JOURNAL OF BACTERIOLOGY, Oct. 1997, p. 6041–6047 Vol. 179, No. 19
0021-9193/97/$04.00+0

Dihydroxyacetone Synthase from a Methanol-Utilizing Carboxydobacterium, *Acinetobacter* sp. Strain JC1 DSM 3803

YOUNG T. RO,[1]† CHI Y. EOM,[1] TAEKSUN SONG,[1] JIN W. CHO,[2] AND YOUNG M. KIM[1]*

Molecular Microbiology Laboratory[1] and Cell Biology Laboratory,[2] Department of Biology, Yonsei University, Seoul 120-749, Korea

Received 14 March 1997/Accepted 22 July 1997

***Acinetobacter* sp. strain JC1 DSM 3803, a carboxydobacterium, grown on methanol was found to show dihydroxyacetone synthase, dihydroxyacetone kinase, and ribulose 1,5-bisphosphate carboxylase, but no hydroxypyruvate reductase and very low hexulose 6-phosphate synthase, activities. The dihydroxyacetone synthase was found to be expressed earlier than the ribulose 1,5-bisphosphate carboxylase. The dihydroxyacetone synthase was purified 19-fold in eight steps to homogeneity, with a yield of 9%. The final specific activity of the purified enzyme was 1.12 μmol of NADH oxidized per min per mg of protein. The molecular weight of the native enzyme was determined to be 140,000. Sodium dodecyl sulfate-gel electrophoresis revealed a subunit of molecular weight 73,000. The optimum temperature and pH were 30°C and 7.0, respectively. The enzyme was inactivated very rapidly at 70°C. The enzyme required Mg^{2+} and thiamine pyrophosphate for maximal activity. Xylulose 5-phosphate was found to be the best substrate when formaldehyde was used as a glycoaldehyde acceptor. Erythrose 4-phosphate, glycolaldehyde, and formaldehyde were found to act as excellent substrates when xylulose 5-phosphate was used as a glycoaldehyde donor. The K_ms for formaldehyde and xylulose 5-phosphate were 1.86 mM and 33.3 μM, respectively. The enzyme produced dihydroxyacetone from formaldehyde and xylulose 5-phosphate. The enzyme was found to be expressed only in cells grown on methanol and shared no immunological properties with the yeast dihydroxyacetone synthase.**

Carboxydobacteria are a group of facultative chemolithoautotrophic bacteria, except for *Streptomyces thermoautotrophicus*, which are able to grow aerobically at the expense of carbon monoxide (CO), in addition to several organic materials, as a sole source of carbon and energy (15, 24). It has been known for a long time that *Pseudomonas gazotropha* is the only carboxydobacterium also capable of growing methylotrophically on methanol as the sole carbon and energy sources and is recognized as the first organism adopting three nutrition types, i.e., organotrophy, autotrophy, and methylotrophy (15, 25, 27, 28, 39). We, however, observed in a previous study that *Acinetobacter* sp. strain JC1 DSM 3803, a carboxydobacterium isolated in Korea (6), also grows methylotrophically on both methanol and methylamine as sole carbon and energy sources.

It is well known that there are four different ways for assimilation of C_1 compounds in methylotrophic organisms (2). The ribulose monophosphate (RuMP) cycle for the assimilation of formaldehyde, the first metabolite of methanol oxidation, the serine pathway for the fixation of one molecule each of formaldehyde and CO_2, the end product of methanol oxidation, and the Calvin reductive pentose phosphate cycle for CO_2 fixation were found to work in methylotrophic bacteria, whereas the xylulose monophosphate (XuMP) cycle for the assimilation of formaldehyde was found only in methylotrophic yeasts (2, 8, 9).

In this study, we report the presence of a novel mechanism for the assimilation of methanol in *Acinetobacter* sp. strain JC1 DSM 3803; i.e., the bacterium grown on methanol was found to exhibit activities of key enzymes for the Calvin and XuMP cycles but no activity of that for the RuMP cycle and very low activity of the serine pathway enzyme. We also report several properties of dihydroxyacetone synthase (DHAS), the key enzyme for XuMP cycle (2, 8, 9), purified from cells of *Acinetobacter* sp. strain JC1 DSM 3803 to learn more about the diversity of DHAS in methylotrophic organisms.

MATERIALS AND METHODS

Organism and cultivation. *Acinetobacter* sp. strain JC1 DSM 3803 (hereafter referred to as JC1) (6) was cultivated at 30°C in a mineral medium (14) supplemented with 0.5% (vol/vol) methanol. For several experiments, *Methylobacterium extorquens* AM1 (NCIB 9133), *Methylophilus methylotrophus* (NCIB 10515), and *Candida boidinii* (KCTC 1712) grown under the same conditions were used as controls. Growth was measured by turbidity determined at 436 nm, using a Hitachi U-2000 spectrophotometer. Cells were harvested at the late exponential growth phase, washed once with 0.05 M potassium phosphate buffer (pH 7.0) (standard buffer), and stored at −20°C.

Protein determination. Protein was determined by the method of Lowry et al. (22), using bovine serum albumin as a standard. The amounts of protein in crude cell extracts were estimated by the same method after boiling in 20% NaOH for 10 min (14).

Enzyme assays. All assays were carried out at 30°C unless otherwise described.

Hydroxypyruvate reductase (HPR) activity was assayed by measuring the hydroxypyruvate-dependent oxidation of NADH by the method of Large and Quayle (20). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of NADH per min.

Hexulose 6-phosphate synthase (HPS) activity was assayed at 37°C according to the method of Ferenci et al. (10) by measuring the decrease in the amount of formaldehyde after the reaction of the added formaldehyde with the ribulose 5-phosphate formed in the reaction mixture. Formaldehyde was determined by the method of Nash (26). One unit of enzyme activity was defined as the amount of enzyme required to consume 1 μmol of formaldehyde per min.

