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UDP-GLUCOSE-4-EPIMERASE FROM *POTERIOOCHROMONAS MALHAMENSIS*

KLAUS-STEN THOMSON*, CHRISTINE JUNG and HEINRICH KAUSS

Fachbereich Biologie, Universität Kaiserslautern, Postfach 3049, 6750 Kaiserslautern, Federal Republic of Germany

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Key Word Index—*Poterioochromonas malhamensis*; Chrysophyceae; UDP-glucose-4-epimerase.

Abstract—UDP-glucose-4-epimerase of *Poterioochromonas malhamensis*, Peterfi has been purified to apparent electrophoretic homogeneity. The enzyme has an apparent MW of 120 000 as determined by gel filtration of the active enzyme. Sodium dodecylsulfate polyacrylamide gel electrophoresis gave a MW of 59 000, thus indicating a dimeric structure. The epimerase does not require external NAD for activity. The apparent K_m values for UDP-glucose and UDP-galactose were calculated to be 1.67 mM and 0.26 mM, respectively. The pH optimum is at pH 8.7 and the isoelectric point is at pH 5.1 ± 0.15.

INTRODUCTION

UDP-glucose-4-epimerase (EC 5.1.3.2), which is essential for the direct interconversion of UDP-glucose and UDP-galactose, has been purified from various sources including bovine tissues (liver, thyroids and lacting mammary glands) [1], calf liver [2], *Escherichia coli* [3] and *Saccharomyces fragilis* [4].

In the golden brown wall-less flagellate *Poterioochromonas malhamensis*, cell water content, and thus volume, is controlled by changes of cellular solutes. On addition of solutes to a cell suspension, the cells shrink within 1–2 min. The water lost is regained during the following 1–2 hr due to the accumulation mainly of isofloridoside (α-galactosyl-1 → 1-glycerol) [5, 6]. One of the two substrates necessary for synthesis of isofloridoside in *P. malhamensis* is UDP-galactose. It was therefore of interest to study the UDP-glucose-4-epimerase of this organism.

This report describes the purification and partial characterization of UDP-glucose-4-epimerase from this organism.

*To whom correspondence should be addressed.

RESULTS AND DISCUSSION

Enzyme purification

Acetone powder was used as the starting material for purification of the UDP-glucose-4-epimerase. Without the preparation of an acetone powder, only 15% of the activity of the enzyme could be found in crude homogenates. The acetone powder can be stored at −20° for at least 2–3 months without any loss of activity.

The epimerase was purified by streptomycin sulfate precipitation, dye–ligand chromatography on Procion yellow H-A-Sepharose 4B, ion exchange chromatography on DEAE-cellulose, gel filtration on Sephadex G 150, and preparative polyacrylamide gel electrophoresis. With the procedure summarized in Table 1 the enzyme could be purified to homogeneity. The average specific activity of five different enzyme preparations was 2333 μkat/mg, and the purification was 1700-fold. The loss of activity during preparative gel electrophoresis is probably due to inefficient cooling of the WTW preparative electrophoresis apparatus. A single band staining for protein was obtained after SDS polyacrylamide gel electrophoresis.

Chromatography on Procion yellow H-A-Sepharose 4B has been used as an efficient purification step.

Table 1. Purification of the UDP-glucose-4-epimerase

Purification step	Total protein (mg)	Total activity* (μkat)	Specific activity* (nkat/mg)	Yield (%)	Purification factor
Extract of acetone powder	3300	4420	1340	100	1
Streptomycin sulfate supernatant	2500	4230	1700	95.8	1.27
Procion Yellow H-A Sepharose 4B	55	3600	65 500	81.5	48.9
DEAE cellulose	11	2430	221 000	55.1	165
Sephadex G 150	2	1900	950 000	43	710
Preparative gel electrophoresis	0.16	367	2 290 000	8.3	1710

*Assay I.

