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3-Ketoglucose Reductase of *Agrobacterium tumefaciens*

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Two kinds of 3-ketoglucose-reducing enzyme were partially purified from the sonic extract of *Agrobacterium tumefaciens* IAM 1525 grown on a sucrose-containing medium. Both enzymes have a specific requirement for reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a hydrogen donor and catalyze the reduction of 3-ketoglucose to glucose but do not reduce 3-ketoglucosides such as 3-ketosucrose, 3-ketoglucose-1-phosphate, 3-ketotrehalose, and 3-ketocellobiose. From the requirement and substrate specificity of the enzymes, the name NADPH: 3-ketoglucose oxidoreductase (trivial name, 3-ketoglucose reductase) was proposed. By diethylaminoethyl-cellulose column chromatography, two reductases were separated, and the early and late eluted enzymes were designated reductase I and II, respectively. K_m values of reductase I and II were as follows: for 3-ketoglucose both had an identical value of 2.5×10^{-5} M, and for NADPH the values were 1.0×10^{-5} M and 1.5×10^{-5} M, respectively. Optimal *p*H values were also identical: *p*H 4.8 to 5.0 in 10^{-2} M phosphate buffer. Intracellular localization of the enzymes is discussed.

In a previous paper (6), the presence of D-aldohexoside-(acceptor) oxidoreductase (trivial name, D-glucoside-3-dehydrogenase) which catalyzes the oxidation of D-glucose to 3-ketoglucose was demonstrated in *Agrobacterium tumefaciens*, a plant tumor-inducing strain, and it was shown that the enzyme requires flavine adenine dinucleotide (FAD) as prosthetic group and transfers electron from FAD to cytochrome C_{552} exclusively (9).

In this paper, we describe the presence of a different enzyme, reduced nicotinamide adenine dinucleotide phosphate (NADPH): 3-ketoglucose oxidoreductase (trivial name, 3-ketoglucose reductase) which catalyzes reduction of 3-ketoglucose to D-glucose in the same organism. Purification and properties of the enzyme are reported.

MATERIALS AND METHODS

Sugars. 3-Ketoglucose was prepared from the enzymatic hydrolysate of 3-ketosucrose (3-ketoglucosyl fructoside) by the method described previously (4). The following 3-ketoglucosides were also prepared in our laboratory: 3-ketosucrose (5), 3-ketoglucose-1-phosphate (1), 3-ketotrehalose (3), and 3-ketocellobiose (7). To obtain radioactive 3-ketoglucose, uniformly labeled 3-ketosucrose (^{14}C) prepared from labeled sucrose (11.7 mCi/mmole) was used.

Microorganism and cultivation. *A. tumefaciens* IAM 1525, a strain of a plant tumor-inducing bacterium, was cultured in the sucrose medium of McIntire, as modified by Fukui et al. (5). Cultivation was at 27 C on a rotary shaker. The cells were collected at mid-logarithmic growth phase by centrifugation at 10,000 × *g* for 20 min, washed twice with cold 50 mM phosphate buffer (*p*H 7.0), and suspended in the same buffer. The suspension was used as resting cells.

Sonic extract. Cells were disrupted by sonic oscillation (10 kc) for 10 min, followed by centrifugation at 10,000 × *g* for 10 min to remove intact cells and cell debris. The supernatant fraction was used as a source of 3-ketoglucose reductase.

Determination of NADP and NADPH. NADP was determined by increase in absorbancy at 340 nm by an NADP-requiring isocitrate dehydrogenase system (11). Amount of NADPH was estimated from absorbancy at 340 nm.

Paper chromatography. For separation of 3-ketoglucose from other sugars, paper chromatography was employed with the following solvent system: acetone-acetic acid-water (4:1.2:1, v/v). The ketosugar and reducing sugars were detected by the urea phosphate reagent (13) and alkaline silver nitrate, respectively. When radioactive 3-ketoglucose was used as substrate, a sample of the reaction mixture was applied on filter paper (Tolyo-filter paper, no. 51 A) and developed overnight at room temperature. After removal of the solvent from the filter paper by air-drying, radioactivity on the chromatogram was

measured by a paper-scanning gas flow counter (Tokyo Musen Co., Ltd. Tokyo).