DHAS activity was assayed, depending on the purpose of the experiment, by one of three methods described previously (12, 37), with several modifications. For routine assay and to test the effects of glycoaldehyde acceptors on DHAS activity, the activity was measured by a modification of the method of Kato et al. (12) (method A). The reaction mixture (1 ml) contained 50 μmol of standard buffer (pH 7.0), 0.5 μmol of xylulose 5-phosphate (Xu5P), 5 μmol of $MgCl_2$, 0.5 μmol of thiamine pyrophosphate (TPP), 0.16 μmol of NADH, 62.6 U triose-

* Corresponding author. Fax: 82-2-312-5657. E-mail: young547@bubble.yonsei.ac.kr.

† Present address: Department of Virology and Immunology, Southwest Foundation for Biomedical Research, San Antonio, TX 78245.

phosphate isomerase (from baker's yeast; Sigma Chemical Co.), 0.26 U of α-glycerophosphate dehydrogenase (from rabbit muscle; Sigma), and cell extracts. To test the effect of glycoaldehyde donors on DHAS activity, the activity was assayed by a method based on the system described by Waits and Quayle (37) (method B). The reaction mixture of method B was the same as that for method A except that the mixture (1 ml) contained 1 μmol ATP and 0.23 U of glycerokinase (from *Candida mycoderma*; Sigma) instead of triosephosphate isomerase. The mixtures for methods A and B were incubated for 90 s to determine endogenous activity. The reaction was started by the addition of 1 μmol of formaldehyde, and the reduction in absorbancy at 340 nm ($\varepsilon_{340} = 6.22 \times 10^3$ M^{-1} cm^{-1} [7]) was measured between 75 and 105 s after addition of formaldehyde. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of NADH per min. To examine the effect of pH and temperature on enzyme activity, the activity was assayed by a method based on the consumption of formaldehyde during the reaction (12) (method C). The reaction mixture (1 ml) for this method was the same as that for the method A except that it did not contain NADH, triosephosphate isomerase, and α-glycerophosphate dehydrogenase. Thirty minutes after addition of formaldehyde, the reaction was stopped by the addition of 100 μl of 4 N HCl. The mixture was then centrifuged at 15,000 × *g* for 20 min, and the supernatant was analyzed for formaldehyde (26). One unit of enzyme activity was defined as the amount of enzyme required to consume 1 μmol of formaldehyde per min.

Dihydroxyacetone kinase (DHAK) activity was assayed by measuring the dihydroxyacetone (DHA)- and ATP-dependent oxidation of NADH according to the method of Kato et al. (11), with modifications. The reaction mixture (1 ml) contained 0.1 μmol of potassium phosphate buffer (pH 7.5), 1.0 μmol of ATP, 16 μmol of $MgCl_2$, 0.15 μmol of NADH, 3 U of glycerol 3-phosphate dehydrogenase, and cell extracts. The reaction was started by the addition of 1 μmol of DHA. One unit of enzyme was defined as the amount of enzyme needed to oxidize 1 μmol of NADH per min.

Fructose 1,6-bisphosphatase (FBPase) activity was assayed by measuring the fructose 1,6-bisphosphate-dependent reduction of NADP at 340 nm by the method of Attwood and van Dijken (3). One unit of enzyme activity was defined as the amount of enzyme needed to reduce 1 μmol of NADP per min.

Ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) activity was assayed by the method of McFadden and Tu (23), with several modifications: the amount of Tris added in the reaction mixture (0.25 ml) was 12.5 μmol instead of 15 μmol, and the enzyme reaction was stopped at 10 min, instead of 5 min, after addition of ribulose 1,5-bisphosphate. One unit of enzyme activity was defined as the amount of enzyme required to incorporate 1 μmol of CO_2 per min.

DHAS purification. All purification steps were carried out at 4°C except when noted otherwise. Samples were concentrated, if needed, with an ultrafiltration membrane (Amicon YM10) under an atmosphere of nitrogen gas.

An 80-g portion of thawed cells was suspended in 240 ml of cold standard buffer and disrupted by sonic treatment (10 s per ml). The suspension was centrifuged at 15,000 × *g* for 30 min. The resulting supernatant (crude extract) was then treated with protamine sulfate to a final concentration of 0.054%, left in ice for 10 min, and then sedimented at 100,000 × *g* for 90 min. The resulting supernatant fluid (soluble fraction) was next made 20% saturated with respect to ammonium sulfate. After 30 min, this fraction was centrifuged at 15,000 × *g* for 30 min. The resulting supernatant was further treated with ammonium sulfate to achieve a final concentration of 60% saturation. After 30 min, the solution was centrifuged again at 15,000 × *g* for 30 min, and the sediment was resuspended in a small volume of cold standard buffer. The suspension was then applied to a Phenyl-Sepharose column (3.2 by 15 cm) preequilibrated with standard buffer containing 2 M ammonium sulfate. Elution was first carried out with 300 ml of standard buffer containing 2 M ammonium sulfate followed by a linear ammonium sulfate gradient (1,200 ml, 2 to 0 M) in standard buffer. Fractions were collected at a flow rate of 7.2 ml/cm^2 per h, and fractions with high specific activity were pooled. The pooled fractions were then applied to a DEAE-Sephacel column (2.6 by 24 cm) preequilibrated with standard buffer, and elution was performed with 290 ml of standard buffer followed by a linear KCl gradient (700 ml, 0 to 1 M) in standard buffer at a flow rate of 5.1 ml/cm^2 per h. Fractions with high specific activity were pooled and concentrated with an ultrafiltration membrane. The concentrate (5 ml) was then mixed with 100 ml of 10 mM potassium phosphate buffer (pH 7.0) and concentrated again with the same membrane. The preparation was applied to a hydroxylapatite column (2.4 by 10 cm) previously equilibrated with 0.01 M potassium phosphate buffer. Elution was carried out with 120 ml of the same buffer followed by a linear potassium phosphate gradient (180 ml, 0.01 to 0.3 M) at a rate of 8.7 ml/cm^2 per h, and fractions with high specific activity were pooled. The pooled fractions were then applied to a Q-Sepharose column (2.2 by 10 cm) preequilibrated with 0.2 M KCl in standard buffer. The column was first washed with 90 ml of standard buffer containing 0.2 M KCl, and elution was performed with a linear KCl gradient (150 ml, 0.2 to 0.5 M) in standard buffer at a rate of 7.1 ml/cm^2 per h. Fractions with high activity were pooled and concentrated with an ultrafiltration membrane. The concentrate was then applied to a Sephacryl S-200 column (1.6 by 120 cm) preequilibrated with standard buffer. Elution was done with standard buffer at a flow rate of 5.5 ml/cm^2 per h. Fractions with the highest specific activity were pooled and stored at −20°C under air.

