchielli [13]. The identification of glycolaldehyde phosphate 14 as a periodate oxidation product confirmed the position of the phosphate ester at C-6 for GlcNAc. Stoichiometry of the reaction was done and results show that the amount, of GlcNAc-6-*P* formed was equal to the amount of ADP in the reaction. Furthermore, the amount of GlcNAc which disappeared during the reaction could be fully accounted for by the appearance of an equivalent amount of GlcNAc-6-*P*.

Discussion

N-Acetylglucosamine kinase was first reported in liver by Leloir and Cardini [15]. Later the enzyme was purified from *Escherichia coli* [16], *Streptococcus pyrogens* [17], hog spleen [12] and human gastric mucosa [18]. Here, we report the purification of this enzyme from yeast (*Candida albicans*), a simple eukaryote. There are a number of differences in the regulatory aspects and properties of this enzyme from various sources. For instance the enzyme is constitutively present in all the systems reported except yeast. In *E. coli* and *S. pyrogens*, the enzyme level remains unchanged when the glucose grown cells were transferred to GlcNAc medium. Furthermore, *S. pyrogens* enzyme, even after 1000-fold purification phosphorylates both GlcNAc and glucose at equal rates [17]. *E. coli* enzyme also phosphorylates glucose but its affinity for GlcNAc is 100-times more than that for glucose [16]. GlcNAc kinase isolated from hog spleen is very specific for GlcNAc [12] but the enzyme from human gastric mucosa can phosphorylate *N*-acetylmannosamine, though to a much lesser extent (15%) as compared to GlcNAc [18]. Like hog spleen kinase, the yeast enzyme as reported here is also highly specific for GlcNAc as phosphate acceptor. Other sugars e.g. glucose, fructose, galactose, *N*-acetylmannosamine and *N*-acetylgalactosamine are ineffective. Not only do the differences exist in sugar specificity among the enzymes isolated from various sources, there are significant differences in K_m values for GlcNAc. Similarly, the enzymes also do not share any of the following properties; pH optima, nucleotide specificity and stability. However, the molecular weight of this enzyme determined only in case of human gastric mucosa (77 000) is comparable to the yeast enzyme (75 000) reported in this paper.

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