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Partial Purification and Properties of the Pyruvate-Uridine Diphospho-N-Acetyl-glucosamine Transferase from *Staphylococcus epidermidis*

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A method for the partial purification of the pyruvate-uridine-diphospho-N-acetylglucosamine transferase from *Staphylococcus epidermidis* is presented. Some properties of the enzyme are discussed.

Detection of the enzymatic transfer of pyruvate to uridine diphospho-N-acetylglucosamine (UDP-GlcNAc) from phosphoenolpyruvate (PEP) was first reported by Strominger (6) in cell-free extracts of *Staphylococcus aureus*.

UDP-GlcNAc + PEP →
UDP-GlcNAc-enolpyruvate + inorganic orthophosphate

More recently, the phosphoenolpyruvate: uridine-5′-diphospho-N-acetyl-2-amino-2-deoxyglucose-3-enolpyruvyltraasferase (pyruvate-UDP-GlcNAc transferase) from *Enterobacter cloacae* has been purified and studied by Gunetileke and Anwar (2, 3). This report describes the partial purification and some properties of the *S. epidermidis* pyruvate-UDP-GlcNAc transferase.

MATERIALS AND METHODS

S. epidermidis strain Texas 26 was cultured in a growth medium containing, per liter: glucose, 4.0 g (sterilized and added separately); yeast extract (Difco), 10.0 g; Bactopeptone (Difco), 5.0 g; ammonium sulfate, 4.6 g; K_2HPO_4, 2.0 g; and Dow Corning silicone Antifoam A spray, 0.05 ml. The cells were grown at 37 C; the generation time was approximately 30 min. The cells were harvested at 60% of the maximal optical density and yielded approximately 5 to 6 g (wet weight) of cells per liter. Cells harvested at later times or during the stationary phase had much lower enzymatic activity.

Preparation of crude enzyme. A paste of 12.0 g (wet weight) of washed cells was suspended in 24 ml of 0.075 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 7.7) containing 4 mM dithiothreitol (DTT) and 0.1 mM ethylenediaminetetraacetic acid (EDTA). Then 20 ml of acid-washed glass beads (120 μm, 3M Co.) was added with 0.05 ml of tri-n-butylcitrate antifoaming agent. The suspension was ground for 30 min at top speed in a Gifford-Wood Mini-Mill at a gap setting of 9.0. The ground suspension was centrifuged at 0 C in a Sorvall refrigerated centrifuge for 10 min at 27,000 × g. The supernatant solution (about 25 ml was removed and centrifuged at 100,000 × g for 1 hr in an International B-60 ultracentrifuge. The supernatant solution from this centrifugation was used as crude enzyme and was immediately frozen and stored in liquid nitrogen.

Assay for pyruvate-UDPGlcNAc transferase. The preparation of ^{14}C-PEP used in the following assay has already been described (7). A typical incubation mixture consisted of 5 μliters of 0.1 M KF (fluoride ion was added to inhibit the enolase reaction and prevent the conversion of ^{14}C-PEP to ^{14}C-2-phosphoglyceric acid), 12.5 μliters of 0.1 M Tris buffer (pH 7.2) 1 μliter of 0.07 M PEP, 2 μliters of 0.035 M UDP-GlcNAc, 2 μliters of ^{14}C-PEP (30 mCi/mmole, 40,000 counts/min), 2 μliters of 0.01 M DTT, and enzyme sample (e.g., 30 μg of crude enzyme or 0.3 μg of enzyme at step 4) and deionized distilled water to a final volume of 40 μliters. The assay was carried out at 37 C for 10 min. The reaction was stopped by the addition of 0.2 ml of 10% trichloroacetic acid. When the assay was performed on crude preparations, protein precipitated by the addition of trichloroacetic acid was removed by centrifugation. Then 0.6 ml of a 5% suspension of acid-washed activated charcoal (Darco KB, Atlas Chemical Industries) was added to the reaction supernatant solution. After standing at room temperature for 20 min with occasional agitation, the charcoal was washed three times with 0.8 ml of 0.1 M ammonium acetate. The adsorbed nucleotides were then eluted with 0.6 ml of a 50% ethanol-water solution containing 0.05 M ammonium hydroxide. After gentle agitation for 20 min

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in the eluting solution, the charcoal was removed by centrifugation, and a sample of the aqueous supernatant solution was counted in a Packard Tri-Carb liquid scintillation spectrometer after solubilization with 0.5 ml of Beckman Biosolv.

A single charcoal-adsorbable radioactive product whose formation was dependent on UDP-GlcNAc and PEP was produced in this assay. Its mobility on paper electrophoresis at *p*H 3.9 and on paper chromatography in isobutyric acid-1.0 N ammonia (5:3) matched that of UDP-GlcNAc-enolpyruvate previously prepared in this laboratory (6). The product of this reaction was also used as substrate for the production of uridine diphospho-*N*-acetylmuramic acid (UDP-MurNAc) as previously described (7).

The production of ^{14}C-UDP-GlcNAc-enolpyruvate was linear with time (Fig. 1A) and was proportional to added enzyme (Fig. 1B) within the limits used in this assay.

