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# Uridine Diphosphate D-Glucose Dehydrogenase of *Aerobacter aerogenes*

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Uridine diphosphate D-glucose dehydrogenase (EC 1.1.1.22) from *Aerobacter aerogenes* has been partially purified and its properties have been investigated. The molecular weight of the enzyme is between 70,000 and 100,000. Uridine diphosphate D-glucose is a substrate; the diphosphoglucose derivatives of adenosine, cytidine, guanosine, and thymidine are not substrates. Nicotinamide adenine dinucleotide (NAD), but not nicotinamide adenine dinucleotide phosphate, is active as hydrogen acceptor. The *p*H optimum is between 9.4 and 9.7; the $K_m$ is 0.6 mM for uridine diphosphate D-glucose and 0.06 mM for NAD. Inhibition of the enzyme by uridine diphosphate D-xylose is noncooperative and of mixed type; the $K_i$ is 0.08 mM. Thus, uridine diphosphate D-glucose dehydrogenase from *A. aerogenes* differs from the enzyme from mammalian liver, higher plants, and *Cryptococcus laurentii*, in which uridine diphosphate D-xylose functions as a cooperative, allosteric feedback inhibitor.

In several different organisms and tissues, uridine 5'-(α-D-glucopyranosyluronic acid pyrophosphate) (UDPGA) is formed by the nicotinamide adenine dinucleotide (NAD)-linked dehydrogenation of uridine 5'-(α-D-glucopyranosyl pyrophosphate) (UDPG) (4). The UDPG dehydrogenase of calf liver, which has been investigated more thoroughly than the enzyme from other sources, recently has been prepared in a state of high purity (23). Although UDPG dehydrogenase activity has been demonstrated in bacteria (11, 18), there has been no report of purification of the enzyme from these organisms.

UDPG dehydrogenase from pea seedlings and bovine liver (14) as well as from *Cryptococcus laurentii* (1) is potently and specifically inhibited by uridine 5'-(α-D-xylopyranosyl pyrophosphate) (UDPXyl). Since kinetic studies demonstrated that the inhibition is allosteric, Neufeld and Hall (14) suggested that UDPXyl serves as a feedback inhibitor of UDPG dehydrogenase and thus regulates the synthesis of UDPGA in certain tissues. It would be of interest to study the properties of UDPG dehydrogenase obtained from a source which does not contain glycosidically bound D-xylose and in which UDPXyl would not be expected to act as a specific allosteric inhibitor. In this paper, we describe the partial purification of, some properties of, and kinetic studies of such a dehydrogenase from *Aerobacter aerogenes*.

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## MATERIALS AND METHODS

*Materials.* Enzyme grade $(NH_4)_2SO_4$ was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio; UDPG, NAD, nicotinamide adenine dinucleotide phosphate (NADP), nitro blue tetrazolium, and phenazine methosulfate were purchased from Sigma Chemical Co., St. Louis, Mo.; calcium phosphate gel, containing 0.22 g of $Ca_3(PO_4)_2$ (dry weight) per ml of suspension in 1 mM phosphate buffer (*p*H 6.8), UDPXyl, adenosine 5'-(α-D-glucopyranosyl pyrophosphate (ADPG), thymide 5'-(α-D-glucopyranosyl pyrophosphate) (TDPG), cytidine 5'-(α-D-glucopyranosyl pyrophosphate) CDPG, and guanosine 5'-(α-D-mannopyranosyl pyrophosphate) (GDPM) were purchased from Calbiochem, Los Angeles, Calif. UDPGA labeled with $^{14}C$ in the D-glucuronosyl moiety was prepared as described previously (5). *A. aerogenes* A3(S1) was obtained from the American Type Culture Collection (ATCC 12658). Pure, homogeneous calf liver UDPG dehydrogenase (specific activity, 2.4 μmoles of UDPG oxidized per min per mg of protein at 30 C) was prepared in our laboratory (J. Zalitis and D. S. Feingold, *unpublished data*).