Immobilized triazine dyes are increasingly important for enzyme purification [7–10]. It could be shown that the epimerase is completely inhibited by the Procion yellow H-A dye (5×10^{-4}M), but elution with UDP-glucose or UDP-galactose from the dye-Sepharose 4B column failed. The mode of inhibition is none of the simple inhibition types [unpublished results].

Storage

The purified enzyme was stable for several months if mixed with an equal volume of glycerol and stored in liquid nitrogen. Repeated freezing and thawing was avoided. More than 80% of the activity of the final enzyme preparation was lost by freeze drying.

K_m *values*

From double reciprocal plots (assay II, 5 min incubations), the apparent K_m values for UDP-galactose and UDP-glucose were calculated to be 0.26 mM and 1.67 mM, respectively. For UDP-galactose as the substrate the K_m was determined by the three different assays, for the coupled assay I: $K_m = 0.26$ mM, for assay II: $K_m = 0.3$ mM, and for assay III: $K_m = 0.26$ mM.

MW determination

Disc gel electrophoresis at pH 8.8 yielded a single protein band and the enzyme activity was associated with this band. SDS-polyacrylamide gel electrophoresis gave a single band of MW 59 000, whereas gel filtration on Sephadex G 150 resulted in a MW of 120 000. These results suggest that the enzyme consists of two identical subunits. Similar results have been reported for the UDP-glucose-4-epimerase of the yeast *Saccharomyces fragilis* [11].

pH optimum

The pH optimum of epimerase was at pH 8.7. The enzyme still shows about 50% of the maximum activity at pH 6.5 or 10, respectively.

Approach to equilibrium

The approach to equilibrium with UDP-galactose as the substrate is shown in Fig. 1. At equilibration about 27% of UDP-galactose and 73% of UDP-glucose are present. For the epimerase of *Saccharomyces fragilis* Ray and Bhaduri [12] showed that this enzyme has Michaelis–Menten kinetics with UDP-glucose. At very low UDP-galactose-concentration as the substrate these authors showed that a unidirectional catalysis takes place and the equilibrium is established only after UDP-galactose is first completely converted to UDP-glucose. In contrast to these results the epimerase from *Poterioochromonas* shows Michaelis–Menten kinetics for both substrates and the equilibrium is established in the normal fashion.

Independence of externally added NAD^+ or $NADP^+$

When NAD^+ or $NADP^+$ were added (final concentration 10^{-3}M) to the final epimerase preparation no increase in activity could be measured. The epimerase of *Saccharomyces fragilis* shows the same properties [12]. *p*-Chloromercuric benzoate has been shown to inactivate

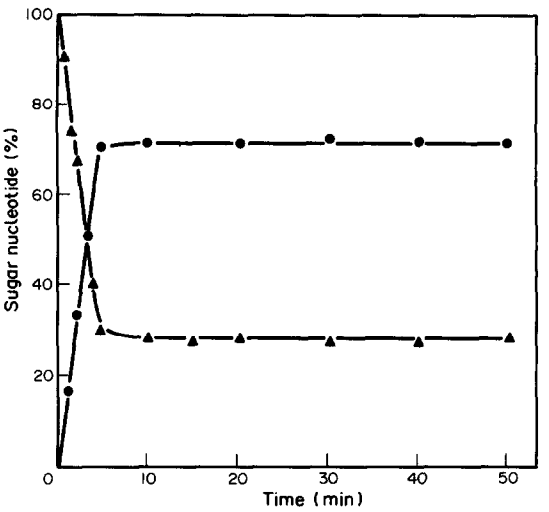

Fig. 1. Approach to equilibrium with UDP-galactose as the substrate. (▲——▲) indicates the per cent of UDP-galactose left after different incubation times whereas (●——●) indicates the amount of UDP-glucose formed. The assay mixture consisted of 20 mM glycylglycine pH 8.7, 0.02 mM UDP-galactose containing 1 μCi UDP-[^{14}C]-galactose and an appropriate amount of the enzyme in a total volume of 2 ml. UDP-galactose and UDP-glucose were separated by paper chromatography.

the yeast-epimerase [12] and activity could be restored by addition of 2-mercaptoethanol and external NAD^+. With the epimerase from *P. malhamensis* we were not able to inactivate the enzyme by titration with *p*-chloromercuric benzoate.