Protein determination. Protein was determined by the method of Lowry et al. (5). Since DTT which was present during the entire procedure interfered with the assay, the protein in each sample was precipitated with 5% trichloroacetic acid. After centrifugation, the supernatant solution was removed, and the precipitated protein was dissolved in 0.1 ml of 1.0 N NaOH. The standard procedure was then followed.

RESULTS

Partial purification and some properties of the pyruvate-UDP-GlcNAc transferase. The following purification procedure was followed; all operations were carried out at 0 to 4 C as rapidly as possible. Crude enzyme (protein content, approximately 18 to 22 mg/ml; 4.0 ml) was added to 2.0 ml of a 1.5% protamine sulfate (Mann Chemical Corp.) solution, *p*H 7.2, containing 4 mM DTT. The precipitate was removed by centrifugation at 0 C, and 0.42 g (dry weight) of calcium phosphate gel in 2.0 ml of water was added to the protamine sulfate supernatant solution. After being mixed well and allowed to stand for 1 hr to 0 C, the gel was removed from the solution by centrifugation. The gel was washed thoroughly three times with 1.5 ml of a solution of 0.01 M Tris buffer, *p*H 7.7, containing 0.01 M sodium pyrophosphate and 4 mM DTT. The enzyme was then eluted by washing three times with 1.50 ml of a solution of 0.04 M Tris-hydrochloride buffer, *p*H 7.7, containing 0.05 M sodium pyrophosphate and 4 mM DTT. The last three fractions were stored in liquid nitrogen.

The third fraction eluted from the calcium phosphate gel, containing the most enzyme of the three fractions, was placed on a Sephadex G-100 column, 2.54 by 27.0 cm, (Fig. 2) which was equilibrated just prior to use with a solution of 0.05 M Tris buffer, *p*H 7.2, containing 0.1 mM EDTA and 4 mM DTT. Since a significant amount of enzyme inactivation occurred at 4 C, the Sephadex G-100 column was precalibrated with a partially purified enzyme fraction so appropriate fractions could be picked and immediately stored in liquid nitrogen before any extensive loss in activity occurred. Small samples of column fractions also stored in liquid nitrogen were thawed and assayed for activity before proceeding with later steps or before use. The results of a typical purification are shown in Table 1.

Attempts at concentration, such as ultrafiltration (Amicon Diaflo, UM-2 membrane), lyophilization, or dialysis, resulted in large losses of activity. Diethylaminoethyl cellulose chromatography resulted in very little apparent purification owing to substantial losses in total activity.

Although no measurable loss in activity in partially purified fractions containing 4 mM DTT occurred during storage in liquid nitrogen for periods of up to 6 weeks, storage at other temperatures, including −70 C, resulted in appreciable losses in activity in less than 2 weeks (Table 2). Crude enzyme preparations, however, were quite stable at −15 C when

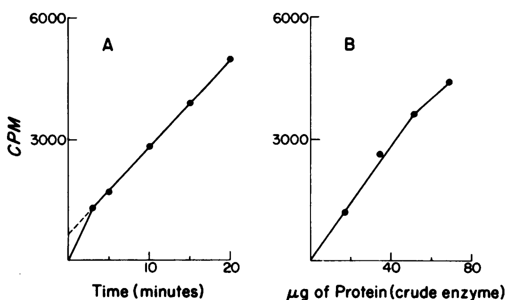

FIG. 1. *Formation of UDP-GlcNAc-enolpyruvate as a function of time (A) and the amount of added enzyme (B). Crude enzyme (36 μg) was used in each assay in part A. The samples were incubated for 10 min in part B.*

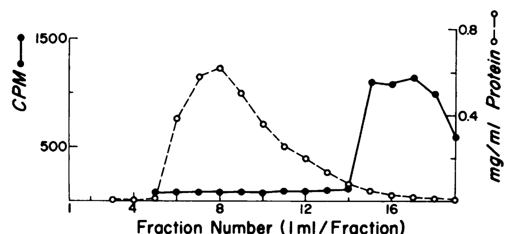

FIG. 2. *Chromatography of the pyruvate-UDP-GlcNAc transferase on Sephadex G-100 (V_0 = 6.5 ml for blue dextran; V_t = 19.0 ml for dinitrophenylglutamic acid.*

TABLE 1. *Purification of pyruvate-UDP-GlcNAc transferase*

Step	Vol (ml)	Total activity (counts/min)	Total protein (μg)	Specific activity[a]	Fold purification
100,000 × g supernatant solution	4.00	130,000	145,000	0.9	1.0
Protamine sulfate supernatant solution	6.00	110,000	142,000	0.8	0.9
Calcium phosphate gel (third eluate)	1.50	12,000	5,100	2.4	2.7
Sephadex G-100 column (peak tubes)	12.00	13,000	200	65.0[b]	72.0

[a] Ratio, total activity to total protein.
[b] This value corresponds to an absolute activity of about 0.2 μmole per min per mg.

