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Serine Transhydroxymethylase Isoenzymes from a Facultative Methylotroph

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Two serine transhydroxymethylase activities have been purified from a facultative methylotrophic bacterium. One enzyme predominates when the organism is grown on methane or methanol as the sole carbon and energy source, whereas the second enzyme is the major isoenzyme found when succinate is used as the sole carbon and energy source. The enzyme from methanol-grown cells is activated by glyoxylate, is not stimulated by Mg^{2+}, Mn^{2+}, or Zn^{2+}, and has four subunits of 50,000 molecular weight each. The enzyme from succinate-grown cells is not activated by glyoxylate and is stimulated by Mg^{2+}, Mn^{2+}, and Zn^{2+}, and sodium dodecyl sulfate-acrylamide gel electrophoresis indicates that this enzyme has subunit molecular weight of 100,000, the same as the molecular weight obtained for the active enzyme. Cells grown in the presence of both methanol and succinate incorporate less methanol carbon per unit time than cells grown on methanol and have a lower specific activity of the glyoxylate-activated enzyme than methanol-grown cells. Adenine, glyoxylate, or trimethoprim in the growth medium causes an increased level of serine transhydroxymethylase in both methanol- and succinate-grown cells by stimulating the synthesis of the glyoxylate-activated enzyme.

Serine transhydroxymethylase (STHM) (EC 2.1.2.1.) catalyzes the interconversion of serine and glycine. In most microorganisms, its major functions are to provide glycine for protein and purine synthesis and N^5,N^{10}-methylene tetrahydrofolate for the one-carbon pool (14). The interrelationships of STHM and the one-carbon pool are shown in Fig. 1. This enzyme has a second function in microorganisms which use reduced, one-carbon compounds as sole sources of carbon and energy and which use the serine pathway for formaldehyde assimilation. It converts glycine to serine, incorporating a one-carbon unit derived from the oxidation of the substrate used for growth (2). It therefore catalyzes an important reaction in the assimilation of carbon for these organisms. The two pathways in which STHM is involved are summarized in Fig. 2.

We are studying an organism which is a facultative methylotroph. It is able to use methane or methanol as a sole carbon and energy source, but unlike other methane-oxidizing bacteria, it can also grow on more complex substrates such as glucose or succinate (16). The regulation of STHM in this organism might pose a difficult problem since it must not only be regulated for amino acid biosynthesis but also for carbon assimilation. The regulation of STHM in other organisms is not clearly understood. Mansouri et al. (10) reported that STHM was repressed by methionine in *Escherichia coli*. The results of Taylor et al. (20) were not in agreement. Meedel and Pizer (13) provided evidence indicating that S-adenosylmethionine, rather than methionine, was the small-molecular-weight compound responsible for regulating the levels of this enzyme in *E. coli*. The results of Stauffer et al. (19) indicated end product control of STHM in *Salmonella typhimurium* by adenine, serine, methionine, and guanine. It is probable that the STHM required for glycine biosynthesis in a facultative methylotroph is also under end product control. If high end product concentrations of glycine inhibited or repressed the enzyme, the organism would be unable to incorporate carbon from methane or methanol. Since glycine does not inhibit the growth of these organisms on methanol (M. L. O'Connor, unpublished data), the possible existence of two isoenzymes was considered. Support for this possibility has come from the isolation of a mutant in the facultative methanol utilizer, *Pseudomonas* AM1, which required glycine and lacked STHM activity when grown on succinate but regained STHM activity and grew as a prototroph when methanol was used as the carbon and energy source (2). However, preliminary attempts to distinguish two isoenzymes in this organism have failed (R. D. Quayle, personal communication).

We have observed two separable STHM activities in extracts of a facultative methylotroph. The two enzymes differ in their physical properties, their regulation in vitro, and their response to additions to the growth medium.

MATERIALS AND METHODS

Organism. Isolate XX (16), ATCC 27886, is a gram-negative, polarly flagellated rod which grows at the expense of methane, methanol, acetate, dicarboxylic acids, monosaccharides, disaccharides, and amino acids. Growth is most rapid on glucose, succinate, malate, or methanol as sole carbon and energy sources.

Media and growth conditions. The modified nitrate mineral salts (NMS) medium previously described (16) was used for all studies. All supplements were dissolved in distilled water, sterilized separately, and added directly to the growth medium at the following concentrations: methanol, 0.5%; succinate, 0.1%; serine, glycine, glyoxylate, trimethoprim, methionine, 0.02%; thymine, adenine, guanine, 0.001%.

Liquid cultures were incubated on rotary shakers at 30 C. Large batches of cells were grown in a New Brunswick Microferm 14L fermenter. A 5% inoculum was added to appropriately supplemented NMS medium (5 to 11 liters). The temperature was maintained at 30 C. The fermenter was continuously gassed with air or with a methane-air mixture (70 ml of methane on 1 liter of air per min). Growth was followed by measuring the absorbance at 525 nm in a Beckman model DB spectrophotometer. The cells were harvested by centrifugation during the mid-exponential phase of growth, washed once with 50 mM potassium phosphate buffer (pH 7.3), and stored frozen at −20 C.

Assays of STHM. STHM was assayed by the method of Taylor and Weissbach (21) as modified by Nakamura and co-workers (15). D,L-[3-^{14}C]serine (Amersham Searle) was used as the substrate. The radioactive N^5,N^{10}-methylene tetrahydrofolate produced was equilibrated with carrier formaldehyde