Osmotic shock. The shock fluid was prepared from resting cells by the osmotic shock technique of Neu and Heppel (10) with some modification (S. Fukui and A. Hirata, *unpublished data*) as follows. A 500-mg amount (wet cell weight) of the resting cells harvested at exponential growth phase in the sucrose medium was suspended in 50 ml of 20% sucrose solution in 30 mM tris(hydroxymethyl)aminomethane (Tris)-chloride buffer (*p*H 8.2) containing 0.5 mM ethylenediaminetetraacetate (disodium salt). A 50-ml amount of the cell suspension in a 300-ml Erlenmeyer flask was incubated at 27 C with shaking for 10 min; cells were then collected by centrifugation. The cells were suspended in 50 ml of 0.5 mM magnesium chloride in 0.5 mM Tris-chloride buffer (*p*H 8.2) in a 300-ml Erlenmeyer flask and were incubated at 0 C for 10 min with shaking. After incubation the suspension was centrifuged, and the supernatant fluid thus obtained was designated as shock fluid. After this treatment, survival of the bacterium was in a range 90 to 100%.

Enzyme assay. For assay of 3-ketoglucose reductase, 1.0 ml of the reaction mixture contained 100 nmoles of 3-ketoglucose, 50 nmoles of NADPH, 10 nmoles of KH_2PO_4, and enzyme solution. The reaction was initiated by addition of enzyme solution, and incubation was at 20 C. Activity is determined by measuring a rate of decrease in absorbancy at 340 nm.

D-Glucoside 3-dehydrogenase activity was estimated by colorimetry with 2,6-dichloroindophenol as a hydrogen acceptor by the method described previously (7). In the present work, glucose-1-phosphate was used as substrate in place of sucrose. The reaction mixture was incubated at 20 C.

Activity of "alkaline phosphatase" was determined as follows. One milliliter of the reaction mixture contained 10 μmoles of glucose-1-phosphate, 5 μmoles of Tris-chloride buffer (*p*H 8.2), and an appropriate amount of the shock fluid. The reaction mixture was incubated at 30 C for 10 min. Glucose thus formed was determined (12).

Activity of 3-ketoglucose-1-phosphate-hydrolyzing enzyme (8) was determined from the rate of liberation of 3-ketoglucose which has a molar extinction coefficient of $3.8 \times 10^3\ M^{-1}\ cm^{-1}$ in 0.1 M phosphate buffer, *p*H 7.0 (2). One milliliter of the reaction mixture contained 10 μmoles of 3KG-1-P, 100 μmoles of phosphate buffer (*p*H 7.0), and enzyme solution. Incubation was at 20 C.

For assay of α-glucosidase, 1.0 ml of the reaction mixture contained 10 μmoles of *p*-nitrophenyl-α-D-glucoside, 100 μmoles of phosphate buffer (*p*H 7.0), and enzyme solution. The reaction mixture was incubated at 30 C for 5 min. After termination of the reaction by addition of 10% Na_2CO_3, *p*-nitrophenol formed was measured by spectrophotometrically at 410 nm.

One unit of enzyme is defined as the amount that catalyzes the formation of 1 μmole of product per min.

RESULTS

Partial purification of 3-ketoglucose reductase. To the sonic extract (150 ml) prepared from the resting cells harvested from a 5-liter culture was added 105 g of solid ammonium sulfate (0.6 saturation). The preparation was stored overnight at 2 C. The precipitate formed was dissolved in 80 ml of 1 mM phosphate buffer, *p*H 7.0, and dialyzed against 5 liters of the same buffer for 1 day. After dialysis, the dialysate was centrifuged at 105,000 × *g* for 2 hr to remove subcellular particles. To the clear supernatant solution (115 ml) was added 370 mg of protamine sulfate dissolved in distilled water. After 30 min, the precipitate formed was removed by centrifugation at 10,000 × *g* for 30 min. A 35-g amount of solid ammonium sulfate was added to the supernatant solution (128 ml), and the precipitate thus formed was discarded by centrifugation. To the supernatant solution was added 17.5 g of ammonium sulfate, and the mixture was allowed to stand overnight at 2 C. The precipitate formed was collected by centrifugation, dissolved in 30 ml of 1 mM phosphate buffer (*p*H 7.0), and dialyzed against 2 liters of the same buffer overnight at 4 C. The dialyzed preparation was applied to a diethylaminoethyl (DEAE)-cellulose column (3.0 by 25 cm) which was equilibrated with 1 mM phosphate buffer (*p*H 7.0) before use. An elution was carried out with 1 liter of KCl solution having a linear concentration gradient of 0 to 0.5 M in 1 mM phosphate buffer (*p*H 7.0) at a flow rate of 2.0 ml per min. An elution profile is presented in Fig. 1. The activity of 3-ketoglucose reductase was recovered as two components in the eluates with approximately 0.15 and 0.2 M KCl. The early and late components were designated reductase I and II, respectively.

