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Purification and Characterization of Hydroxypyruvate Reductase from the Facultative Methylotroph *Methylobacterium extorquens* AM1

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Hydroxypyruvate reductase was purified to homogeneity from the facultative methylotroph *Methylobacterium extorquens* AM1. It has a molecular mass of about 71 kDa, and it consists of two identical subunits with a molecular mass of about 37 kDa. This enzyme uses both NADH (K_m = 0.04 mM) and NADPH (K_m = 0.06 mM) as cofactors, uses hydroxypyruvate (K_m = 0.1 mM) and glyoxylate (K_m = 1.5 mM) as the only substrates for the forward reaction, and carries out the reverse reaction with glycerate (K_m = 2.6 mM) only. It was not possible to detect the conversion of glycolate to glyoxylate, a proposed role for this enzyme. Kinetics and inhibitory studies of the enzyme from *M. extorquens* AM1 suggest that hydroxypyruvate reductase is not a site for regulation of the serine cycle at the level of enzyme activity.

Methylobacterium extorquens AM1 is a gram-negative facultative methylotroph capable of growth on methanol, methylamine, and a variety of multicarbon compounds which assimilates C_1 units via the serine cycle during growth on C_1 substrates (12, 17). A number of enzymes have been shown to be specifically involved in dissimilation and assimilation of C_1 substrates in *M. extorquens* AM1, and these are known to be inducible by growth on the appropriate C_1 substrates. In addition, the assimilatory enzymes are repressed by the presence of multicarbon compounds such as succinate or glucose, but the dissimilatory enzymes are not (2, 14).

Although intensive work on the methanol oxidation system in *M. extorquens* AM1 is beginning to shed light on both the biochemistry and the regulatory mechanisms involved (1, 13, 15, 16, 18), the serine cycle is less well understood. This cycle functions by the condensation of methylene tetrahydrofolate and glycine to generate serine, conversion of serine to phosphoenolpyruvate via several steps, and carboxylation of phosphoenolpyruvate to generate oxaloacetate. The oxaloacetate is then converted to malyl coenzyme A (CoA), which is cleaved to acetyl-CoA and glyoxylate. The acetyl-CoA is oxidized to glyoxylate, and this is then converted to the acceptor molecule, glycine. However, the route of acetyl-CoA oxidation in this last stage is unknown. Isocitrate lyase is not present in this organism, and a novel, as yet undetermined pathway must carry out this oxidation (2).

Hydroxypyruvate reductase catalyzes the reduction of hydroxypyruvate to glycerate, one of the key steps in the conversion of serine to phosphoenolpyruvate in the serine cycle. It has also been suggested to carry out the reduction of glyoxylate to glycolate in cell extracts, but the significance of this reaction in vivo is not known (2, 4). Mutants in hydroxypyruvate reductase would be expected to be defective in growth on C_1 compounds, but should grow normally on other compounds. However, mutant 20BL, which lacks hydroxypyruvate reductase, is unable to grow on both C_1 and C_2 compounds (4). This suggests a second role for hydroxypyruvate reductase involving C_2 compounds, most likely, the oxidation of glycolate to glyoxylate (4). This reaction may be important in the serine cycle, in the oxidation of acetyl-CoA to glyoxylate, and during growth on C_2 compounds (4). However, the conversion of glycolate to glyoxylate is apparently not essential during growth on C_4 compounds that are metabolized by the C_2 pathway (19). If hydroxypyruvate reductase plays a dual role in the serine cycle, it has the potential to be a key regulatory step during growth on both C_1 and C_2 compounds. Hydroxypyruvate reductase has never been purified from *M. extorquens* AM1, and nothing is known about its regulation by effectors. In addition, although several genes encoding enzymes involved in both dissimilatory and assimilatory C_1 metabolism have been cloned and studied from this organism (13), the gene for hydroxypyruvate reductase has not, and the precise lesion in mutant 20BL is unknown. We are interested in defining the metabolic role of this enzyme in *M. extorquens* AM1 and determining mechanisms of its regulation. We now report the first step in these studies, the purification and characterization of hydroxypyruvate reductase from *M. extorquens* AM1.