Electrophoresis. Nondenaturing polyacrylamide gel electrophoresis (PAGE) was performed by a procedure described by Laemmli (17), but without sodium dodecyl sulfate. Denaturing PAGE was carried out by the method of Laemmli (17). Proteins were stained with Coomassie brilliant blue R-250 by a modification (14) of the method of Weber and Osborn (38).

Effect of pH and temperature. To determine the optimal temperature for DHAS activity, the enzyme was assayed by method C at various temperatures (20, 25, 30, 35, 40, 50, and 60°C). The optimal pH was determined by the same method at 30°C except that the 1-ml reaction mixture contained 50 μmol of succinate buffer (pH 5.0 and 6.0), 50 μmol of potassium phosphate buffer (pH 6.0, 6.5, 7.0, 7.5, and 8.0), or 50 μmol of Tris-HCl buffer (pH 8.0 and 9.0) instead of 50 μmol of standard buffer. Thermal stability was tested after incubation of the enzyme for 1 min at various temperatures (30, 40, 50, 60, and 70°C), and then the remaining activity of the heat-treated enzymes at 30°C was measured by method C.

Detection of DHA. DHA in the reaction mixture after reaction of DHAS with Xu5P and formaldehyde was identified by thin-layer chromatography as described by Waits and Quayle (35), with slight modification. The DHAS reaction was started by the addition of purified enzyme into 1 ml of reaction mixture containing 50 μmol of standard buffer, 5 μmol of $MgCl_2$, 0.5 μmol of TPP, 2 μmol of Xu5P, and 2 μmol of formaldehyde. After incubation for 1 h at 30°C, the reaction was terminated by the addition of 1 ml of acetone and the mixture was centrifuged for 5 min at 500 × *g*. The resulting precipitate was dried at 60°C in an oven and resuspended in 50 μl of acetone. The suspension was then applied to a silica gel plate (20 by 20 cm; Merck) together with three reference samples for ascending chromatography in a rectangular glass chamber saturated with a solvent mixture of ethylmethyl ketone-acetic acid-boric acid (4%, wt/vol) (9:1:1, vol/vol/vol). One of the references was the product of the above-specified mixture containing no DHAS. The second reference contained, in addition to the above-specified mixture, 0.16 μmol of NADH, 62.6 U of triosephosphate isomerase, 0.26 U of α-glycerophosphate dehydrogenase, and purified DHAS. The third reference was 50 μmol of authentic DHA (Sigma) dissolved in standard buffer. Spots separated on the silica gel plate were then visualized with a mixture containing equal volumes of 0.1 M aniline and 0.1 M orthophosphoric acid solutions.

Immunological test. Antiserum against the purified DHAS was raised in a New Zealand White rabbit as described previously (14). Double-immunodiffusion assays were performed in 1.2% agarose gel by a modification (16) of the method of Ouchterlony and Nilson (30).

Immunoblotting. The expression patterns of DHAS and RuBisCO in cells growing on methanol were analyzed as described in the ECL (enhanced chemiluminescence) Western blotting protocols (Amersham, Little Chalfont, England) after transfer to a nitrocellulose membrane (Hybond-ECL; Amersham) of the proteins in cell extracts prepared from cells growing at various growth phases. To prepare the cell extracts, a 100-ml culture of *Acinetobacter* sp. strain JC1 grown in a mineral medium (14) supplemented with 0.2% (wt/vol) pyruvate was inoculated into 5 liters of the same medium and cultivated at 30°C. Cells were harvested at the late stationary phase under sterile condition, suspended in a small volume of the mineral medium, and reinoculated into 5 liters of the same medium supplemented with 0.5% (vol/vol) methanol. Cells were collected at appropriate intervals during cultivation and stored immediately at −70°C. The thawed cells were resuspended in standard buffer and disrupted by sonic treatment (15 s per ml). The suspensions were centrifuged at 25,000 × *g* for 30 min at 4°C, and proteins in the resulting supernatants were then subjected to nondenaturing PAGE prior to transfer to the nitrocellulose membrane (33). Antiserum against purified RuBisCO of *Acinetobacter* sp. strain JC1 (13) was raised in a New Zealand White rabbit in the same manner as the antiserum against the purified DHAS.

RESULTS

Enzymes for assimilation of methanol. *Acinetobacter* sp. strain JC1 grown on methanol showed very low HPS, the key enzyme for RuMP cycle, and no HPR, the key enzyme for serine pathway, activities (Table 1). The cells instead exhibited DHAS and DHAK activities, which are known to be present only in methylotrophic yeasts. FBPase activity was also present in methanol-grown cells. Cells grown on methanol also exhibited RuBisCO activity (0.016 U per mg of protein) similar to that of the cells grown on CO (0.019 U per mg of protein). Cells grown with pyruvate or glucose did not show DHAS, DHAK, and RuBisCO activities. *M. extorquens* AM1 and *M. methylotrophus* showed high activities of HPR and HPS, respectively.

Purification of DHAS. The purification steps for DHAS of *Acinetobacter* sp. strain JC1 grown on methanol are summarized in Table 2. The enzyme was purified 19-fold in eight steps to homogeneity, with a yield of 9% and a specific activity of 1.12 U per min per mg of protein.