Fractions of reductase I (tube no. 40 to 44) and II (tube no. 50 to 55) were separately combined, and the enzymes were precipitated by salting out with 0.6 saturation of ammonium sulfate. Each precipitate of reductase I and II was dissolved in 5 ml of 1 mM phosphate buffer (*p*H 7.0) and dialyzed against 1 liter of the same buffer overnight. With the dialyzed preparations, a second DEAE-cellulose column chromatography was performed as follows. The enzyme preparation was charged on a column of DEAE-cellulose (1.5 by 15 cm) which was previously equilibrated with 1 mM phosphate buffer (*p*H 7.0), followed by elution with 500 ml of KCl solution having a linear concentration

gradient of 0 to 0.3 M in the same buffer at an elution rate of 1.0 ml per min. Enzyme activity was recovered as a single component. In disc-gel electrophoresis, fractions showing the highest specific activity (see Table 1) were still composed of three or four protein bands. Dialysis and column chromatography were performed in a cold room (2 C). The entire procedure is summarized in Table 1.

Identification of reaction product from 3-ketoglucose and stoichiometry in the reaction. Characterization of the chemical reaction catalyzed by 3-ketoglucose reductases obtained here was achieved by identifying the reaction product from 3-ketoglucose. A 3.0-ml amount of reaction mixture contained 3 μmoles of radioactive 3-ketoglucose, approximately 3 μmoles of NADPH, 30 μmoles of KH_2PO_4 and 1.0 unit of the enzyme I (specific activity 2,020) or II (specific activity 1,200). The reaction mixture was incubated at 20 C for 30 min. Then, 3-ketoglucose, NADPH, and glucose in the reaction mixture were determined. As seen in Table 2, the 3-ketoglucose that disappeared was quantitatively converted to glucose by both enzymes I and II. Paper chromatography of the reaction mixture also indicated the formation of glucose from 3-ketoglucose (Fig. 2).

The NADPH that disappeared in the reac-

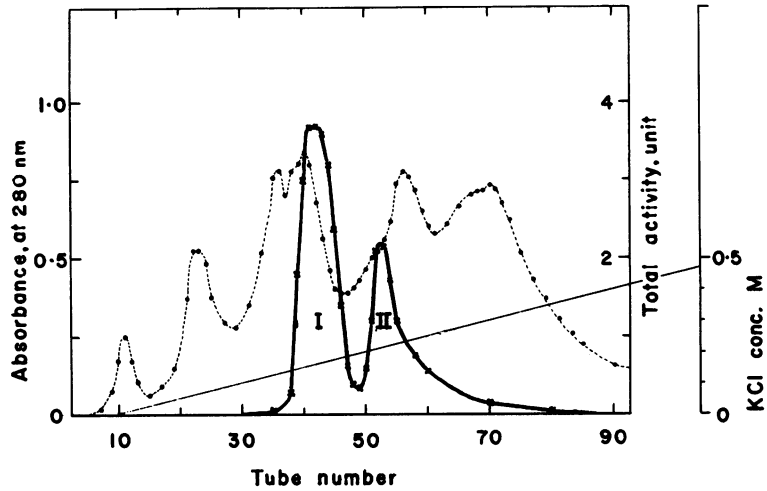

FIG. 1. *Elution profile of 3-ketoglucose reductase on DEAE-cellulose column chromatography (each tube, 10 ml). Solid line, enzyme activity; broken line, absorbance at 280 nm.*

TABLE 1. *Purification of 3-ketoglucose reductase*

Fraction	Reductases	Total vol (ml)	Total activity (units)	Total optical density (A_{280})[a]	Specific activity (units/A_{280})[a]
Sonic extract	I[b] + II[b]	150	91	21×10^3	0.0043
Ammonium sulfate fractionation-ultracentrifugation	I + II	115	—	6.7×10^3	—
Protamine treatment	I + II	128	67	1.3×10^3	0.051
1st DEAE-cellulose column chromatography	I	50	15.7	33.3	0.47
	II	50	8.3	29.5	0.28
2nd DEAE-cellulose column chromatography	I	10	8.7	4.3	2.0
	II	17.5	2.9	2.4	1.2
Fraction giving the highest specific activity in the 2nd chromatography	I	2.5	3.1	1.1	2.8
	II	2.5	0.71	0.36	2.0

[a] A_{280}, Absorbance per centimeter at 280 nm.
[b] See text.

tion was recovered as NADP. A 0.1-ml amount of the reaction mixture after 30 min of incubation was added to 2.0 ml of an NADP-requiring isocitrate dehydrogenase system composed of 10^{-3} M isocitrate, 10^{-4} M $MnCl_2$, 10^{-2} M phosphate buffer (*p*H 7.7), and one unit of isocitrate dehydrogenase (Sigma). An increase of optical density of approximately 0.3 at 340 nm was observed. Thus, an equation of the reaction catalyzed by 3-ketoglucose reductase is as follows: 3-Ketoglucose + NADPH $\rightleftharpoons$ Glucose + NADP. The reaction rate was not affected by the addition of 2.5×10^{-3} M glucose and NADP to the assay system in which 3-ketoglucose was 1.0×10^{-4} M (Fig. 3). The findings described here indicate that the forward reaction is much faster than the backward reaction. This means the equilibrium constant is very small.