*Methods.* Chromatography of nucleotide sugars was performed on Whatman no. 1 paper with 95% ethyl alcohol−1 M ammonium acetate, *p*H 7.5 (7:3, v/v) (15). Paper electrophoresis was done on oxalic acid-washed sheets of Whatman 3 MM or no. 1 filter paper at *p*H 5.8 (5), with the solvent-cooled high voltage Electrophorator (Gilson Medical Electronics, Middleton, Wis.). Electrophoresis of proteins was performed on cellulose polyacetate strips (Sepraphore III, Gelman Instrument Co., Ann Arbor, Mich.) in a Gelman electrophoresis chamber

at 4 C at 1.5 ma per strip (2.7 cm) for 2 hr in 0.05 M tris(hydroxymethyl)aminomethane acetate (*p*H 7.5), 2 mM in ethylenediaminetetraacetic acid (EDTA). UDPG dehydrogenase activity was located on the strips by the phenazine methosulfate-nitro blue tetrazolium staining technique (3). The strips were incubated for 20 min at 37 C (in a moist chamber in the dark) after they had been covered with a thin layer of the following staining solution: 0.8 ml of 0.5 M glycylglycine buffer (*p*H 8.3), 1.0 ml of 0.2% nitro blue tetrazolium in the same buffer, 0.1 ml of 0.2% phenazine methosulfate, 0.05 ml of 50 mM NAD, and 0.05 ml of 0.05 M UDPG. When the activity of the sample was high, the blue bands could be seen almost immediately.

Protein was estimated by the method of Lowry et al. (10) with crystalline serum albumin as a standard. UDPG dehydrogenase was assayed essentially as described by Strominger et al. (19). Assay mixtures contained 2 μmoles of UDPG, 1.25 μmoles of NAD, 0.1 mmole of glycylglycine buffer (*p*H 8.4), and enzyme in a total volume of 1 ml. The increase in absorbancy at 340 nm was measured with the Gilford model 2000 automatic spectrophotometer equipped with a thermospacer to keep the cuvette temperature of the sample at 30 C. A unit of enzyme activity is defined as the amount of enzyme required to produce an increase in absorbancy per minute of 0.001 at 340 nm with a 1-cm light path in 1 ml of reaction mixture at 30 C (6,220 units of enzyme are equivalent to the amount of enzyme required to reduce 1 μmole of NAD per min).

*Separation of proteins by density gradient centrifugation.* A linear gradient of sucrose (3 to 36%) in 0.01 M phosphate buffer (*p*H 7.0), 1 mM in both EDTA and dithiothreitol (DTT), was prepared with the gradient mixing device of Martin and Ames (12). The tubes were centrifuged at 4 C for 16 hr at 114,743 × *g* in the SW-39 rotor of a Spinco model L2 ultracentrifuge; the protein solution was applied to the top of the gradient with the aid of band-forming caps (7). After centrifugation, 0.25-ml fractions were collected with an ISCO (Instrument Specialties Co., Lincoln, Neb.) model D density gradient fractionator with an ISCO model UA-2 ultraviolet analyzer to measure absorbancy at 280 nm.

Each tube was assayed for activity as described. The approximate molecular weight of the dehydrogenases separated by centrifugation was determined by comparing their sedimentation behavior with that of other enzymes of known molecular weight according to the procedure of Martin and Ames (12).

## RESULTS

*Growth of cells. A. aerogenes* A3(S1) was grown in 0.2% glucose (w/v), 0.3% $KH_2PO_4$, 0.7% $K_2HPO_4$, 0.1% $(NH_4)_2SO_4$, and 0.01% $MgSO_4 \cdot 7H_2O$ in 10-liter batches at 30 C in the Microferm Laboratory Fermentor (New Brunswick Scientific Co., New Brunswick, N.J.). Air flow was set at 9 liters/min and the agitator speed was set at 400 rev/min. When 1 liter of cells in the stationary phase was used as a starter for 9 liters of medium, maximal turbidity (about 350 Klett units, filter 42) was reached after 5 to 6 hr. Enzyme yield and specific activity were approximately the same whether we used cells from the end of the exponential phase or from the stationary phase (after 18 hr of incubation). The cells were harvested with the DeLaval Gyrotester (DeLaval Separator Co., Poughkeepsie, N.Y.) and were washed once with 0.2 M phosphate buffer, *p*H 7.0.

*Preparation of cell-free extract.* (All subsequent operations were performed at 0 to 4 C.) The packed cells (about 40 g per 10 liters of culture) were suspended in 0.02 M phosphate buffer (pH 7.0) to a final volume of about 160 ml, and were disrupted by sonic treatment at 0 to 6 C in a cooling cell (16) with the Branson Sonifier (Branson Instrument Inc., Stamford, Conn.) at an output of 10 amp for 4 min. The broken-cell suspension was centrifuged at 27,000 × *g* for 15 min; the turbid supernatant fluid then was centrifuged at 80,000 × *g* for 45 min and the clear supernatant fluid was retained (crude extract).