EXPERIMENTAL

Materials. Bovine serum albumin, β-nicotinamide adenine dinucleotide, UDP-galactose and UDP-glucose were purchased from Sigma. Calibration proteins were taken from the Combithek manufactured by Boehringer, Mannheim. Procion Yellow H-A (the dye component of Matrex gel orange A, Amicon Corporation) was a gift from Imperial Chemical Industries, Organics Division, Manchester. Streptomycin sulfate and reagents for preparative and sodium dodecyl sulfate polyacrylamide gel electrophoresis were from Serva, Heidelberg. Ampholytes were obtained from LKB. Sephadex G 150 and Sepharose 4B were products of Pharmacia, Upsala. DE 32 DEAE cellulose was from Whatman. UDP-[^{14}C(U)]-galactose (sp. act. 302 mCi/mM) and UDP-[^{14}C(U)]-glucose (sp. act. 281 mCi/mM] were from New England Nuclear, Boston.

Cell growth. Poterioochromonas malhamensis, Peterfi (syn. *Ochromonas malhamensis*, Pringsheim) from Algensammlung, Göttingen, Germany, were grown in a defined medium [13] with 10 g glucose per l. Fifty ml of a 3 day culture were inoculated in 800 ml of medium under sterile conditions, and grown in the dark at 25° under aeration. The cells were harvested by centrifugation and Me_2CO powder was prepared by the method of ref. [14].

Enzyme purification. The purification procedure was carried out at 0–4°. Acetone powder (20 g) was stirred for 2 hr with 2 l. of 30 mM Tris–HCl, pH 8, containing 5 mM 2-mercaptoethanol, and 0.05% (w/v) NaN_3. The suspension was centrifuged at 15 000 *g* for 20 min, and the residue was discarded. To the supernatant a soln of streptomycin sulfate was added dropwise to give a final concn of 1% (w/v). This suspension was stirred for 30 min and centrifuged (15 000 *g*, 20 min). The residue was

discarded. The supernatant was loaded on a Procion yellow H-A-Sepharose 4B column, 4 × 12 cm, which had previously been equilibrated with 30 mM Tris–HCl, pH 8, containing 5 mM 2-mercaptoethanol, and 0.05% (w/v) NaN_3. The column was washed with 1 l. of the above buffer at a rate of 250 ml per hr. The enzyme was then eluted with the same buffer containing 1.5 M NaCl. The activity-containing fractions were concd to 30 ml in a Diaflon cell using a Berghof BM 1000 membrane, dialysed overnight against 2 l. (3 changes) of 30 mM Tris–HCl, pH 7.8, containing 5 mM 2-mercaptoethanol and 0.05% NaN_3, and loaded on a DEAE-cellulose column (1 × 18 cm), which had been equilibrated with the same buffer. The column was then washed with 250 ml of buffer at a rate of 50 ml/hr, the enzyme was eluted with a linear gradient of 0–0.4 M of NaCl in the above buffer. Total vol. of the gradient was 200 ml. 2.5 ml fractions were collected. The active fractions of previous step were pooled, concd in an ultra-thimble UH 100/75 (Schleicher and Schuell, Dassel) and loaded on a Sephadex G 150 column, 1.5 × 120 cm, which had been equilibrated with 30 mM Tris–HCl, pH 7.8 containing 5 mM 2-mercaptoethanol and 0.05% NaN_3. The enzyme was eluted at a rate of 7 ml/hr and 1.8 ml fractions were collected. The activity containing fractions of the gel filtration were pooled and concd in an ultrathimble to about 2 ml, and dialysed for 2 hr against sample buffer. Preparative gel electrophoresis was performed in a WTW electrophoresis apparatus with 10 ml of a 6% polyacrylamide gel at 20 mA as described by Davis [15]. Elution was performed with running buffer at a rate of 20 ml/hr and 1 ml fractions were collected.