Some properties of the purified enzymes. Both reductases I and II required NADPH as a hydrogen donor and 3-ketoglucose as a hydrogen acceptor with high specificity. From Lineweaver-Burk plots at *p*H 7.0, K_m values were calculated as follows: for NADPH, reductase I and II gave 1.0×10^{-5} M and 1.5×10^{-5} M, respectively, and for 3-ketoglucose both gave an identical value of 2.5×10^{-5} M. NADH and 3-ketoglucosides, such as 3-ketosucrose, 3-ketoglucose-1-phosphate, 3-ketotrehalose and 3-ketocellobiose, were inactive. The *p*H dependency curves of reductases I and II also showed an identical optimum at *p*H 4.8 to 5.0 in 10^{-2} M phosphate buffer.

Localization of 3-ketoglucose reductase. By means of osmotic shock, "alkaline phosphatase" and glucoside-3-dehydrogenase were released from the resting cells, but no activity of the reductase was detected in the shock fluid as shown in Table 3. When cells were disrupted by sonic oscillation, by grinding with sea sand, or by treatment with a Ribi cell fractionator (20,000 psi at 2 C), the reductases were recovered in the supernatant fluid after ultracentrifugation at 105,000 × *g* for 1 hr. These findings indicate that the reductases are not surface enzymes and might be cytoplasmic enzymes.

TABLE 2. *Stoichiometry in the reaction*[a]

Enzyme	Incubation	NADPH (μmoles)	3-Ketoglucose (μmoles)	Glucose (μmoles)
Reductase I	Before	3.5	3.1	0
	After	0.6	0.1	2.8
Reductase II	Before	3.8	3.0	0
	After	0.9	0.1	2.8

[a] Incubation was at 20 C for 30 min. Reaction mixture was 3.0 ml. Glucose was determined by "Glucostat" (Worthington Biochemical Corp., Freehold, N.J.).

DISCUSSION

Evidence has been presented for the existence of two kinds of an enzyme that catalyzes the reduction of 3-ketoglucose to glucose in cells of *A. tumefaciens*. Based upon studies on cofactor requirements, substrate specificity, and stoichiometry of the reaction, the enzymes should be named NADPH:3-ketoglucose oxidoreductase (trivial name, 3-ketoglucose reductase) according to the rules presented by the Enzyme Commission.

In a previous paper (6) we described the presence of D-glucoside-3-dehydrogenase which

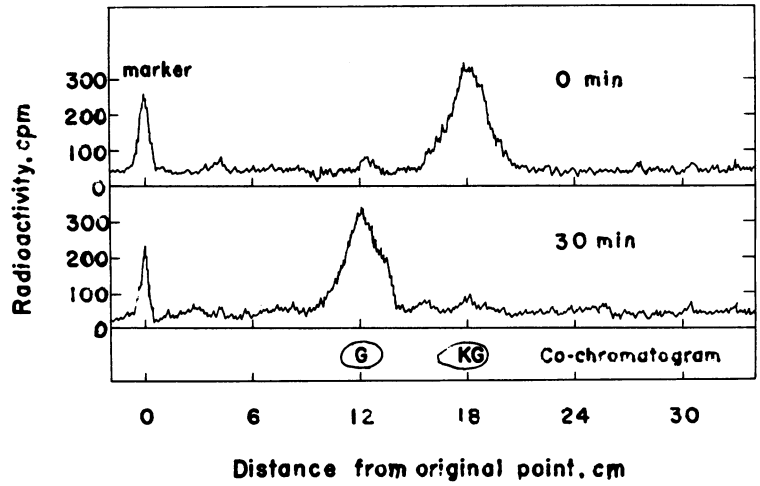

FIG. 2. *Paper chromatography of reaction mixture. Reaction conditions are described in the text.*