MATERIALS AND METHODS

Growth conditions. *M. extorquens* AM1 was grown in an ammonium mineral salts medium (5) at 30°C. Methanol (0.5%, vol/vol) was used as a growth substrate. Cells were harvested in mid-log phase by centrifugation at 11,000 × *g* and 2°C and washed with 50 mM Tris HCl buffer, pH 7.5.

Hydroxypyruvate reductase assay. The standard assay mixture (total volume, 1 ml) contained 50 mM MOPS (morpholinepropanesulfonic acid) buffer (pH 6.6), 0.2 mM NADH or 0.3 mM NADPH, 3.5 mM lithium hydroxypyruvate, and an appropriate amount of the enzyme. The reaction was started with hydroxypyruvate when crude enzyme solution was used or with enzyme solution when pure enzyme was used and was followed spectrophotometrically (340 nm) at 30°C. The assay mixture for the reverse reaction contained 200 mM Tris HCl buffer (pH 9.0), 4 mM NAD, 5 mM D-(−)-glycerate, and an appropriate amount of the enzyme. Spectrophotometric methods (8, 23) were used for protein deter-

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mination. One unit of enzyme was defined as the amount of the enzyme that catalyzed the oxidation of 1 μmol of NAD(P)H per min under the above conditions. Possible inhibition by lithium was tested by including 3.5 mM lithium chloride in the reaction mixture, and this had no effect on activity.

Enzyme purification. All isolation steps were performed at 0 to 4°C. The buffer used for purification procedures was 50 mM Tris HCl, pH 7.5, containing 10% glycerol and 1 mM dithiothreitol (buffer A), unless otherwise specified.

(i) Step 1. Preparation of a cell extract. Washed cells collected from 4 liters of culture were suspended in 50 mM Tris HCl buffer, pH 7.5, and passed through a French pressure cell four times at 20,000 lb/in^2. The cell debris was centrifuged at 40,000 × *g* for 40 min to obtain a crude cell extract.

(ii) Step 2. Ammonium sulfate fractionation. The supernatant from step 1 was taken to 40% of saturation with $(NH_4)_2SO_4$. After equilibration, the mixture was centrifuged (40,000 × *g*, 5 min) and the resulting supernatant was taken to 80% of saturation with $(NH_4)_2SO_4$. After equilibration, the mixture was centrifuged again and the pellet was redissolved in a minimum volume of buffer A.

(iii) Step 3. Sephadex G-75 chromatography. The redissolved pellet was applied to a column of Sephadex G-75 (1.5 by 35 cm) equilibrated previously with buffer A. The column was eluted with the same buffer. Fractions were collected, and those showing significant hydroxypyruvate reductase activity were pooled.

(iv) Step 4. Ion-exchange chromatography. The pooled fractions from step 3 were applied to a column (1 by 15 cm) of DEAE-Sepharose equilibrated previously with buffer A. The column was washed with approximately 3 bed volumes of the starting buffer and then eluted with a linear gradient of 0 to 0.5 M KCl in buffer A. Fractions containing hydroxypyruvate reductase activity were pooled.

(v) Step 5. Hydroxylapatite chromatography. The pooled fractions from step 4 were applied to a hydroxylapatite column (2 by 8 cm), equilibrated with buffer A. Elution was carried out with a linear gradient of potassium phosphate buffer (0 to 100 mM), pH 7.5. The fractions containing the majority of the hydroxypyruvate activity were pooled and concentrated with an Amicon Centricon (Diaflow Corp., Amicon) YM10 filter.