TABLE 1. Activities of several enzymes in methylotrophic microorganisms grown on methanol

Methylotroph	Sp act[a]				
	HPR[b]	HPS[c]	DHAS[d]	DHAK[e]	FBPase[f]
Acinetobacter sp. strain JC1	—[g]	<0.001	0.11	0.04	0.24
M. extorquens AM1	0.18	—	—	—	0.27
M. methylotrophus	—	0.12	—	—	0.30
C. boidinii	NT[h]	NT	0.19	0.21	0.29

[a] Mean of five tests.
[b] Micromoles of NADH oxidized per milligram of protein per minute.
[c] Micromoles of formaldehyde consumed per milligram of protein per minute.
[d] Micromoles of NADH oxidized per milligram of protein per minute.
[e] Micromoles of NADH oxidized per milligram of protein per minute.
[f] Micromoles of NAD reduced per milligram of protein per minute.
[g] —, no activity.
[h] NT, not tested.

Molecular weight and structure. The molecular weight of the native enzyme was estimated to be 140,000 by gel filtration on a Sephacryl S-300 column (1.8 by 105 cm), using the method of Andrews (1) with reference proteins of known molecular weights. Denaturing PAGE revealed the presence of a single subunit in the native enzyme. The molecular weight of the subunit was determined to be 73,000 by denaturing PAGE with several proteins of known molecular weights as references.

Optimal pH and temperature and thermal stability. The purified enzyme was most active at pH 7.0 and 30°C. When the enzyme was left at various temperatures for 1 min, over 50 and 95% of the original activity were inactivated at 60 and 70°C, respectively.

Effect of composition of the assay mixture. To examine the effect on DHAS activity of the individual components present in the assay mixture, the enzyme activity was determined by method A, but in the absence of Xu5P, formaldehyde, TPP, or $MgCl_2$. The enzyme revealed no activity in the absence of Xu5P or formaldehyde. The enzyme exhibited 42, 40, and 27% of the activity found in the complete mixture in the absence of $MgCl_2$, TPP, and both of the two components, respectively.

Substrate specificity. When the enzyme was assayed with Xu5P as a glycoaldehyde donor, erythrose 4-phosphate, glycolaldehyde, formaldehyde, glyceraldehyde, and acetaldehyde, but not DHA, glutaraldehyde, or glucose 6-phosphate, were able to act as glycoaldehyde acceptors (Table 3). Erythrose 4-phosphate and glycolaldehyde, especially, were found to be better acceptors than formaldehyde. Xu5P served as the best glycoaldehyde donor among those tested, though hydroxypyruvate, ribulose 5-phosphate, and fructose 6-phosphate also served in this capacity, in an assay mixture of the method B containing formaldehyde as a glycoaldehyde acceptor (Table 3).

TABLE 2. Purification of DHAS from *Acinetobacter* sp. strain JC1 grown on methanol

Purification step	Total protein[a] (mg)	Sp act[b]	Purification (fold)	Total activity[c]	Recovery (%)
Crude extract	4,364	0.06	1	261.8	100
Soluble fraction	3,960	0.08	1.3	316.8	121
Ammonium sulfate (25–60%)	2,969	0.11	1.8	326.6	125
Phenyl-Sepharose	809	0.22	3.7	178.0	86
DEAE-Sephacel	291	0.23	3.8	67.0	26
Hydroxylapatite	59	0.50	8.3	29.5	11
Q-Sepharose	30	0.78	13.0	23.4	9
Sephacryl S-200	20	1.12	18.7	22.4	9

[a] Method of Lowry et al. (22).
[b] Micromoles of NADH oxidized per milligram of protein per minute.
[c] Micromoles of NADH oxidized per minute.

TABLE 3. Substrate specificity of purified DHAS for the acceptors and donors of the glycoaldehyde group

Substrate	Concn (mM)	Relative activity[a] (%)
Acceptor[b]		
Formaldehyde	2	100.0
Acetaldehyde	2	14.3
Glycolaldehyde	2	180.0
Glyceraldehyde	2	77.1
Glutaraldehyde	2	0.0
Dihydroxyacetone	2	0.0
D-Erythrose 4-phosphate	1	194.3
D-Glucose 6-phosphate	2	0.0
Donor[c]		
D-Xylulose 5-phosphate	2	100.0
Hydroxypyruvate	2	41.4
D-Ribose 5-phosphate	2	0.0
D-Ribulose 5-phosphate	2	44.8
D-Glucose 6-phosphate	2	0.0
D-Fructose 6-phosphate	2	37.9

[a] The activity with formaldehyde and Xu5P as the acceptor and donor, respectively, was set as 100%.
[b] 0.5 mM Xu5P was used as a glycoaldehyde donor.
[c] 2 mM formaldehyde was used as a glycoaldehyde acceptor.

Kinetic properties. The rates of assimilation of Xu5P and formaldehyde were proportional to the amount of enzyme added. Methods A and B were used to measure the dependence of the rate of NADH oxidation on the concentration of formaldehyde and Xu5P, respectively, to determine the K_m and V_{max} of the purified DHAS, except that various amounts of formaldehyde and Xu5P were added to the reaction mixture. The rate of NADH oxidation followed Michaelis-Menten kinetics. On a Lineweaver-Burk (21) plot, the K_m and V_{max} for Xu5P were 33.3 μM and 0.44 μmol of Xu5P per min per mg of protein, respectively, and those for formaldehyde were 1.86 mM and 0.66 μmol of formaldehyde per min per mg of protein, respectively.