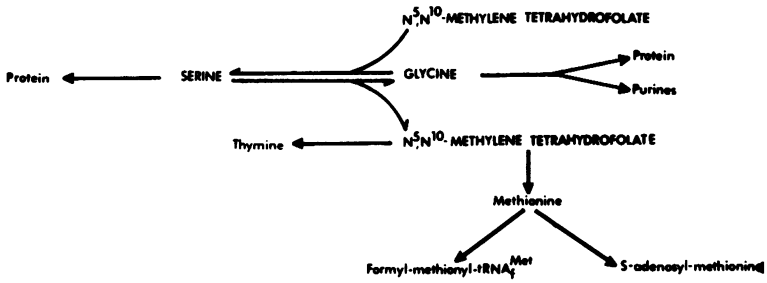

FIG. 1. *The role of STHM in metabolism of bacteria.*

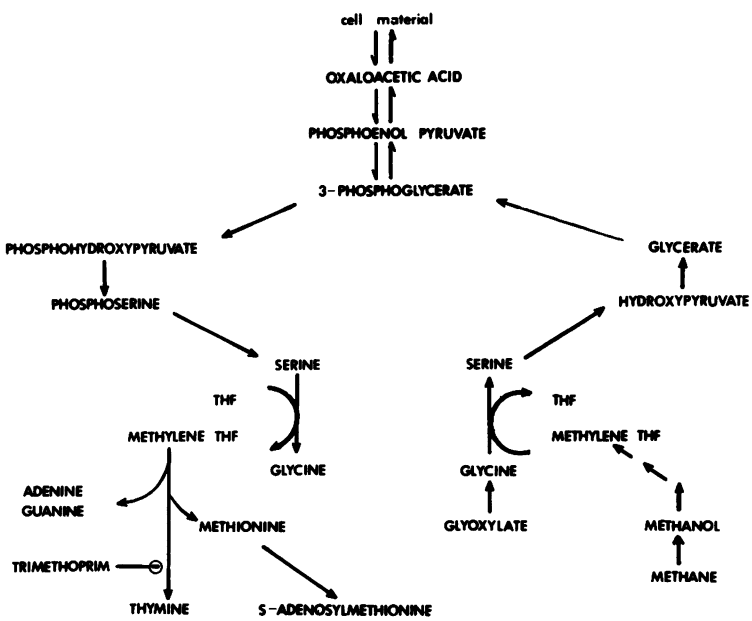

FIG. 2. *The two pathways requiring STHM in bacteria that grow on one-carbon compounds.*

and converted to its dimedon (5,5-dimethyl-1,3-cyclohexadione) derivative. The labeled dimedon derivative was extracted in toluene, and the radioactivity in the toluene layer was determined in a Packard model 3375 scintillation spectrophotometer.

D,L-[3-^{14}C]Serine was normally used at a specific activity of 10^5 dpm/μmol and a final concentration of 2 mM. Recovery and counting efficiency were determined using a control with 0.1 μmol of [^{14}C]formaldehyde of known specific activity. A zero time control was included with each assay, and the toluene-extractable counts from this control were subtracted from the gross counts for each sample tested. The total reaction volume was 0.5 ml. Tetrahydrofolate (Sigma) was stored under argon at −20 C. All activities are reported as micromoles of formaldehyde produced per hour.

Purification procedure from methanol- and methane-grown cells. All buffers contained 5×10^{-8} M pyridoxal-5′-phosphate (PLP), 5×10^{-6} M dithiothreitol, and 10^{-3} M ethylenediaminetetracetic acid tetrasodium salt. All buffers also contained 30% glycerol unless otherwise indicated.

(i) Preparation of crude extracts. Frozen cells were thawed and resuspended in twice their weight of the same buffer and disrupted by three passes through a French pressure cell at 6,000 lb/in^2. The extract was centrifuged at 20,000 × g for 20 min to remove intact cells and debris. Magnesium chloride was added to the soluble fraction to give a concentration of 1 mM, and ribonuclease and deoxyribonuclease, each at 50 μg/ml, were added. This preparation was dialyzed overnight at 4 C against three changes of 50 mM potassium phosphate buffer (pH 7.3) plus 1 mM $MgCl_2$.

(ii) DEAE-cellulose column 1. Diethylaminoethyl (DEAE)-cellulose (Whatman DE-52, W. C. R. Balston Ltd., Kent, England) was prepared by swelling the cellulose in 50 mM potassium phosphate buffer (pH 7.3) followed by repeated defining. The cellulose slurry was poured into a jacketed column (2 cm in diameter), allowed to settle to a height of 10 cm, and equilibrated with 50 ml of buffer. The dialyzed extract was layered beneath the buffer. The buffer flow rate was maintained at 0.5 ml/min with a peristaltic pump. Fractions (5 ml) were collected and held at 4 C in a refrigerated fraction collector. The enzyme activity was found in fractions 2 to 7 and was stimulated by glyoxylate.

(iii) DEAE-cellulose column 2. The fractions from the first column containing high specific activity were pooled, diluted fivefold with distilled water, and layered onto a second DEAE column (2 by 10 cm) which had been equilibrated with 50 ml of 10 mM potassium phosphate buffer (pH 7.3). The solution was allowed to enter the column, and 50 ml of the same buffer was pumped through at 0.5 ml/min. The protein was eluted with a linear potassium acetate gradient (0 to 0.25 M in the same buffer). The tubes containing high specific activity were pooled and concentrated at 0 C using an Amicon Diaflo ultrafiltration unit (Amicon Corporation, Lexington, Mass.) equipped with an XM-50 membrane.

(iv) Preparative electrophoresis. Sephadex G-25 (Pharmacia, Uppsala, Sweden) was equilibrated with 60 mM tris(hydroxymethyl)aminomethane (Tris) buffer, adjusted to pH 8.0 with hydrochloric acid, and poured into a stoppered Buchler Fractophorator (Buchler Instruments, Inc., Fort Lee, N.J.) water-jacketed column (13 by 1.3 cm) at 4 C. One milliliter of the enzyme concentrate was applied to the top of the column, and the buffer flow was started to allow the enzyme preparation to enter the resin column. The top of the column was then sealed with a 1-cm layer of 0.5% agar, and the column was inserted into the Fractophorator apparatus. Electrophoresis was performed at 10 mA, with 60 mM Tris-hydrochloride buffer (pH 8.0, without glycerol) in the electrode reservoir chambers. After 20 to 24 h, the enzyme band could be distinguished near the middle of the column. The current was turned off, the agar seal was broken, and the band was eluted off with the same buffer. The fractions containing the highest specific activity were frozen at −20 C.

Purification procedure for succinate-grown cells. (1) Preparation of extracts. The procedure for preparation of extracts was identical to that described above for cells grown on methane and methanol.

(ii) DEAE-cellulose column 1. The dialyzed extract was loaded onto a DEAE-cellulose column prepared as described above. The column was washed with 50 ml of buffer. Ten percent of the total activity was eluted in this wash. The enzyme activity eluted was stimulated by glyoxylate. The remainder of the enzyme (90%) was then batch eluted with 0.2 M potassium acetate in buffer. This enzyme fraction was not stimulated by glyoxylate.

(iii) DEAE-cellulose column 2. The fractions of the glyoxylate-insensitive enzyme containing the highest specific activity were pooled and dialyzed overnight at 4 C against three changes of 10 mM Tris-citrate buffer (pH 7.8). A second DEAE column (2 by 10 cm) was prepared using the same buffer. The dialyzed sample was loaded on the column, the column was washed with 50 ml of the same buffer and then 50 ml of 0.1 M potassium acetate in buffer, and the enzyme was eluted with 0.2 M potassium acetate in buffer. Fractions containing the highest specific activity were pooled and concentrated as described above.