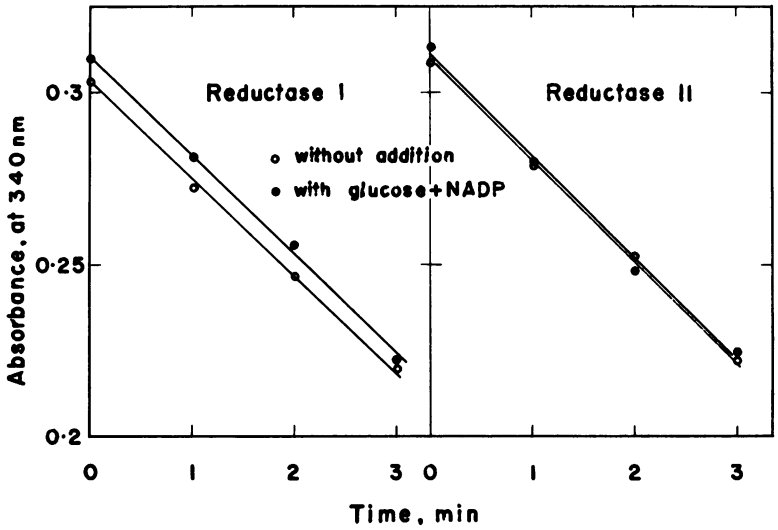

FIG. 3. *Effect of glucose and NADP on NADPH-oxidizing rate by 3-ketoglucose reductases; 2.5 μmoles of glucose and 250 nmoles of NADP were added to the assay system for 3-ketoglucose reductase. Assay system is described in the text.*

TABLE 3. *Release of enzymes from resting cells by osmotic shock*[a]

Enzymes	Sonic extract (units)[b]	Osmotic shock	
		Shock fluid (units)[b]	Yield[c] (%)
"Alkaline phosphatase"	1.2	0.8	66.7
D-Glucoside-3-dehydrogenase	4.5	2.4	53.3
α-Glucosidase	12.1	0	0
3-Ketoglucosidase	6.3	0	0
3-Ketoglucose reductase	2.8	0	0

[a] After osmotic shock, survival was 98% in this experiment.

[b] Enzyme activity is expressed as units per 5 g of wet cells.

[c] Yield is a percentage of activity in the shock fluid per activity in the sonic extract. Activity in the sonic extract shows total activity.

catalyzes the oxidation of glucose to 3-ketoglucose in the cells of the same strain of the bacterium used here. From their catalytic properties, it was found that the reactivity of 3-ketoglucose reductases is opposite to that of D-glucoside-3-dehydrogenase in the direction of biochemical oxidoreduction between glucose and 3-ketoglucose. If the intracellular locations of these enzymes are identical, it would be very difficult to explain the physiological significance of the enzymes. However, the locus of the reductase is considered to be different from that of the dehydrogenase. The reductase was not released from resting cells by osmotic shock, whereas the dehydrogenase was readily released. By the designation of Neu and Heppel (10), the loci of the former and latter enzymes are cytoplasm and periplasm, respectively.

Resting cells as well as a sonic extract of the bacterium could metabolize 3-ketoglucose with consumption of molecular oxygen. However, it was not determined whether the reductase is essential for 3-ketoglucose metabolism in vivo. On the other hand, we obtained the following interesting results on glycogen synthesis by growing cells of the bacterium. When labeled glucose (a mixture of glucose-*1*-^{14}C and glucose-*3*-^{3}H) was used as carbon source, the glycogen that accumulated in the cytoplasmic space consisted of glucose-*1*-^{14}C which had a specific radioactivity value quite close to that of the substrate glucose-*1*-^{14}C. On the other hand, specific radioactivity of tritium in glucose of the bacterial glycogen was much lower than that of the substrate glucose-*3*-^{3}H. These findings strongly suggest that glucose was once oxidized to 3-ketoglucose, then reduced to glucose by 3-ketoglucose reductase, and then polymerized to glycogen. In other words, both glucoside-3-dehydrogenase and 3-ketoglucose reductase are essential for glycogen synthesis from glucose by the growing cells (C. K. Chern and S. Fukui, *unpublished data*).

As seen in Fig. 1, the reductase activity was recovered in two fractions (reductases I and II) after DEAE-cellulose column chromatography. During the purification, interconversion be-

tween reductases I and II was not observed under the conditions used here. The activity ratio of reductases I and II (I/II) in the sonic extract was not constant, but varied from 1/1 to 5/1 in this work. Total activity of the reductases in the sonic extract prepared from the cells at early logarithmic growth phase was found to be several times the activity in the cells at stationary growth phase. This tendency in functional activity during cultivation was also observed with glucoside-3-dehydrogenase and glucoside transport (8). The tendency was opposite in 3-ketoglucoside transport (8).

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