(vi) Steps 6, 7, and 8. 5′-AMP–Sepharose 4B chromatography. The concentrated enzyme solution from step 5 was applied to a column (1 by 8 cm) of 5′-AMP–Sepharose 4B, equilibrated with buffer A. The column was eluted with the same buffer. The second half of the hydroxypyruvate reductase activity peak was collected, and the fractions were pooled and concentrated with an Amicon YM10 filter. Although hydroxypyruvate reductase did not bind to 5′-AMP–Sepharose (in a variety of conditions used), it provided partial separation of the enzyme from impurities. After the column was washed, the chromatography was repeated twice.

Electrophoresis and isoelectrofocusing (IEF). Sodium dodecyl sulfate (SDS)-gel electrophoresis was performed in 12% polyacrylamide, using a Tris-glycine system (11). The gels were stained with Fast Stain (Zoion Research Inc.). The mass of the subunit of the enzyme was determined by comparison with the mobilities of standard proteins (low range; Bio-Rad).

Native gel electrophoresis was performed with PhastGel Gradient 8-25 gels and the PhastSystem (Pharmacia LKB) in accordance with the recommendations of the manufacturer. Gels were stained with Fast Stain, silver, or activity stains (see below). Standard proteins (high range; Bio-Rad) were used for determination of molecular mass of the enzyme.

IEF of crude or pure enzyme preparations was performed with PhastGel IEF 4-6.5 gels and PhastSystem in accordance with the recommendations of the manufacturer (Pharmacia LKB). Gels were stained with Fast Stain or an activity stain (see below). Pharmacia LKB IEF standards were used for determination of the pI of the enzyme.

The reaction mixture for activity staining of hydroxypyruvate reductase, using the reverse reaction, contained 200 mM Tris HCl buffer (pH 9.5), 5 mM D-(−)-glycerate, 2 mM NAD, 0.5 mM phenazine methosulfate, and 1 mM nitroblue tetrazolium.

N-terminal amino acid sequence determination. The N-terminal amino acid sequence of purified enzyme was analyzed by a gas-phase peptide sequenator (Applied Biosystems 477A).

TABLE 1. Summary of purification of hydroxypyruvate reductase from *M. extorquens* AM1

Step	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Purification (fold)	Yield (%)
1. Cell extract	300	675	2.25	1	100
2. Ammonium sulfate	125	594	4.75	2.1	88
3. Sephadex G75	90	563	6.25	2.8	83
4. DEAE-Sepharose	14	525	37.5	16.7	78
5. Hydroxyl-apatite	1.6	260	162.5	72.2	39
6. 5′-AMP–Sepharose 1	0.57	257	450	200	38
7. 5′-AMP–Sepharose 2	0.22	138	625	278	20
8. 5′-AMP–Sepharose 3	0.1	68	675	300	10

RESULTS

Purification of hydroxypyruvate reductase. A typical summary of hydroxypyruvate reductase purification is shown in Table 1. The overall purification of the enzyme was approximately 300-fold, with a yield of 10%. As judged by SDS (Fig. 1) and native (data not shown) polyacrylamide gel electrophoresis, the purified hydroxypyruvate reductase preparation was homogeneous. A preliminary report on this enzyme had suggested that two forms might be present that could be separated by DEAE-cellulose column chromatography, but which had similar properties (10). We have now shown that the original preparations of these two forms are, in fact, identical, and under appropriate conditions both behave in a similar manner on ion-exchange columns.

Molecular mass and subunit structure. The molecular mass of hydroxypyruvate reductase was estimated to be 71 kDa on the basis of its mobility in gradient polyacrylamide gels relative to those of reference proteins. The subunit molecular mass was estimated to be 37 kDa on the basis of mobility in SDS-polyacrylamide gels. These data suggest that the enzyme consists of two identical subunits.

Isoelectric point. The isoelectric point of the enzyme was about 4.75 on the basis of its mobility in PhastGel IEF 4-6.5.