*Enzyme purification.* To the stirred crude extract (140 ml), we added 60 ml of 2% protamine sulfate in 0.025 M acetate buffer (*p*H 5.0) to obtain a ratio of 0.33 mg of protamine sulfate per mg of protein in the crude extract. Stirring was continued for 10 min; the precipitate was centrifuged and discarded (protamine sulfate supernatant fluid). Solid $(NH_4)_2SO_4$ was added to the supernatant fluid to 35% saturation, the precipitate was discarded, and the supernatant solution was brought to 52% saturation with solid $(NH_4)_2SO_4$. The precipitate was dissolved in 0.02 M phosphate buffer (*p*H 7.0) to yield 20 ml of solution [$(NH_4)_2SO_4$ fraction]. A 10-ml amount of the above solution was applied to a 400-ml Sephadex G-100 column (2.5 cm in diameter) which had been equilibrated with 0.01 M phosphate buffer (*p*H 7.0), 1 mM in EDTA and 0.1 mM in DTT. The column was eluted with the same buffer, and the active fractions were pooled (Sephadex fraction). To 86 ml of Sephadex fraction (protein concentration, 4 mg/ml), we added enough calcium phosphate gel (about 1 ml of suspension per 16 mg of protein) to adsorb about 90% of the enzyme. The gel was centrifuged and the supernatant fluid was discarded. The gel was washed once with 60 ml of 0.01 M phosphate buffer (*p*H 7.0) and then was extracted with 50-ml portions of phosphate buffer (*p*H 7.6; 1 mM in EDTA and 0.1 mM in DTT), ranging in molarity by stepwise 0.01 M increments from 0.01 to 0.06 M. Extracts containing enzyme having a specific activity higher than 1,000 units per mg of protein (usually extracted with 0.03 to 0.05 M buffer) were pooled; protein was precipitated by addition of solid $(NH_4)_2SO_4$ to 80% saturation and was taken up

in enough 0.01 M phosphate buffer (*p*H 7.0; containing EDTA and DTT as above) to give a final volume of 3.9 ml (concentrated $Ca_3(PO_4)_2$ eluate). The concentrated $Ca_3(PO_4)_2$ eluate was dialyzed overnight against 4 liters of 0.01 M phosphate buffer (*p*H 7.2; 1.0 mM in EDTA and 0.3 mM in DTT); 2.5 ml of the dialyzed enzyme (25,000 units) was applied to a 10-ml column (1 cm in diameter) of diethylaminoethyl (DEAE) cellulose (DE 23, Whatman) which had been washed with HCl and NaOH and equilibrated with 0.01 M phosphate buffer (*p*H 7.4; 1 mM in EDTA and 0.3 mM in DTT). The column then was washed with about 20 ml of the same buffer and was eluted with a linear gradient of NaCl; this gradient was obtained with 60 ml of 0.6 M NaCl in the above buffer in the reservoir bottle and with 60 ml of buffer in the mixing bottle. The active fractions were pooled; protein was precipitated by the addition of solid $(NH_4)_2SO_4$ to 80% saturation and was dissolved in a small volume of 0.01 M phosphate buffer (pH 7.0) containing EDTA and DTT as above (concentrated DEAE-C eluate). Approximately 40-fold purification was achieved, with 23% recovery (Table 1). Both the concentrated $Ca_3(PO_4)_2$ eluate and the concentrated DEAE-C eluate could be stored frozen at −10 C for at least 1 month with little loss of activity.

*Reaction products.* An 0.4-μmole amount of UDPG, 0.5 μmole of NAD, and 0.25 mg of enzyme [$Ca_3(PO_4)_2$ eluate] in 0.1 M glycylglycine buffer (*p*H 8.5) in a total volume of 50 μliters were incubated at 30 C for 1 hr. The reaction products were mixed with $^{14}$C-labeled UDPGA and were separated by electrophoresis at *p*H 5.8. In addition to residual NAD and UDPG, two products were detected. One had the mobility of reduced nicotinamide adenine dinucleotide and fluoresced under ultraviolet illumination; the other had the mobility of the authentic $^{14}$C-UDPGA, as shown by coelectrophoresis of authentically labeled (detected by autoradiography) and unlabeled (detected by ultraviolet quenching) UDPGA. The UDPGA was further characterized by cochromatography on paper with authentic $^{14}$C-UDPGA in the solvent of Paladini and Leloir (15).

*Substrate specificity.* When UDPG was replaced in the reaction mixture by ADPG, CDPG, or GDPM, the rate of reduction of NAD was less than 1% of the rate with UDPG. With TDPG, the rate of NAD reduction was about 2% of that with UDPG. No dehydrogenase activity was observed when NADP was used in place of NAD in the standard assay mixture.