Assays. UDP-glucose-4-epimerase was assayed by three different methods: *Assay I* was coupled spectrophotometric assay [16]. An excess of UDP-glucose-dehydrogenase (prepared from calf liver to step V, following the method of ref. [17]) and NAD^+ was present as the indicator system. The vol. of the assay was 0.5 ml. The final assay mixture contained 200 mM glycylglycine pH 8.7, 2 mM NAD^+, 0.01 unit UDP-glucose, dehydrogenase and the requisite amount of UDP-glucose-4-epimerase. After a constant *A* at 340 nm was reached, the reaction was started with UDP-galactose as the substrate (final concn 2 mM) and the rate was measured as *A*) increase at 340 nm for 10 min at 22°. Dilutions of UDP-glucose-4-epimerase were made in 50 mM Tris–HCl pH 6.5, containing 0.1% w/v bovine serum albumin.

Assay II was used to determine the K_m values with UDP-galactose or UDP-glucose as substrate. The vol. of the assay was 0.5 ml. The final assay mixture consisted of 20 mM glycylglycine, pH 8.7, 0.05 to 2 mM UDP-galactose or UDP-glucose containing 1 μCi UDP-[^{14}C]-galactose or 1 μCi UCP-[^{14}C]-glucose, respectively. The reaction was started by addition of UDP-glucose-4-epimerase. The assays were incubated at 25° for 5 min and the reaction stopped by addition of 0.5 ml of $CHCl_3$ with vigorous stirring. After complete separation of the layer about 0.35 ml of the upper layer was carefully transferred with a Pasteur pipette. $CHCl_3$ was removed by heating the tubes for 2 min at 65°. UDP-glucose and UDP-galactose were separated by paper chromatography (see below).

Assay III was additionally used to determine the K_m value with UDP-galactose as substrate. This assay is a two step procedure and was modified according to ref. [12]. For the first step, in a final assay mixture of 1 ml containing 0.1 M glycylglycine buffer, pH 8.7, the requisite amount of the enzyme, and varying concns of UDP-galactose were mixed. The reaction was allowed to proceed for 5 min at 25° and stopped by the addition of 1 ml of $CHCl_3$ with rapid stirring. The aq. layer was transferred and $CHCl_3$ removed by heating the tubes at 65° for 2 min. An aliquot was transferred to an assay mixture of 1 ml final vol. containing 0.1 M glycylglycine, pH 8.7 and 0.01 unit of UDP-glucose-dehydrogenase. The initial *A* of this sample at 340 nm was noted and the reaction then started with 10 μl of 50 mM NAD^+ and recorded until a constant value at 340 nm was obtained. One unit of the enzyme was defined as the amount of enzyme needed to convert 1 mol/sec of UDP-galactose to UDP-glucose under standard conditions of assay I. This assay was mainly used for measuring the enzyme activity during the purification procedure.

Paper chromatography. The method of ref. [18] was used.

Preparation of Procion yellow H-A Sepharose 4B. Procion yellow H-A was coupled to Sepharose 4B according to ref. [19].

Isoelectric focussing. Isoelectric focussing was performed as described in ref. [20] with the 110 ml column of LKB. Ampholines in the pH range 3–7 were used.

Polyacrylamide gel electrophoresis. Gel electrophoresis in the presence of sodium dodecylsulfate was performed as described in ref. [21] at pH 8.8 using 7.5% w/v of acrylamide. Preparative polyacrylamide gel electrophoresis was carried out in a WTW electrophoresis apparatus (Weinheim, Germany) using 6% w/v of acrylamide and the system of ref. [15].

Protein determination. Protein was determined by the Coomassie blue method of ref. [22] with bovine serum albumin as a standard.

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