(iv) Glycerol density gradient. A linear 10 to 30% glycerol gradient was prepared in polyallomer tubes and precooled to 4 C. One-half milliliter of the concentrated enzyme preparation from the second DEAE column was layered on top of the gradients and centrifuged with a Beckman model L-2 centrifuge at 38,000 rpm in a type SW41 rotor for 24 h at 4 C. The tubes were punctured with an 18-gauge needle, and 12-drop fractions were collected. The fractions containing the highest specific activity were pooled and frozen at −20 C.

Enzyme homogeneity. The homogeneity of enzyme preparations was determined by polyacrylamide disc gel electrophoresis using the method of Hedrick and Smith (4). Eight percent gels were used at a pH of 7.3. Protein was stained with 0.025% Coomassie brilliant blue in methanol-water-acetic acid (5:5:1) for 4 h and then destained in 7.5% acetic acid. Acrylamide and methylene-bis-acrylamide

were obtained from Bio-Rad Laboratories (Richmond, Calif.). Enzymatic activity was localized by slicing separate gels, crushing the slices in 10 mM potassium phosphate buffer (pH 7.3), and assaying each sample for activity.

Molecular weight determinations. Molecular weights were estimated by the sucrose density centrifugation technique of Martin and Ames (11) and the slope molecular weight technique of Hedrick and Smith (4). For the first method, centrifugation was carried out with a Beckman model L-2 centrifuge at 45,000 rpm in a type SW 65L titanium rotor for 3 h at 3 C. For the second method, 6, 7, 8 and 10% gels were used for each determination, and the slope of the logarithm of the relative mobility versus the percent gel was plotted against the molecular weight. Subunit molecular weights were determined with sodium dodecyl sulfate (SDS)-acrylamide gels, using the procedure described by Laemmli (7). Hemoglobin, molecular weight 68,000 (5), yeast alcohol dehydrogenase, molecular weight 150,000 (3), catalase, molecular weight 250,000 (6), and apoferritin, molecular weight 450,000 (17), were used as standards for the molecular weights of the active enzymes. Lysozyme, molecular weight 14,300 (22), trypsin, molecular weight 23,800 (22), pepsin, molecular weight 37,500 (1), and cellulase, molecular weight 76,000 (23), were used as standards for the subunit molecular weights.

Protein determination. Protein was determined by the Folin phenol reagent method (9) with crystalline bovine serum albumin (Sigma, fraction V) as a standard. Absorbance was measured at 500 nm on a Beckman model DB spectrophotometer.

Incorporation of labeled compounds. Incorporation of labeled substrates into cold trichloroacetic acid-precipitable material was determined as previously described (16).

Determination of enzyme levels in vivo. A 250-ml portion of appropriately supplemented NMS media was inoculated with either methanol-grown cells for media containing methanol or succinate-grown cells for media containing succinate or succinate plus methanol. The cells were incubated with shaking at 30 C for 2 days, harvested, and washed by centrifugation (50 mM potassium phosphate buffer, pH 7.3). The cells were resuspended in 1.5 ml of the same buffer and sonified at maximum intensity for 2 min (Bronwill Biosonik sonifier, Bronwill Scientific, Rochester, N.Y.). The extract was cooled in an ice bath during sonification. Each extract was centrifuged at 20,000 × *g* for 20 min. The supernatants were assayed with and without 1 mM glyoxylate.

RESULTS

Purification of two STHMs. Table 1 summarizes the purification of STHM from methanol- and succinate-grown cells. Figure 3 shows the acrylamide gel banding patterns for each purified enzyme. After the last step, one band is present for the enzyme from cells grown on methanol (the glyoxylate-activated enzyme), and a major and minor band are present for the enzyme from cells grown on succinate (the glyoxylate-insensitive). All bands on the acrylamide gels for the last purification step exhibited enzymatic activity. SDS gels showed only one band for each enzyme.

The elution profiles of the DEAE-cellulose columns are shown in Fig. 4A, B, and C. The two enzymes are separated on the first DEAE-cellulose column. The minor isoenzyme in each case is about 10% of the major one. In Fig. 4B and C, it can be seen that the STHM is contained within one of the protein peaks separated by the columns. The profiles of the preparative electrophoresis column and the glycerol gradient are shown in Fig. 4D and E.

Properties of the STHMs. Several properties of the two enzymes are summarized in Table 2. The partially purified enzyme preparation used was the concentrated activity from the second DEAE column in all cases.

(i) pH optima. Enzyme activity was determined over a pH range of 8.0 to 9.5. Both enzyme preparations showed little change in activ-

TABLE 1. *Purification of the two STHMs from isolate XX, methanol- and succinate-grown cells*

Step	U[a]/ml	Total U[a]	mg/ml	Sp act[b]	% yield	Fold purification
Methanol-grown cells						
Crude extract	83	950	32	2.6	100	
DEAE column 1	9.6	560	3.8	2.7	59	1.04
DEAE column 2	3.2	220	1.0	3.2	23	1.23
Electrophoresis	7.2	72	0.2	36	7.6	11.2
Succinate-grown cells						
Crude extract	29.1	582	21.5	1.35	100	
DEAE column 1	3.8	250	1.0	2.56	44	1.9
DEAE column 2	13.3	133	3.2	4.16	23	3.1
Glycerol gradient	6.2	62	0.2	31.15	9.4	23.0

[a] Expressed as micromoles of formaldehyde/hour.
[b] Expressed as micromoles of formaldehyde/hour per milligram of protein.