DHA was produced as a reaction product. DHA was found to be synthesized as a product of the reaction of the purified DHAS with Xu5P and formaldehyde (Fig. 1, lane b). Slightly more DHA was detected from a reaction mixture containing triosephosphate isomerase, α-glycerophosphate dehydrogenase, and NADH in addition to the components present in the reaction mixture for the lane b (Fig. 1, lane c). This may be caused by the consumption of glyceraldehyde 3-phosphate, another product of the DHAS reaction, by the three extra additives, which resulted in the increased production of DHA. The product, however, was not found in the mixture containing no DHAS (Fig. 1, lane a). The R_f value of DHA was 0.62.

Immunological properties. Double immunodiffusion revealed that the DHAS of *Acinetobacter* sp. strain JC1 was expressed only in cells growing on methanol or methylamine, not in cells growing with CO, pyruvate, or glucose (Fig. 2). The enzyme was also found to share no immunological properties with DHAS in cell extracts prepared from methanol-grown cells of *C. boidinii* (data not shown).

Expression pattern of DHAS. DHAS in *Acinetobacter* sp. strain JC1 was expressed at the very early stage during growth

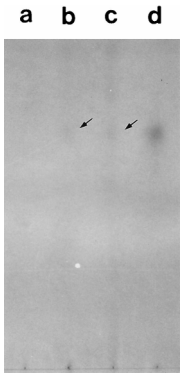

FIG. 1. Thin-layer chromatography of the reaction product of purified DHAS. The product of each of reaction mixture containing 50 μmol of standard buffer, 5 μmol of $MgCl_2$, 0.5 μmol of TPP, 2 μmol of Xu5P, and 2 μmol of formaldehyde (standard mixture) (lane a), standard mixture plus purified enzyme (80 μg) (lane b), and standard mixture plus purified enzyme (80 μg) supplemented with 0.16 μmol NADH, 62.6 U of triosephosphate isomerase, and 0.26 U of α-glycerophophate dehydrogenase (lane c) was analyzed by thin-layer chromatography using a silica gel plate and a solvent mixture of ethylmethyl ketone-acetic acid-boric acid (4%, wt/vol) (9:1:1, vol/vol/vol) as described in Materials and Methods. Lane d contains 50 μmol of standard buffer plus 50 μmol of authentic DHA. Arrows indicate DHA spots.

on methanol; i.e., the bacterium was found to produce DHAS in 5 min after inoculation of the cells grown on pyruvate into the methanol-containing medium (Fig. 3B). The RuBisCO, however, was expressed 15 min later than DHAS (Fig. 3A).

DISCUSSION

The presence of no HPR and very low HPS activities in *Acinetobacter* sp. strain JC1 grown on methanol indicated that the bacterium does not synthesize cellular materials from C_1 units through the serine and RuMP pathways, the most common routes for C_1 assimilation in methylotrophic bacteria (2).

Detection of DHAS and DHAK, the key enzymes for XuMP cycle, activities from *Acinetobacter* sp. strain JC1 is quite interesting since no methylotrophic bacteria have been reported to exhibit the two enzyme activities. These findings together with the result that *Acinetobacter* sp. strain JC1 grown on methanol also showed FBPase, another key enzyme for the XuMP cycle, activity suggest that the XuMP cycle is involved in C_1 assimilation during growth of the cells on methanol. The observation that DHAS purified from *Acinetobacter* sp. strain JC1 produced DHA with Xu5P and formaldehyde as substrates strongly supports this indication since the DHAS found in methylotrophic yeasts catalyzes the transfer from Xu5P of the glycolaldehyde group to formaldehyde, resulting in the production of DHA and glyceraldehyde 3-phosphate (34).

In addition to the XuMP cycle, *Acinetobacter* sp. strain JC1 was suggested to operate another route, the Calvin cycle, for C_1 assimilation since cells grown on methanol were found to exhibit high RuBisCO activity. Conversion of CO_2 to organic materials through the Calvin cycle requires 2 mol of NADPH plus H^+ per mol of CO_2 fixed, which is a big burden for the bacterium. This problem, however, can be partly overcome by the so-called dissimilatory XuMP cycle, which is also assumed to operate in this bacterium since the cell was found to show high activities of NADP-dependent glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (31), the two key enzymes for the dissimilatory XuMP cycle (19). *P. gazotropha*, another carboxydobacterium, was reported to assimilate methanol through the serine pathway (32).

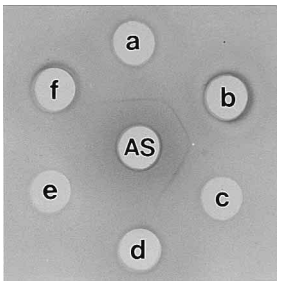

FIG. 2. Double-immunodiffusion patterns for DHASs in cells grown on different substrates. Immunodiffusion assays were performed in 1.2% agarose gel for 24 h at 30°C, followed by staining with Coomassie brilliant blue R-250 as described in Materials and Methods. Wells: AS, antiserum raised against purified enzyme (20 μl); a, b, and d to f, cell extracts of *Acinetobacter* sp. strain JC1 grown on methanol (152 μg) (a), methylamine (185 μg) (b), pyruvate (102 μg) (d), CO (178 μg) (e), and glucose (298 μg) (f); c, purified DHAS (32 μg).

The earlier expression of DHAS than of RuBisCO in cells of *Acinetobacter* sp. strain JC1 growing on methanol suggests that the assimilation of the C_1 metabolites in *Acinetobacter* sp. strain JC1 may first begin with the XuMP cycle, followed by both the XuMP and Calvin cycles. This is quite feasible since it may be advantageous for the bacterium at the very early stage of adaptation to the methanol medium to employ the XuMP cycle, which requires three molecules of ATP and no reducing power to make a glyceraldehyde 3-phosphate molecule, than to use the Calvin cycle, which consumes nine and six molecules, respectively, of ATP and NADPH to synthesize the C_3 compound. The 15-min interval between the expression of DHAS and RuBisCO implies that *Acinetobacter* sp. strain JC1 grown on pyruvate may require at least 20 min to be fully adapted to the new environment. It seems that the RuBisCO may play a more active role than the DHAS after the early stage of growth since the RuBisCO was found to be one of the major proteins produced in cells growing on methanol (13).