Stability. The enzyme retained at least 90% of the initial activity when stored for 2 months at 4°C in 50 mM Tris HCl buffer, pH 7.5, containing 10% glycerol and 1 mM dithiothreitol. Only 40% of the initial activity was retained after 15 days when the enzyme was stored without glycerol and dithiothreitol. Glycerol alone, dithiothreitol alone, and glycerol plus hydroxypyruvate (1 mM) had a less stabilizing

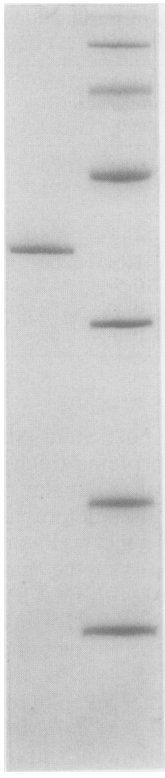

FIG. 1. SDS-polyacrylamide gel electrophoresis of the purified hydroxypyruvate reductase from *M. extorquens* AM1 (left lane). Electrophoresis was carried out in the presence of 12% polyacrylamide with 10 μg of protein. The gel was stained with Fast Stain (Zoion Research, Inc.). Molecular mass markers (right lane) were (bottom to top) hen egg white lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), bovine carbonic anhydrase (31.0 kDa), hen egg white ovalbumin (45.0 kDa), bovine serum albumin (66.2 kDa), and rabbit muscle phosphorylase *b* (97.4 kDa).

effect. The enzyme was unstable in both potassium phosphate (pH 7.0) and MOPS (pH 7.0) buffer.

Substrate specificity and kinetics. As summarized in Table 2, purified hydroxypyruvate reductase had maximal activity with hydroxypyruvate as substrate and NADH as cofactor. It also used NADPH as a cofactor, at a slightly lower rate but with a K_m for hydroxypyruvate that was over a magnitude higher than that with NADH. The K_m values for the two cofactors were similar. Glyoxylate was also used as a substrate but at much reduced rates and higher K_m values. No activity was detected with either pyruvate or oxaloacetate with NADH or NADPH.

TABLE 2. Reaction properties of purified hydroxypyruvate reductase from *M. extorquens* AM1

Substrate	Cofactor[a]	Rate (% of maximum)[b]	K_m (mM)	
			Substrate	Cofactor
Direct reaction				
Hydroxypyruvate (3 mM)	NADH	100	0.1	0.04
	NADPH	80	1.25	0.06
Glyoxylate (10 mM)	NADH	15	1.5	ND[c]
	NADPH	1.5	6.25	ND
Reverse reaction				
Glycerate (5 mM)	NAD^+	1.5	2.6	ND
	$NADP^+$	0		
Glycolate (5 mM)	NAD^+	0		
	$NADP^+$	0		

[a] NADH, 0.2 mM; NADPH, 0.3 mM; NAD^+ and $NADP^+$, 3 mM each.
[b] Rate with hydroxypyruvate and NADH is set at 100%.
[c] ND, not determined.

Hydroxypyruvate reductase catalyzed the reverse (oxidation) reaction at a low rate with D-(−)-glycerate as substrate and NAD^+ as cofactor. The enzyme did not exhibit any detectable activity with glycerate and $NADP^+$ or with glycolate and either NAD^+ or $NADP^+$ at a variety of pH values.

Effect of pH. The pH profile for the reduction of hydroxypyruvate showed a broad optimum between 4.0 and 6.5, with a slight maximum at pH 4.5. The pH curve for NADPH-linked hydroxypyruvate activity showed a sharper peak (about 55% of maximal activity was attained at pH 7.0 in MOPS buffer) than the pH curve for NADH-linked activity (about 90% of maximal activity was attained at pH 7.0 in MOPS buffer). The optimal pH for D-(−)-glycerate oxidation was between 9 and 11.