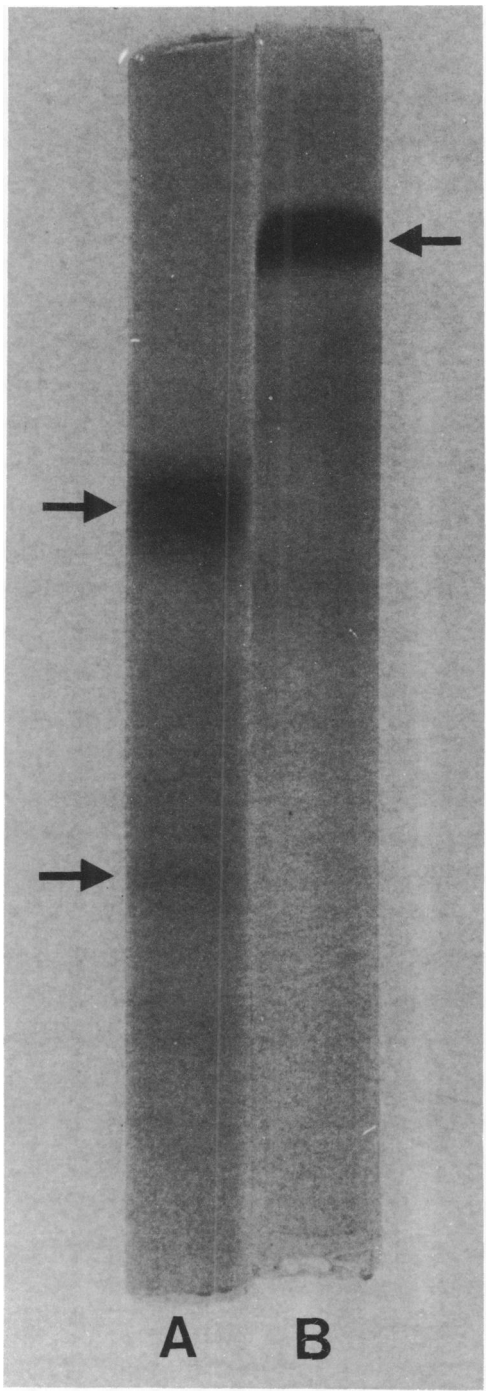

FIG. 3. *Polyacrylamide disc gel electrophoresis of the two purified STHMs. (A) STHM purified from succinate-grown cells, 25 μg of protein. (B) STHM purified from methanol-grown cells, 35 μg of protein. Electrophoresis was performed on 8% gels by the procedure of Hedrick and Smith (4). Gels were stained with 0.025% Coomassie brilliant blue for 4 h and destained with 7.5% acetic acid. Arrows indicate areas which showed STHM activity when separate gels were sliced and assayed.*

ity over a pH range of 8.5 to 9.0 (crude extract and partially purified). The glyoxylate-stimulated enzyme showed maximum activity at pH 8.7 to 8.8, whereas the activity of the glyoxylate-insensitive enzyme was greatest at pH 8.5 to 8.6.

(ii) Enzyme kinetics. The apparent Michaelis constants for serine were determined for each partially purified enzyme using the double reciprocal method of Lineweaver and Burk (8) (Fig. 5). A K_m of 1.25 mM was found for the glyoxylate-stimulated enzyme, and the glyoxylate-insensitive enzyme had a K_m of 1.0 mM. Maximum velocities were 6.1 and 5.1 μmol/h per mg of protein for the glyoxylate-stimulated and glyoxylate-insensitive enzyme, respectively.

(iii) Cation stimulation. Both partially purified enzymes were stimulated by 1 mM Ca^{2+}, K^+, and Na^+, but only the glyoxylate-insensitive enzyme was stimulated by 1 mM Mg^{2+}, Mn^{2+}, and Zn^{2+}.

(iv) Molecular weights. The major enzyme from cells grown on succinate had a molecular weight of 100,000 (± 5,000) as determined by SDS-acrylamide gel electrophoresis (Fig. 6). This value was confirmed when the molecular weight was determined by sedimentation on sucrose density gradients and relative mobility on acrylamide gels with increasing percentages of acrylamide (Fig. 7). The active enzyme from cells grown on methanol had a molecular weight of 200,000 (± 10,000) as determined by sucrose density gradient centrifugation and relative mobility on acrylamide gels (Fig. 7). From SDS-acrylamide gel electrophoresis (Fig. 6), the subunit molecular weight was found to be 50,000, indicating the presence of four subunits. The molecular weights of the active enzymes were the same in both crude and purified preparations, as determined by enzymatic activity on sucrose density gradients.

(v) Electrophoretic mobility. The enzyme from methanol-grown cells migrated on acrylamide gels with a mobility of 0.17 relative to a bromphenol blue marker. The relative mobility of the enzyme from succinate-grown cells was higher (0.22).

(vi) Specific activities. The specific activity of the glyoxylate-stimulated enzyme in crude extracts of methanol-grown cells was consistently higher than the specific activity of the glyoxylate-insensitive enzyme from crude extracts of succinate-grown cells (Table 2).