It is known that some methylotrophic bacteria possess key enzymes of more than one C_1 assimilation pathway, except the XuMP cycle, and that no methylotrophic yeasts contain key enzymes for the bacterial C_1 assimilation pathways (18). It therefore is very interesting that *Acinetobacter* sp. strain JC1 possesses the key enzymes for the XuMP cycle, which have been detected only in yeasts. It, however, is more interesting that the bacterium contains RuBisCO in addition to the yeast enzyme, indicating that *Acinetobacter* sp. strain JC1 may be the first bacterium possessing the eucaryotic C_1 assimilation pathway and also the first organism possessing both the procaryotic and eucaryotic C_1 assimilation enzymes. In a previous study, *Methylococcus capsulatus* Bath, which assimilates carbon via

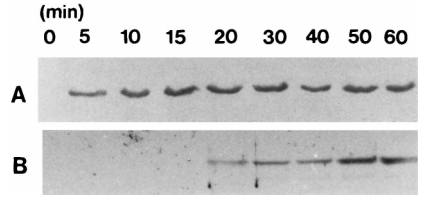

FIG. 3. Immunoblotting of DHAS and RuBisCO. The expression patterns of DHAS and RuBisCO in cells of *Acinetobacter* sp. strain JC1 growing on methanol were analyzed by ECL Western blotting protocols. Cells harvested at the indicated time intervals after inoculation of the pyruvate-grown cells into methanol-containing medium were disrupted, subjected to nondenaturing PAGE, and immunoblotted with antiserum raised against purified DHAS (A) and RuBisCO (B) as described in Materials and Methods.

TABLE 4. Comparison of some properties of DHASs from different sources

Properties	*Acinetobacter*	*C. boidinii*		
		CBS 5777	KD1	2201
Sp act	1.12[a]	0.77[a]	4.0[b]	3.88[a]
Mol wt	140,000	107,500	145,000	190,000
Subunit structure	73,000 × 2	65,500 × 2	76,000 × 2	55,000 × 4
Temp optimum (°C)	30	ND[c]	ND	35
pH optimum	7.0	7.4–7.6	6.8–7.1	7.0
Stability	Unstable	Very unstable	Very unstable	Very unstable
Cofactors				
TPP requirement	+	+	+	+
Mg^{2+} requirement	+	+	+	+
Donor substrates				
Xylulose 5-phosphate	+	+	+	+
Hydroxypyruvate	+	+	NT[d]	+
Fructose 6-phosphate	+	+	±	+
Ribulose 5-phosphate	+	–[e]	–	–
Ribose 5-phosphate	–	–	NT	–
Glucose 6-phosphate	–	–	NT	–
Acceptor substrates				
Formaldehyde	+	+	+	+
Glycolaldehyde	+	+	NT	+
Glyceraldehyde	+	+	–	+
Acetaldehyde	+	+	NT	+
Erythrose 4-phosphate	+	+	–	NT
Ribose 5-phosphate	NT	+	–	+
Glucose 6-phosphate	–	–	–	NT
Glutaraldehyde	–	–	NT	NT
K_m (mM)				
Formaldehyde	1.86	1.14	NT	0.43
Xylulose 5-phosphate	0.033	0.72	NT	1.0
Reference	This work	37	5	12

[a] Micromoles of NADH oxidized per milligram of protein per minute.
[b] Micromoles of triose phosphate produced per milligram of protein per minute.
[c] ND, not determined. Activity, however, was measured at 30°C.
[d] NT, not tested.
[e] –, no activity.

the RuMP pathway, was found to produce DHA as a response to formaldehyde accumulation in the cell, but the enzyme responsible for the production of DHA was not identified (4).

DHAS was purified to homogeneity by conventional methods. The specific activity of the enzyme was higher than that of the *C. boidinii* CBS 5777 enzyme (37) but lower than that of the *C. boidinii* 2201 (12) and *C. boidinii* KD1 (5) enzymes (Table 4).

The presence of only one kind of subunit in the purified enzyme together with the molecular weights of the native enzyme (140,000) and subunit (73,000) indicates that the DHAS of *Acinetobacter* sp. strain JC1 consists of two identical subunits, which is the same as for the *C. boidinii* CBS 5777 and *C. boidinii* KD1 enzymes except for the differences in the molecular sizes of the native enzyme and peptide subunit (Table 4). The molecular size and structure of the purified enzyme, however, were completely different from those of the *C. boidinii* 2201 enzyme, which has a molecular weight of 190,000 and is composed of four identical subunits with a molecular weight of 55,000.

The optimal pH and temperature of the purified DHAS were found to be similar to those of the yeast enzymes (Table 4). The stability of the purified enzyme was also found to be similar to that of the very unstable yeast enzymes since the purified enzyme lost over 50% and almost all of the original activity in 1 min of incubation at 60 and 70°C, respectively, indicating that both procaryotic and eucaryotic DHASs are unstable enzymes.

The purified enzyme was found to require both TPP and Mg^{2+} ion for maximal activity since the activity was reduced by 58 and 60%, respectively, in the absence of either component. This requirement is the same as that of the yeast enzymes exhibiting reduced level of activity without the components (Table 4).

The DHAS present in cells grown only on methanol is a special transketolase able to use formaldehyde as the acceptor for the glycoaldehyde group from the donor substrates (5, 12, 37). The classical transketolase found in cells grown on C_1 compounds as well as on multicarbon substrates, however, is known to exhibit strong activity on aldose phosphates such as

erythrose 4-phosphate and ribose 5-phosphate, but little or no activity with formaldehyde, as a glycoaldehyde acceptor (5, 12, 29, 35, 36). The DHASs from *C. boidinii* CBS 5777 and *C. boidinii* 2201 were found to have both the DHAS and classical transketolase activities; i.e., the enzymes exhibited transketolase activity with aldose phosphates, in addition to formaldehyde, as the acceptor for the glycoaldehyde group (12, 37). *C. boidinii* KD1, however, was found to have DHAS which uses formaldehyde as the only glycoaldehyde group acceptor among those aldehydes and aldose phosphates tested (5), indicating that the DHAS of this bacterium is more specific than those of the other two *C. boidinii* strains.