*Effect of enzyme concentration.* Under the assay conditions employed, the reaction was linear for at least 3 min. A linear relation existed between activity and amount of protein over the range 2.5 to 30 μg per ml of reaction mixture.

TABLE 1. *Summary of purification of UDPG dehydrogenase*

| Fraction[a] | Volume | Total units | Specific activity | Recovery |
|---|---|---|---|---|
| | (*ml*) | | *units/mg* | |
| Crude extract... | 140 | 203,000 | 56 | 100 |
| Protamine sulfate supernatant fluid..... | 195 | 215,000 | 82 | 106 |
| $(NH_4)_2SO_4$ fraction..... | 20 | 150,000 | 104 | 74 |
| Sephadex fraction..... | 89 | 142,000 | 420 | 70 |
| Concentrated $Ca_3(PO_4)_2$ eluate..... | 3.9 | 74,000 | 1,380 | 36 |
| Concentrated DEAE-C eluate..... | 1.7 | 47,000 | 2,200 | 23 |

[a] See text for description of the fractions.

*Effect of pH.* The *p*H optimum for activity of the purified enzyme was between 9.4 and 9.7 (Fig. 1). The reaction rate was determined with either 0.1 M glycine or glycylglycine buffer; since the enzyme is most stable at a neutral *p*H, subsequent kinetic studies were carried out at an intermediate *p*H value (8.4) in 0.1 M glycylglycine buffer.

*Some physical properties of the enzyme.* Although the enzyme was completely excluded from columns of Sephadex G-75, it readily penetrated columns of Sephadex G-100, indicating a molecular weight in the range of $7 \times 10^4$ to $15 \times 10^4$. To compare the *Aerobacter* enzyme with the liver dehydrogenase (which has a molecular weight of about $3 \times 10^5$) the two enzymes were mixed, applied to a linear sucrose gradient, and separated by centrifugation. Details and results of the centrifugation are given in Fig. 2. The two dehydrogenases were completely separated by gradient density centrifugation; the molecular weight of the *Aerobacter* enzyme determined by this method was about $7 \times 10^4$ to $10 \times 10^4$. The enzymes also had different mobilities when examined by electrophoresis on cellulose polyacetate strips. Both migrated toward the anode, but the liver dehydrogenase had about one-third the mobility of the *Aerobacter* enzyme.

*Effect of substrate concentration.* The effect of UDPG and NAD concentration on initial velocity was determined by use of the standard assay procedure with appropriate variations in substrate concentration. Apparent $K_m$ values,

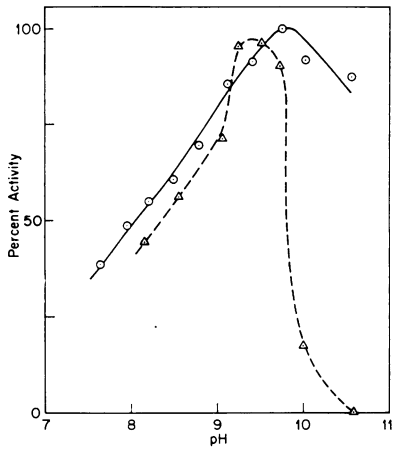


FIG. 1. *Dependence on activity on pH. Experimental conditions are those described in the text for assay, except that the pH and buffer were varied. Symbols: △, 0.1 M glycine-NaOH; ⊙, 0.1 M glycylglycine-NaOH.*

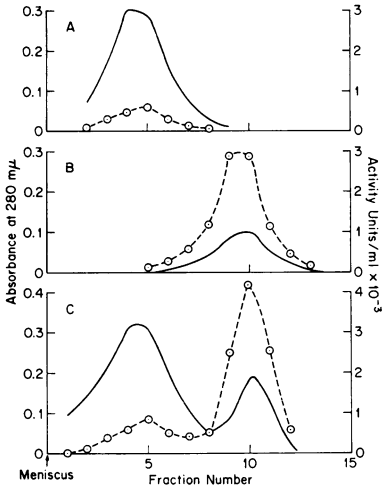


FIG. 2. *Sucrose density gradient. Sedimentation patterns of UDPG dehydrogenase from A. aerogenes and from calf liver. (A) An 0.5-mg amount of the $Ca_3(PO_4)_2$ fraction from Aerobacter in 0.05 ml of solution. (B) An 0.1-mg amount of purified liver enzyme in 0.05 ml of solution. (C) A mixture of each of the above. Symbols: ●, activity; solid line, absorbance at 280 nm.*

determined from these data by the method of Lineweaver and Burk (9), were 0.6 mM for UDPG and 0.06 mM for NAD. The $K_m$ of UDPG of the *Aerobacter* enzyme was the same as that of UDPG dehydrogenase of *C. laurentii* (1) but was much higher than that of beef liver dehydrogenase (0.02 mM at *p*H 8.7) (6).