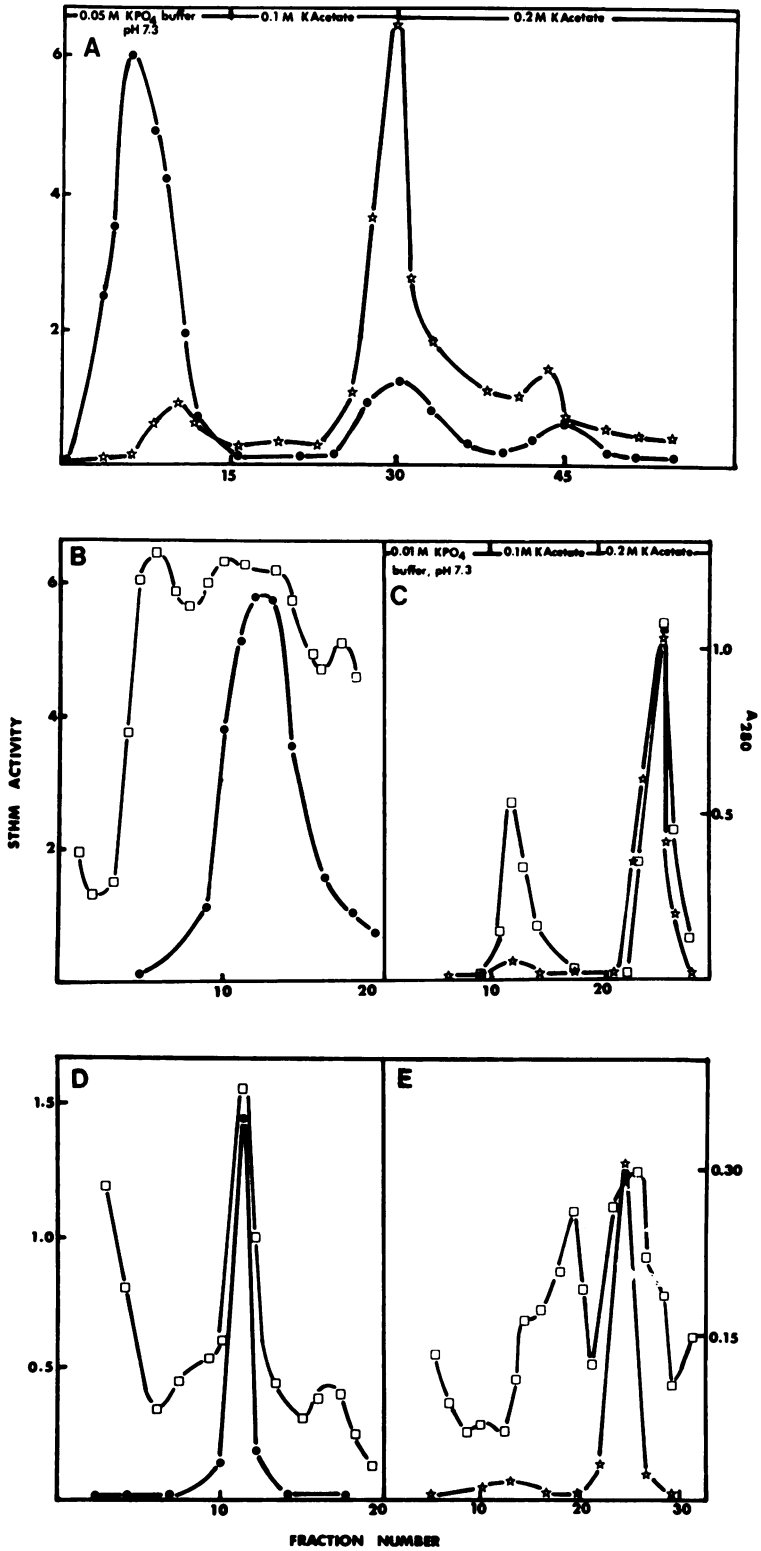

FIG. 4. *Purification of the two STHMs from isolate XX. (A) Step gradient elution profile of STHM from methanol-grown cells and succinate-grown cells from the first DEAE column. Eluting buffers were changed at the points indicated. Fractions 2 to 7 were pooled and used in (B); fractions 28 to 34 were pooled and used in (C). (B) Linear gradient (0 to 0.25 M potassium acetate in 0.01 M potassium phosphate buffer, pH 7.3) elution profile for the second DEAE column. Fractions 9 to 16 were pooled for use on (D). (C) Elution profile for second DEAE column. Fractions 20 to 26 were pooled for use on (E). (D) Preparative electrophoresis. Fraction 11 frozen. (E) Glycerol gradient. Fractions 21 to 23 were pooled and frozen. Symbols: ●, STHM activity, methanol-grown cells; ☆, STHM activity, succinate-grown cells; □, absorbance at 280 nm.*

TABLE 2. *Properties of the two STHMs*

Property	Cells grown on:	
	Methanol	Succinate
K_m	1.25 mM	1.00 mM
V_{max}[a]	6.1	5.1
pH optimum	8.7–8.8	8.5–8.6
Cation stimulation (10^{-3} M)	Ca^{2+}, K^+, Na^+	Ca^{2+}, K^+, Na^+, Mg^{2+}, Mn^{2+}, Zn^{2+}
Molecular weight		
Total	200,000	100,000
Subunit	50,000	100,000
Sp act of extracts[a]	3.8	2.1
Electrophoretic mobility[b]	0.17	0.22
Cofactor requirements[c] for maximum activity		
PLP	2×10^{-5} M	2×10^{-5} M
THF	2×10^{-4} M	2×10^{-4} M
Stability (% activity retained)		
2 months, −20 C	150	50
12 h, 4 C	100	20

[a] Expressed as micromoles of formaldehyde/hour per milligram of protein.
[b] Relative mobility of active enzyme in an 8% gel, pH 8.0.
[c] The purified enzymes had no detectable activity in the absence of tetrahydrofolate (THF) and PLP.

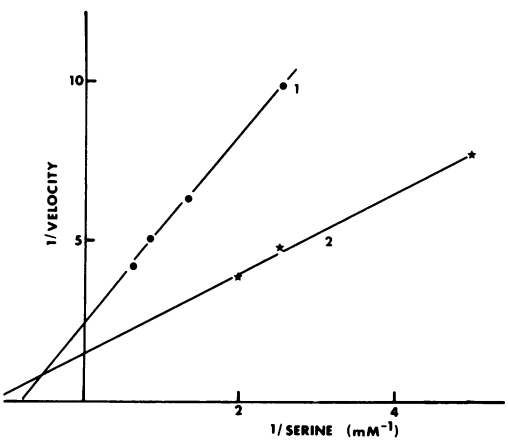

FIG. 5. *Lineweaver-Burk plot for the two STHMs from isolate XX. (1) Enzyme from methanol-grown cells. (2) Enzyme from succinate-grown cells.*

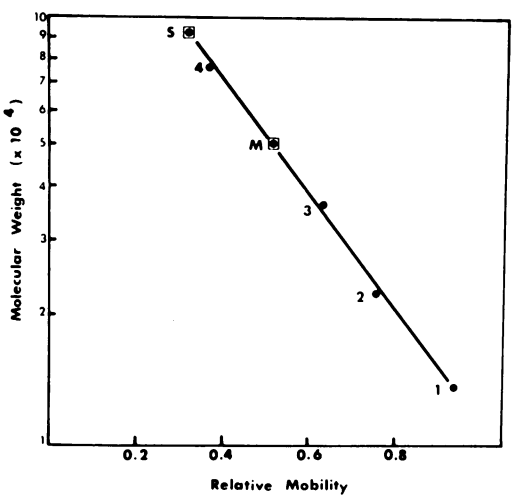

FIG. 6. *Determination of molecular weight by SDS-acrylamide gel electrophoresis. (1) Lysozyme (14,300); (2) trypsin (23,800); (3) pepsin (37,500); (4) cellulase (75,000); (S) STHM from succinate-grown cells; (M) STHM from methanol-grown cells.*

(vii) Cofactor requirements. The cofactor requirements were similar for both enzymes. Both enzymes showed no activity when PLP or tetrahydrofolate was omitted from the assay mixture. Maximum activity was obtained for both enzymes at 2×10^{-5} M PLP and 2×10^{-4} M tetrahydrofolate (Fig. 8 and Table 2).

(viii) Stability in crude extract. The glyoxylate-activated enzyme from cells grown on methanol was quite stable at −20 C, showing a 50% increase in activity after storage for 2 months. The glyoxylate-insensitive enzyme lost activity (50%) after 2 months at −20 C. The glyoxylate-activated enzyme lost no activity during storage for 24 h at 4 C, whereas the glyoxylate-insensitive enzyme lost 80% activity under the same conditions.