The broad substrate specificity of the purified enzyme of *Acinetobacter* sp. strain JC1 for glycoaldehyde donor and acceptor was very similar to that of the *C. boidinii* CBS 5777 and *C. boidinii* 2201 enzymes except that ribulose 5-phosphate was not used as a glycoaldehyde donor by the *C. boidinii* CBS 5777 and *C. boidinii* 2201 enzymes (Table 4). Considering that the DHAS of *C. boidinii* KD1 used formaldehyde and Xu5P as the only acceptor and, possibly, the only donor, respectively, for glycoaldehyde group among those aldehydes and aldose phosphates tested (5), this result indicates that the DHAS of *Acinetobacter* sp. strain JC1 is less specific than the *C. boidinii* KD1 enzyme, like the other two *C. boidinii* enzymes. The DHASs from *Acinetobacter* sp. strain JC1, *C. boidinii* CBS 5777, and *C. boidinii* 2201 showed similar orders of preference of the acceptor and donor substrates and used Xu5P as the best glycoaldehyde donor. The three enzymes, however, did not use formaldehyde as the preferred glycoaldehyde acceptor. Erythrose 4-phosphate was found to be the best acceptor for *Acinetobacter* sp. strain JC1 enzyme, while glycolaldehyde was preferred for the two *C. boidinii* enzymes (12, 37).

The affinity to formaldehyde of the purified enzyme seems to be lower than that of the DHASs from *C. boidinii* strains since the K_m for formaldehyde of the purified enzyme is higher than that of the DHASs from *C. boidinii* CBS 5777 and *C. boidinii* 2201 (Table 4). The K_m for Xu5P of the purified enzyme, on the other hand, is much lower than that of the *C. boidinii* CBS 5777 and *C. boidinii* 2201 enzymes, indicating that the affinity on Xu5P of *Acinetobacter* sp. strain JC1 DHAS is higher than that of the yeast enzymes.

The absence of proteins in cells of *Acinetobacter* sp. strain JC1 grown on glucose or pyruvate cross-reacting with the antiserum raised against the purified DHAS indicates that DHAS in *Acinetobacter* sp. strain JC1 is expressed only in cells growing on methanol. This result is further supported by the observation that DHAS activity was detected only in cells grown on methanol. These findings together with the substrate specificity and the ability to produce DHA of the purified enzyme indicate that the DHAS expressed in *Acinetobacter* sp. strain JC1 is an enzyme specific for the assimilation of methanol in this bacterium.

The properties of the purified enzyme mentioned above suggest that the first step for the assimilation of methanol through the XuMP cycle is mediated by different enzymes of the synthase type. Taken together with the facts that the DHAS, which has been known to be present only in methylotrophic yeasts, was also found in a methylotrophic bacterium and there was no antigenic sites in the purified DHAS cross-reacting with the enzyme in methylotrophic yeast, this observation suggests either that the ability to assimilate methanol through the XuMP cycle evolved independently in different evolutionary lines or at a very early time before divergence occurred, or that genetic exchange by wide-ranging mechanisms dispersed the gene for a common ancestral DHAS to many different groups of methylotrophic organisms at some remote time.

ACKNOWLEDGMENTS

This work was supported by a research grant for Basic Science Research Institute (BSRI 96-4419) from the Ministry of Education, Korea.