Enzyme inhibition. The inhibitory effect of substrates, products, and some substrate or product analogs was investigated in standard assay conditions. All substrates caused inhibition when used in high concentrations. Hydroxypyruvate inhibited NADH-linked activity at concentrations of >4 mM and NADPH-linked activity in concentrations of >10 mM. Both NADH and NADPH were inhibitory when used in concentrations of >0.5 mM. D-(−)-Glycerate and NAD were inhibitory for the reverse reaction in concentrations of >10 and 5 mM, respectively. Glyoxylate had a strong inhibitory effect at high concentration on the NADH-linked activity of the enzyme in the direct reaction and also on the reverse reaction with NAD^+ (Table 3), while very little inhibition of the NADPH-linked activity was observed. This finding correlates with differences in substrate affinity of the NADH- and NADPH-linked activities. The direct reaction products D-(−)-glycerate, glycolate, NAD^+, and $NADP^+$ when used in low concentrations had no or only slight inhibitory effects on NADH-linked activity of the enzyme, while the NADPH-linked activity was affected more dramatically. The substrate analogs pyruvate, oxaloacetate, and citrate were in general only slightly inhibitory, except for pyruvate. Hydroxypyruvate caused 99% inhibition of the reverse reaction at 0.5 mM. Phosphoenolpyruvate (5 mM) and acetyl-CoA (0.1 mM) had no affect on activity.

N-terminal amino acid sequence. Two determinations of the N-terminal amino acid sequence both gave the following 22-residue sequence, beginning with residue 2: K-K-V-V-F-L-D-R-E-S-L-D-A-T-V-R-E-F-N-N-P-H. In these preparations it was not possible to determine the first residue. Only a single sequence was obtained, confirming the presence of two identical subunits.

DISCUSSION

Hydroxypyruvate reductase (also called glycerate dehydrogenase, hydroxypyruvate dehydrogenase, or glyoxylate reductase) is an enzyme found in higher plants, algae, mammalian tissue, and bacteria (6, 9, 20–22, 24). In most cases, it has been postulated to function in converting hydroxypyruvate to glycerate for a variety of metabolic pathways, but most enzymes also carry out the reduction of glyoxylate to glycolate. The two enzymes from *Pseudomo-*

TABLE 3. Inhibition of purified hydroxypyruvate reductase by products and substrate or product analogs

Inhibitor	Concn (mM)	% Inhibition		
		Direct reaction		Reverse reaction (NAD linked)
		NADH linked	NADPH linked	
Glyoxylate	2	25	0	
	5	40		50
	10	55	15	80
Pyruvate	5	30	10	75
	10	40		
Oxaloacetate	1	0		
	2.5	10	20	
	5			25
Citrate	5	20	25	0
Hydroxypyruvate	0.5			99
D-(−)-Glycerate	1	0	15	
	5	20	40	
Glycolate	5	0	15	0
NAD^+	2	0	40	
	4	10	60	
$NADP^+$	2	10	35	
	4	15	40	8

nas aminovorans are exceptions to this (Table 4). In the serine cycle methylotrophs, hydroxypyruvate reductase plays a central role in assimilation of carbon. Not only does it convert hydroxypyruvate to glycerate as a key step in the serine cycle, but also it may play an important role in C_2 reactions, by interconverting glyoxylate and glycolate (4).

We have purified hydroxypyruvate reductase from *M. extorquens* AM1 to study its regulation by effectors and its substrate specificity and as a prelude to cloning the hydroxypyruvate reductase gene. This enzyme shows some similarities to other bacterial hydroxypyruvate reductase enzymes characterized so far and some differences (Table 4). It is most similar to the hydroxypyruvate reductase from *P. denitrificans*, although that enzyme uses NADPH as the preferred cofactor and its molecular mass is unknown. The other enzymes show different substrate specificities, being unable to utilize glyoxylate or unable to carry out the reverse reaction with glycerate.