Centrifugation on a glycerol density gradient. A mixture of the two enzymes (both partially purified) was layered onto a glycerol density gradient prepared as described for the final step of the purification of the enzyme from

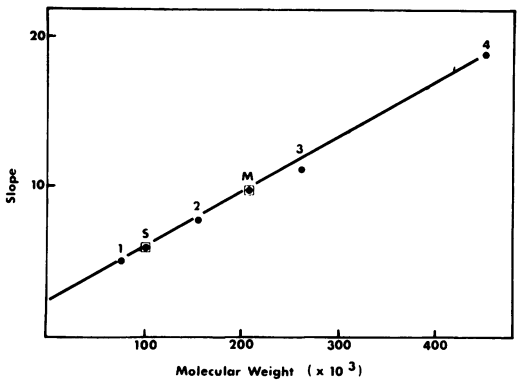

FIG. 7. *The slope molecular weight relationship of standard proteins and the STHMs from succinate- and methanol-grown cells as determined by discontinuous electrophoresis. Slopes for each protein were calculated from a plot of relative mobility versus percentage of acrylamide concentrations. (1) Hemoglobin (68,000); (2) yeast alcohol dehydrogenase (150,000); (3) catalase (250,000); (4) apoferritin (450,000); (S) STHM from succinate-grown cells; (M) STHM from methanol-grown cells.*

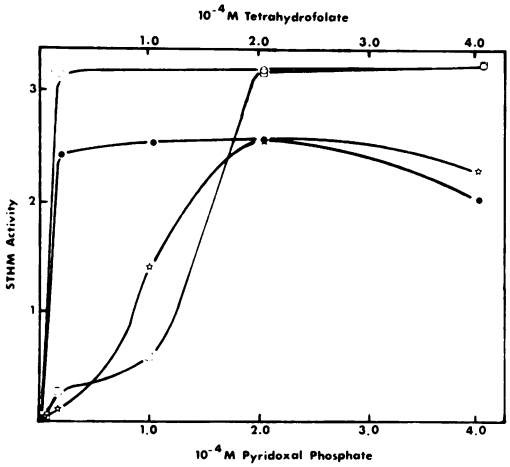

FIG. 8. *Cofactor requirements of the two STHMs from isolate XX. Symbols: ●, PLP, methanol-grown cells; ☆, tetrahydrofolate, methanol-grown cells; ○, PLP, succinate-grown cells; □, tetrahydrofolate, succinate-grown cells.*

succinate-grown cells. The gradient was centrifuged for 24 h as described above. The profile of enzymatic activities for the gradient is shown in Fig. 9. The two activity peaks were distinguishable by their inhibition or activation by glyoxylate. The gradient fractions were first assayed without glyoxylate to determine the location of enzymatic activity. One fraction from each peak was then assayed with and without glyoxylate to determine whether activation or inhibition occurred. The larger enzyme (the activity peak closest to the bottom of the gradient) was activated by 1 mM glyoxylate, whereas the smaller enzyme (the activity peak closest to the top of the gradient) was slightly inhibited (Fig. 9).

The effect of small molecules on enzyme activity. Several compounds were tested for effects on the in vitro activity of the two enzymes. The enzyme preparations used were the concentrated fractions from the second DEAE column in each case. Table 3 shows the activity observed when several compounds were added singly to the assay solution. The most pronounced effects were those of the activation of the enzyme from methanol-grown cells by glyoxylate and the inhibition of both enzymes by glycine. The inhibition was found to be competitive with glycine for both enzymes (the product of the reaction in this assay was glycine) and uncompetitive for glyoxylate with the enzyme from succinate-grown cells (Fig. 10). The weak uncompetitive inhibition of the enzyme from succinate grown cells by glyoxylate may be due to metal chelation, since 5×10^{-3} M ethylenediaminetetraacetate caused a similar inhibition. Ethylenediaminetetraacetate (5×10^{-3} M) had no detectable effect on the enzyme from methanol-grown cells. Glyoxylate had less effect on the enzymes in crude extracts. It activated the enzyme from methanol-grown cells 26 to 28% and had little or no effect on the unpurified enzyme from succinate-grown cells.

The effect of small molecules on enzyme synthesis. Table 4 shows the activity of each enzyme when cells were grown in the presence of the compounds listed. Extracts were assayed with and without glyoxylate. The preceding data has shown that cells grown on methanol contain about 10% of the STHM activity that is not stimulated by glyoxylate, and 90% of the

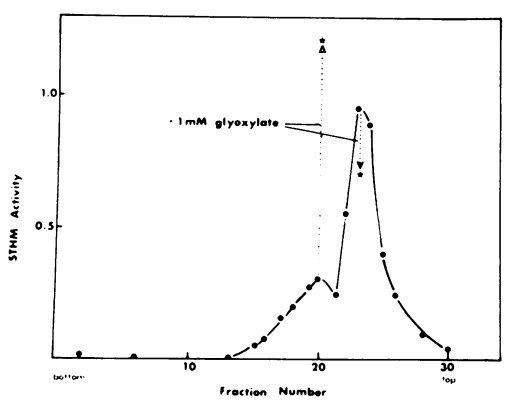

FIG. 9. *Glycerol gradient; mixture of the enzymes from succinate- and methanol-grown cells.*

TABLE 3. *Effect of small-molecular-weight compounds on the activity of the STHMs from methanol- and succinate-grown cells*[a]

Assay supplement[b]	Enzyme act[c]		% of Control[d]	
	Methanol-grown cells	Succinate-grown cells	Methanol-grown cells	Succinate-grown cells
None	0.10	0.21	100	100
AMP	0.126	0.19	126	90
ATP	0.10	0.15	100	72
Methionine	0.10	0.21	100	100
S-adenosylmethionine	0.10	0.21	100	100
Phosphoenolpyruvate	0.10	0.21	100	100
Guanine	0.123	0.19	123	90
Thymine	0.122	0.19	122	90
Glycine	0.048	0.095	48	45
Glyoxylate	0.45	0.19	450	90

[a] All compounds tested were at a final concentration of 5 mM except guanine (0.25 mM) and thymine (1 mM).

[b] AMP, Adenosine 5'-monophosphate; ATP, adenosine 5'-triphosphate.

[c] Expressed as micromoles of formaldehyde/hour (partially purified enzyme, DEAE column 2).