*Inhibition by UDPXyl.* UDPXyl inhibited UDPG dehydrogenase 50% at a concentration of 0.1 mM, when the concentration of UDPG (and of NAD) was 2 mM. Plots of the reciprocal of initial velocity versus the reciprocal of UDPG concentration at different inhibitor concentrations resulted in curves, linear in respect to slope, which are typical for mixed-type inhibition; $K_i$ = 0.08 mM (Fig. 3). When the results of the inhibition studies were plotted as log $[v/(v_0-v)]$ versus log (UDPXyl), the set of parallel lines obtained yielded a value of $n$ not significantly different from 1.0 (Fig. 4), indicating that increased inhibitor concentration does not exert a cooperative effect.

## DISCUSSION

In *A. aerogenes*, as in all other organisms hitherto investigated, UDPGA is formed by the NAD-linked dehydrogenation of UDPG. In higher plants and vertebrates (14), as well as in *C. laurentii* (1), UDPXyl is an allosteric inhibitor of UDPG dehydrogenase and the interaction of inhibitor with enzyme is cooperative. In each of these organisms, in which the nucleotide serves as a donor of the D-xylosyl moiety in the synthesis of D-xylose-containing macromolecules, UDPXyl functions as a feedback inhibitor of the synthesis of UDPGA. Although D-xylose is well known in the plant (22) and animal (8, 17, 21) kingdoms, it is considerably less widespread among bacteria. The strain of *A. aerogenes* used in this study produces an extracellular polysaccharide which con-

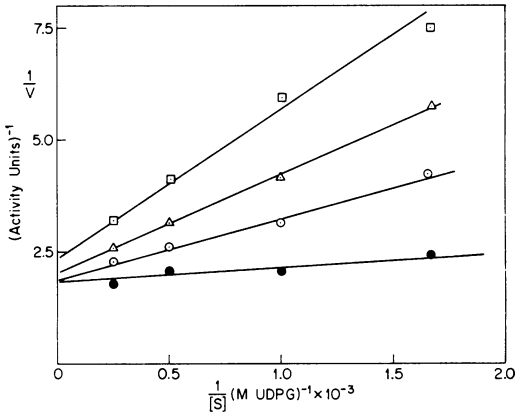


FIG. 3. *Dependence of reaction rate on UDPG concentration at different concentrations of UDPXyl. The conditions of assay are those described in the text, except that the concentration of UDPG was varied. UDPXyl was added to the complete reaction mixtures prior to addition of enzyme. UDPXyl concentrations: ●, no UDPXyl added; ⊙, 0.025 mM; △, 0.05 mM; ⊡, 0.1 mM.*

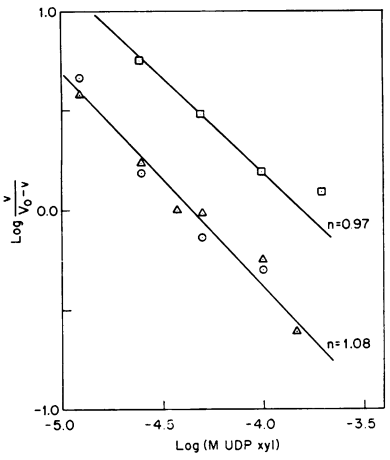


FIG. 4. *Inhibition of UDPG dehydrogenase as a function of UDPXyl concentration. The following concentrations of UDPG were employed:* ⊙, *0.6 mM;* △, *1.0 mM;* ⊡, *4.0 mM. Theoretical curves for n = 0.97 and n = 1.08 are given.*

tains no D-xylose (2); in addition, there is no evidence for the presence of the pentose in the cell wall of the organism (20). Since it is unlikely that *A. aerogenes* contains UDPGA carboxy-lyase or UDPXyl, its UDPG dehydrogenase should not be subject to the particular type of feedback inhibition found in D-xylose-containing organisms. The noncooperative mixed-type inhibition of *A. aerogenes* UDPG dehydrogenase by UDPXyl differs from the inhibition pattern of other UDPG dehydrogenase. The kinetic data, although not conclusive, suggest that, in *A. aerogenes*, UDPXyl may not play the significant role of physiological feedback inhibitor that it plays in *C. laurentii*, plants, and vertebrates.

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