TABLE 2. *Stability of pyruvate-UDP-GlcNAc transferase stored at various temperatures*[a]

Storage temp (C)	UDP-GlcNAc-pyruvate formed (counts/min)
−15	197
−70	973
−196 (liquid nitrogen)	1,530
Fresh control	1,250

[a] Partially purified enzyme samples were stored for 14 days in the presence of 4 mM DTT and 0.1 mM EDTA at the temperature specified. Assays were carried out as described.

TABLE 3. *Activation of pyruvate-UDP-GlcNAc transferase with DTT*[a]

System	UDP-GlcNAc-pyruvate formed (counts/min)
Fresh control	2,300
Stored control (−15 C, 24 hr)	1,530
+EDTA (1 mM)[b]	1,790
+DTT (2 mM)[b]	2,700
+DTE[c] (2 mM)[b]	2,650

[a] Assays were performed as described with the additions noted.
[b] Stored (−15 C, 24 hr) crude enzyme samples were preincubated with the specified reagent for 1 hr at 0 C before being assayed.
[c] Dithioerythritol.

TABLE 4. *Sensitivity of pyruvate-UDP-GlcNAc transferase to thiol reagents*[a]

Additions	UDP-GlcNAc-pyruvate formed (counts/min)
None	2,280
N-ethylmaleimide (0.20 M)	0
DTNB[b] (0.01 M)	0

[a] Assays were performed as described with the additions noted.
[b] DTNB, 5,5′-dithiobis (2-nitrobenzoic acid).

TABLE 5. *Optimal pH for pyruvate-UDP-GlcNAc transferase*[a]

System	UDP-GlcNAc-pyruvate formed (counts/min)
Maleate	
pH 6.5	1,560
pH 6.8	1,450
Tris-hydrochloride	
pH 7.0	2,090
pH 7.4	2,310
pH 7.7	2,060
pH 8.0	1,700
pH 8.4	1,540

[a] Assays were performed as described in the specified buffer.

stored in the presence of 4 mM DTT. The enzyme activity, even in the presence of DTT, became quite temperature-sensitive after the first purification steps.

The enzyme activity was also very susceptible to impurities found in distilled water. Although this inhibition could finally be overcome by the addition of 0.01 M EDTA, all operations were performed in deionized distilled water, which produced no inhibitory effects.

The enzymatic activity which could be maintained only in the presence of DTT (Table 3) was sensitive to reagents that react with thiol groups (Table 4).

The pH optimum of the activity was quite broad (Table 5) and appeared to have a maximum somewhere between 7.0 and 7.7.

The elution profile of enzymatic activity from Sephadex G-100 suggested that the enzyme was adsorbed to Sephadex, as it eluted in the salt region.

The enzyme was not at all inhibited by UDP-MurNac-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala (1 mM) or by UDP-MurNAc-L-Ala-D-Glu (3 mM). However, it was inhibited 40% by 1 mM UDP-MurNAc and 70% by 3 mM UDP-MurNAc. UDP-MurNAc is closely related in structure to the product of the reaction, UDP-

GlcNAc-pyruvate enol ether, and thus inhibition by UDP-MurNAc may represent an example of inhibition by a product analogue.

DISCUSSION

The pyruvate-UDP-GlcNAc transferase has been purified 70-fold from a crude extract prepared from *S. epidermidis*. The specific activity of this partially purified preparation (12 μmoles of UDP-GlcNAc-enolpyruvate formed per hr per mg of protein) was similar to the specific activity (16 μmoles of UDP-GlcNAc-enolpyruvate formed per hr per mg of protein) reported for the pyruvate-UDP-GlcNAc transferase purified 326-fold from *E. cloacae* (3). The specific activity of the transferase in the crude extract from *S. epidermidis* was four times higher than that in the crude preparation from *E. cloacae* and provided a richer source of enzyme.

Both the transferase activity reported here and that reported by Gunetileke and Anwar (3) were purified in the presence of DTT to maintain activity. Both enzymes were sensitive to *N*-ethylmaleimide and other reagents which react with thiol groups. Neither preparation was inhibited by the presence of 0.01 M EDTA. These experiments suggest that the pyruvate-UDP-GlcNAc transferase from *S. epidermidis* is similar to the *E. cloacae* transferase in that it requires a thiol group for activity but does not require added divalent metal ion. Phosphonomycin, (-)(1R,2S)-1,2-epoxypropylphosphonic acid (1), a natural antibiotic produced by *Streptomyces* species, irreversibly inhibits the pyruvate-UDP-GlcNAc transferase from several gram-positive and gram-negative microorganisms (4). The covalent attachment site appears to be a thiol group (F. M. Kahan, *personal communication*).

Pyruvate-UDP-GlcNAc transferase catalyzes the first irreversible step in the pathway which leads to synthesis of UDP-MurNAc-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala, the last soluble end product of the sequence leading to synthesis of peptidoglycan. It is of some interest that this enzyme is not end product-inhibited, as indeed might have been predicted from the fact that UDP-MurNAc-pentapeptide accumulates in staphylococci inhibited by penicillins and several other antibiotics. This accumulation has also been observed in the strain of *S. epidermidis* used in the present study (*unpublished data*). Accumulation could not have occurred if the enzyme were susceptible to end product inhibition.

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