Surprisingly, this enzyme shows some significant differences from the other hydroxypyruvate reductase that has been purified from a serine cycle methylotroph, the enzyme from *Hyphomicrobium methylovorans* GM2 (Table 4). The *H. methylovorans* enzyme has a pH optimum near neutral, does not use NADPH as a cofactor, and does not carry out the reverse reaction with glycerate. In addition, the K_m of the *H. methylovorans* enzyme for glyoxylate is an order of magnitude higher than that for the *M. extorquens* AM1 enzyme. This difference suggests that the *H. methylovorans* enzyme may not be involved in glyoxylate reduction in vivo, while in *M. extorquens* AM1 the K_m is more consistent with this role. *H. methylovorans* is an obligate methylotroph, and strain GM2 is a glycine-resistant mutant that excretes serine (7). It is possible that the differences in the two hydroxypyruvate reductase enzymes reflect the different physiology of these two serine cycle methylotrophs.

The activity with glyoxylate and glycolate is an important characteristic because of the uncertainty concerning the role of hydroxypyruvate reductase and glycolate in both C_1 and C_2 metabolism in *M. extorquens* AM1 (2, 4, 19). No reverse reaction with glycolate could be detected in this study with purified hydroxypyruvate reductase, nor has it been detected in cell extracts of *M. extorquens* AM1 (4). It is possible that the lack of glycolate oxidation could be due to inappropriate conditions in vitro; for instance, as suggested previously, it is possible that glyoxylate must be removed to low levels for activity to occur (4). It is also possible that the defect in mutant 20BL affects more than one activity, and it is important to characterize this mutation more carefully. One approach is to use molecular genetic analyses. However, we have been unable to complement the mutation in strain 20BL by using a genomic clone bank (unpublished data). Now that the enzyme has been purified from *M. extorquens* AM1, it should be possible to isolate the gene by

TABLE 4. Comparison of hydroxypyruvate reductase enzymes from bacteria[a]

Source	Mass (kDa)	Subunits	pH optimum	Cofactor(s)	Substrate (K_m, mM)[b]		
					Hydroxypyruvate	Glyoxylate	Glycerate[c]
Methylobacterium extorquens AM1	71	2 × 37	4.5	NADH,[d] NADPH	+ (0.10)	+ (1.5)	+ (2.6)
Hyphomicrobium methylovorum GM2	70	2 × 38	6.8	NADH	+ (0.175)	+ (10.8)	−
Pseudomonas acidovorans							
Inducible	85	2 × 44	5.5–8.0	NADH,[d] NADPH	+ (8)	−	+ (0.76)
Constitutive	75.6		5.3	NADH, NADPH[d]	+ (0.13)	−	+
Paracoccus denitrificans[e]			5	NADH, NADPH[d]	+ (0.10)	+	+

[a] References 3, 7, 9, and 22.
[b] Determined with the preferred cofactor.
[c] Reverse reaction; K_m values determined with NAD^+, pH 9.
[d] Preferred.
[e] Partially purified.

using an oligonucleotide probe based on the N-terminal amino acid sequence and to construct a well-defined null mutant in hydroxypyruvate reductase. The phenotype of such a mutant should clarify the role of this enzyme in C_2 metabolism and in the serine cycle.

Studies of potential effectors did not reveal any physiologically significant activators or inhibitors of the enzyme studied here. Some substrate inhibition was observed, as described earlier for the enzymes from *P. denitrificans* (3) and *Chlamydomonas reinhardtii* (6), and mild product inhibition was observed, as shown for *H. methylovorum* GM2 (7). However, for the reduction reaction, none of these effects are strong at physiologically significant concentrations. Therefore, hydroxypyruvate reductase is probably not regulated in a major way at the level of enzyme activity. The most probable sites for regulation of the serine cycle have been proposed to be the glyoxylate-activated serine transhydroxymethylase and the acetyl-CoA-independent phosphoenolpyruvate carboxylase (2). Regulation of hydroxypyruvate reductase is probably carried out at the level of enzyme synthesis, since it is upregulated by a product(s) of C_1 substrate oxidation and downregulated during growth on succinate (4, 14). Once the hydroxypyruvate reductase structural gene has been cloned, it will be possible to study the regulation of its expression.

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