[d] Value obtained for the control assay condition (no additions) represents 100%.

activity is found in a separable enzyme that is stimulated by glyoxylate. These crude extracts were consistently found to have a STHM activity which was stimulated 26 to 28% by 1 mM glyoxylate in several experiments. Succinate-grown cells, which were shown to contain 10% of the enzyme that is activated by glyoxylate, showed a glyoxylate stimulation of 1 to 3%. A 30% increase in activity when glyoxylate was added to the reaction mixture was taken to indicate the presence of glyoxylate-activated enzyme as the major STHM isoenzyme, whereas no increase was used to indicate the presence of the glyoxylate-insensitive enzyme. Compounds present in the enzyme preparation were assumed to have no significant effect on the activity, since all enzyme preparations were diluted 50- to 100-fold in the assay solution.

Intermediate levels of activation indicate a mixture of isoenzymes. We have, therefore, used the extent of glyoxylate activation as a measure of the proportion of each isoenzyme. The specific activity of the glyoxylate activated enzyme was calculated using the formula: specific activity of the glyoxylate-activated enzyme = ([specific activity with glyoxylate − specific activity without glyoxylate] × 100/[specific activity without glyoxylate × 30]) total specific activity.

The specific activity of the glyoxylate-insensitive enzyme that is predominant in succinate-grown cells was calculated from (1 − fraction of glyoxylate-activated enzyme) total specific activity. Whereas this method for determining the enzyme levels is crude and somewhat inaccurate, we believe it is useful for demonstrating major changes in the proportions of each enzyme in crude extracts.

The most significant effects of compounds added to the growth medium were caused by trimethoprim (an inhibitor of C-1 transfer [19]), adenine, and glyoxylate. All three compounds stimulate the synthesis of STHM in methanol-

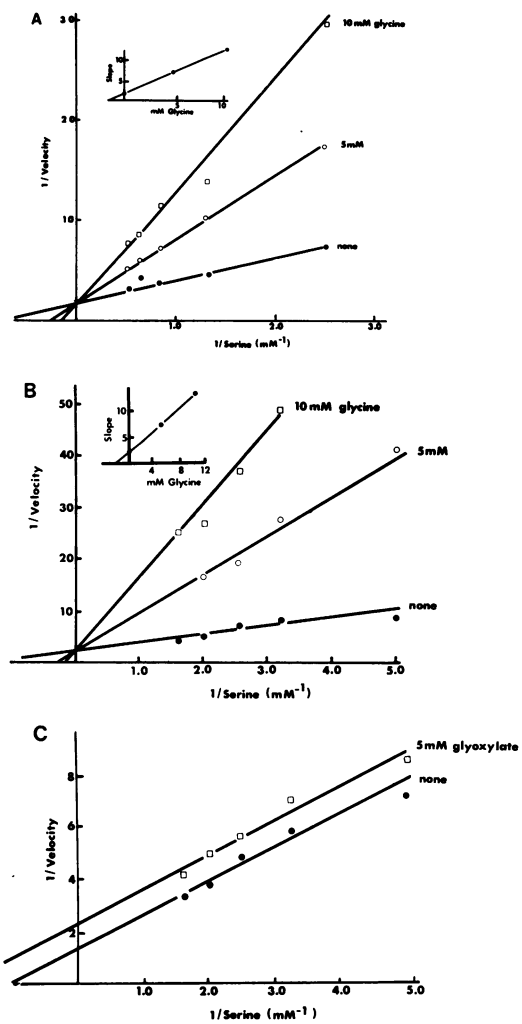

FIG. 10. *Inhibition of STHM by glycine and glyoxylate. (A) STHM from methanol-grown cells, varying glycine. (B) STHM from succinate-grown cells, varying glycine. Replots of the slope versus millimolar glycine are shown in the insets. (C) STHM from succinate-grown cells, varying glyoxylate.*

TABLE 4. *Effect of small-molecular-weight compounds on the levels of the two STHMs in methanol- and succinate-grown cells*

Additions to growth medium	Total sp act[a]	Sp act[a]	Sp act[a]
		Glyoxylate-insensitive enzyme	Glyoxylate-activated enzyme
Extracts from succinate-grown cells			
None	3.3	3.0	0.3
Glycine (Gly)	2.3	1.0	1.3
Serine (Ser)	4.1	3.4	0.7
Glyoxylate	4.5	0.0	4.5
Methionine (Met)	2.6	0.1	2.5
Guanine (Gu)	2.6	2.5	0.15
Adenine (Ad)	6.8	1.5	5.3
Thymine (Th)	5.2	4.3	0.9
Trimethoprim	8.5	2.85	5.9
Gly + Ser + Met	2.0	1.8	0.2
Glu + Ad + Th	3.3	2.9	0.4
Gly + Ser + Met + Gu + Ad + Th	3.5	3.5	0.0
Extracts from methanol-grown cells			
None	6.5	0.7	5.8
Glycine	7.6	0.2	7.4
Serine	11.0	5.8	5.2
Glyoxylate	9.1	0.5	8.6
Methionine	5.9	0.0	5.9
Guanine	5.3	0.1	5.2
Adenine	11.5	0.0	11.5
Thymine	4.4	1.6	2.8
Trimethoprim	13.9	0.0	13.9
Gly + Ser + Met	11.1	3.9	7.2
Gu + Ad + Th	14.1	3.3	10.8
Gly + Ser + Met + Gu + Ad + Th	10.6	0.0	10.6
Extracts from methanol + succinate-grown cells			
None	6.6	2.8	3.8

[a] Expressed as micromoles/hour per milligram of protein.

grown cells and appear to primarily affect the synthesis of the glyoxylate-activated enzyme. In cells grown on succinate, these three compounds also appear to stimulate the synthesis of glyoxylate-activated enzyme. Combinations of amino acids and nucleotides did not result in additive effects, indicating a complex interaction. The levels of the glyoxylate-insensitive enzyme in cells grown on media containing both succinate and methanol were similar to the levels found in cells grown on media containing succinate alone. The specific activity of the glyoxylate-activated enzyme in cells grown on media containing both succinate and methanol was lower than the specific activity of this enzyme in cells grown on methanol alone (Table 4). These data are consistent with the radioactive incorporation study summarized in Table 5. When cells were grown in media containing both methanol and succinate, succinate was incorporated into cold trichloroacetic acid-precipitable material at a rate similar to that observed in cells growing on succinate, but methanol was incorporated into cold acid-precipitable material at a rate less than that observed in cells growing on methanol. Growth rates were similar on methanol and succinate. Generation times were observed to vary from 4 h (in the fermenter) up to 5.5 h (in shake flasks).

Figure 11 shows the levels of the two enzymes when methanol-grown cells were washed in unsupplemented NMS media and resuspended in media containing succinate and when succinate-grown cells were washed in unsupplemented NMS media and resuspended in media containing methanol. In each case, the major isoenzyme decreases, and the minor isoenzyme increases. The activity of the minor isoenzyme reaches a maximum within 12 h. Twelve hours corresponds to three generations.