REFERENCES

1. **Andrews, P.** 1965. The gel filtration behavior of proteins related to their molecular weights over a wide range. Biochem. J. **146:**289–298.
2. **Anthony, C.** 1982. The biochemistry of methylotrophs. Academic Press, New York, N.Y.
3. **Attwood, M. M., and J. P. van Dijken.** 1982. Characteristics of fructose 1,6-bisphosphatase from the methanol utilizing yeast *Hansenula polymorpha*. J. Gen. Microbiol. **128:**2313–2317.
4. **Botte, H. F.** 1992. Formaldehyde-dependent dihydroxyacetone production by *Methylococcus capsulatus* (Bath): a novel regulatory mechanism in C_1-assimilation, abstr. A26. *In* Programme and abstracts of the 7th International Symposium on Microbial Growth on C_1 Compounds. Organizing Committee of the 7th International Symposium on Microbial Growth on C_1 Compounds, Warwick, United Kingdom.
5. **Bystrykh, L. V., A. P. Sokolov, and Y. A. Trotsenko.** 1981. Purification and properties of dihydroxyacetone synthase from the methylotrophic yeast *Candida boidinii*. FEBS Lett. **132:**324–328.
6. **Cho, J. W., H. S. Yim, and Y. M. Kim.** 1985. *Acinetobacter* isolates growing with carbon monoxide. Kor. J. Microbiol. **23:**1–8.
7. **Dawson, R. M. C., D. C. Elliott, W. H. Elliott, and K. M. Jones.** 1986. Data for biochemical research, 3rd ed., p. 122–123. Oxford Science Publications, New York, N.Y.
8. **de Koning, W., and W. Harder.** 1992. Methanol-utilizing yeasts, p. 207–244. *In* J. C. Murrell and H. Dalton (ed.), Methane and methanol utilizers. Plenum Press, New York, N.Y.
9. **Dijkhuizen, L., P. R. Levering, and G. E. de Vries.** 1992. The physiology and biochemistry of aerobic methanol-utilizing Gram-negative and Gram-positive bacteria, p. 149–181. *In* J. C. Murrell and H. Dalton (ed.), Methane and methanol utilizers. Plenum Press, New York, N.Y.
10. **Ferenci, T., T. Strom, and J. R. Quayle.** 1974. Purification and properties of 3-hexulose phosphate synthase and phospho-3-hexuloisomerase from *Methylococcus capsulatus*. Biochem. J. **144:**477–486.
11. **Kato, N., H. Yoshikawa, K. Tanaka, M. Shimao, and C. Sakazawa.** 1988. Dihydroxyacetone kinase from a methylotrophic yeast, *Hansenula polymorpha* CBS 4732. Arch. Microbiol. **150:**155–159.
12. **Kato, N., T. Higuchi, C. Sakazawa, T. Nishizawa, Y. Tani, and H. Yamada.** 1982. Purification and properties of a transketolase responsible for formaldehyde fixation in a methanol utilizing yeast, *Candida boidinii* (*Kloeckera* sp.) no. 2201. Biochim. Biophys. Acta **715:**143–150.
13. **Kim, E. Y., Y. T. Ro, and Y. M. Kim.** 1997. Purification and some properties of ribulose 1,5-bisphosphate carboxylases/oxygenases from *Acinetobacter* sp. strain JC1 and *Hydrogenophaga pseudoflava*. Mol. Cells **7:**380–388.
14. **Kim, Y. M., and G. D. Hegeman.** 1981. Purification and some properties of carbon monoxide dehydrogenase from *Pseudomonas carboxydohydrogena*. J. Bacteriol. **148:**904–911.
15. **Kim, Y. M., and G. D. Hegeman.** 1983. Oxidation of carbon monoxide by bacteria. Int. Rev. Cytol. **81:**1–32.
16. **Kwon, M. O., and Y. M. Kim.** 1985. Relationship between carbon monoxide dehydrogenase and a small plasmid in *Pseudomonas carboxydovorans*. FEMS Microbiol. Lett. **29:**155–159.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
18. **Large, P. J.** 1983. Methylotrophy and methanogenesis. American Society of Microbiology, Washington, D.C.
19. **Large, P. J., and C. W. Bamforth.** 1988. Methylotrophy and biotechnology. Longman Scientific & Technical, Harlow, England.
20. **Large, P. J., and J. R. Quayle.** 1963. Microbial growth on C_1 compounds. 5. Enzyme activities in extracts of *Pseudomonas* AM1. Biochem. J. **87:**386–396.
21. **Lineweaver, H., and D. Burk.** 1934. The determination of enzyme dissociation constants. J. Am. Chem. Soc. **56:**658–666.
22. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
23. **McFadden, B. A., and C. C. L. Tu.** 1967. Regulation of autotrophic and heterotrophic carbon dioxide fixation in *Hydrogenomonas facilis*. J. Bacteriol. **93:**886–893.
24. **Meyer, O.** 1989. Aerobic carbon monoxide-oxidizing bacteria, p. 331–350. *In* H. G. Schlegel and B. Bowien (ed.), Autotrophic bacteria. Springer-Verlag, Berlin, Germany.
25. **Meyer, O., and H. G. Schlegel.** 1983. Biology of aerobic carbon monoxide oxidizing bacteria. Annu. Rev. Microbiol. **37:**277–310.
26. **Nash, T.** 1953. The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. Biochemistry **55:**416–421.

27. **Nozhevnikova, A. N., and L. N. Yurganov.** 1978. Microbiological aspects of regulating the carbon monoxide content in the earth's atmosphere. Adv. Microb. Ecol. **2:**203–244.
28. **Nozhevnikova, A. N., and G. A. Zavarzin.** 1974. On the taxonomy of CO oxidizing Gram-negative bacteria. Izv. Akad. Nauk SSSR Ser. Biol. **3:**436–440.
29. **O'Connor, M. L., and J. R. Quayle.** 1980. Pentose phosphate-dependent fixation of formaldehyde by methanol-grown *Hansenula polymorpha* and *Candida boidinii*. J. Gen. Microbiol. **120:**219–225.
30. **Ouchterlony, O. J., and L. A. Nilson.** 1978. Immunodiffusion and immunoelectrophoresis, p. 19.1–19.44. *In* D. M. Wier (ed.), Handbook of experimental immunology. Blackwell Scientific Publications, Oxford, England.
31. **Ro, Y. T., J. G. Seo, J. Lee, I. K. Chung, and Y. M. Kim.** 1997. Growth on methanol of a carboxydobacterium, *Acinetobacter* sp. strain JC1 DSM 3803. J. Microbiol. **35:**30–39.
32. **Romanova, A. K., I. Y. Vedenina, K. A. Zykalova, Z. M. Ermolenko, and L. R. Semenova.** 1979. Pathways of assimilation of carbon in methylotrophy of *Pseudomonas gazotropha*. Microbiology **47:**492–498.
33. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
34. **van Dijken, J. P., W. Harder, and J. R. Quayle.** 1981. Energy transduction and carbon assimilation in methylotrophic yeasts, p. 191–201. *In* H. Dalton (ed.), Microbial growth on C_1 compounds. Heydon, London.
35. **Waites, M. J., and J. R. Quayle.** 1980. Dihydroxyacetone: a product of xylulose 5-phosphate-dependent fixation of formaldehyde by methanol-grown *Candida boidinii*. J. Gen. Microbiol. **118:**321–327.
36. **Waites, M. J., and J. R. Quayle.** 1981. The interrelationship between transketolase and dihydroxyacetone synthase activities in the methylotrophic yeast *Candida boidinii*. J. Gen. Microbiol. **124:**309–316.
37. **Waites, M. J., and J. R. Quayle.** 1983. Dihydroxyacetone synthase: a special transketolase for formaldehyde fixation from the methylotrophic yeast *Candida boidinii* CBS 5777. J. Gen. Microbiol. **129:**935–944.
38. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244:**4406–4412.
39. **Zavarzin, G. A., and A. N. Nozhevnikova.** 1977. CO oxidizing bacteria, p. 207–213. *In* H. G. Schlegel et al. (ed.), Microbial production and utilization of gases. E. Goltze KG, Göttingen, Germany.