Properties of STHM from methane-grown cells. STHM was purified from cells grown on methane by the same procedure described for methanol-grown cells. The enzymes from cells grown on methane and methanol had the same relative mobility on acrylamide gels, and the partially purified enzyme from cells grown on methane was stimulated fourfold by glyoxylate.

DISCUSSION

We have separated two STHM activities from a facultative methylotroph, previously described by Patt et al. (16). The separable enzymes have similar pH optima, maximum velocities, Michaelis constants for serine, and cofactor requirements. However, they differ in many respects.

TABLE 5. *Incorporation of substrates into cold trichloroacetic acid-precipitable material by isolate XX*

Growth condition	Incubation conditions	Incorporation[a]
Methanol	[^{14}C]methanol (0.5%)	20.4
Methanol + succinate	[^{14}C]methanol (0.5%) + [^{12}C]succinate (0.1%)	4.5
Succinate	[^{14}C]succinate (0.1%)	24.0
Succinate + methanol	[^{14}C]succinate (0.1%) + [^{12}C]methanol (0.5%)	22.2

[a] Expressed as micromoles of carbon from ^{14}C-labeled substrate/hour per milligram of protein.

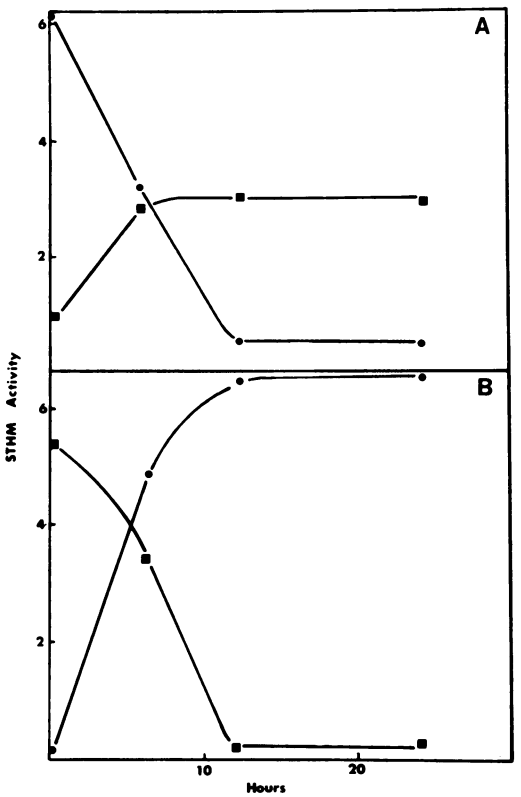

FIG. 11. *Activity of the two STHMs. (A) Methanol-grown cells resuspended in media containing succinate as the sole carbon and energy source. (B) Succinate-grown cells resuspended in media containing methanol as the sole carbon and energy source. Symbols: ●, activity of the glyoxylate-activated enzyme; ■, activity of the glyoxylate-insensitive enzyme.*

The enzyme from methanol-grown cells is more positively charged than the enzyme from succinate-grown cells (at pH 7.3) as evidenced by its smaller affinity for DEAE-cellulose, a cationic resin. The enzyme from methanol-grown cells has twice the molecular weight of the enzyme from succinate-grown cells, migrates slower on acrylamide gels, and sediments faster on a glycerol gradient. Both enzymes are stimulated by K^+, Na^+, and Ca^{2+}, but only the enzyme from succinate-grown cells is stimulated by Mg^{2+}, Mn^{2+}, and Zn^{2+}. The specific activities in crude extracts differ, as well as their stabilities at 4 and −20 C.

Both partially purified enzymes were inhibited competitively by glycine. The partially purified enzyme from methanol-grown cells was activated over fourfold by glyoxylate, whereas the partially purified enzyme from succinate-grown cells was weakly inhibited. Since glyoxylate is a precursor to glycine in the C-1 assimilation pathway, it is not surprising that it would affect the enzyme from methanol-grown cells in a positive manner.

The synthesis of the two isoenzymes responded differently to compounds added to the growth medium. Glyoxylate, adenine, and trimethoprim increased the level of the glyoxylate-activated enzyme in both methanol- and succinate-grown cells. The other precursors and end products tested (guanine, thymine, glycine, serine, and methionine) had less significant effects on the two enzymes.

Glyoxylate might also be expected to affect the synthesis of the enzyme from methanol-grown cells positively since it is a precursor in the C-1 assimilation pathway. Adenine is an end product of a pathway in which methylene tetrahydrofolate is an intermediate, and its effect would have been difficult to predict. Trimethoprim has been shown by Stauffer et al. (19) to derepress the STHM from *Salmonella typhimurium*, possibly as a result of decreased thymine pools in the cell.

The enzymes show both similarities and differences when compared to STHMs purified from other sources. The pH optima were similar to that found for the yeast STHM (15), but rabbit liver and *E. coli* enzymes showed lower optima (10, 18). The K_m's for serine were slightly higher than the range of 6.6×10^{-4} to 8.0×10^{-4} M found for the yeast, rabbit liver, and *E. coli* enzymes (10, 15, 18).

The specific activities of the purified STHM isoenzymes from isolate XX compare favorably with the same enzyme from *E. coli* (28 μmol of formaldehyde/h per mg of protein [10]) and *Saccharomyces cerevisiae* (40 μmol of formaldehyde/h per mg of protein [15]).

Stimulation by mono- and divalent cations has been reported for the yeast enzyme (15) which responds to K^+, Na^+, Ca^{2+}, Mg^{2+}, and Mn^{2+}.

The enzyme from cells grown on methanol has approximately the same molecular weight as the rabbit liver and yeast enzymes (200,000), although the *E. coli* enzyme is a bit smaller, at 170,000 (10, 15, 18). The rabbit liver STHM has been found to consist of four subunits, like the enzyme from cells grown on methanol (18). The enzyme from cells grown on succinate is smaller than other STHMs isolated (10, 15, 18).

The enzyme levels in extracts of cells grown on methanol plus succinate are roughly proportional to the fraction of carbon incorporated from each substrate. Succinate is incorporated into cold trichloroacetic acid-precipitable material at about the same rate as in succinate-grown cells, and the glyoxylate-insensitive en-

zyme is present at about the same level as in succinate-grown cells. However, methanol is incorporated at a lower rate than in methanol-grown cells, and the glyoxylate-stimulated enzyme is present at a lower level than in methanol-grown cells.

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