Characterization of the Assimilatory and Dissimilatory Pathways of Carbon Metabolism during Growth of *Methylophilus methylotrophus* on Methanol

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The enzyme profile of methanol-grown *Methylophilus methylotrophus* has been determined. It shows that the organism uses a variant of the ribulose monophosphate cycle of formaldehyde fixation that involves cleavage of hexose phosphate by 2-keto-3-deoxy-6-phosphogluconate aldolase and a rearrangement sequence involving transketolase and transaldolase. The organism possesses high concentrations of a glucose-6-phosphate dehydrogenase active with both NADP⁺ and NAD⁺, and two separate 6-phosphogluconate dehydrogenases, one active with both NADP and NAD⁺ and the other active only with NAD⁺. In addition, the organism contains methanol dehydrogenase, and NAD⁺-linked formaldehyde and formate dehydrogenases, thus possessing the enzymic potential necessary for both cyclic and linear sequences for oxidation of the formaldehyde derived from methanol. Hexulose phosphate synthase, phosphohexulose isomerase, glucose-6-phosphate dehydrogenase and the two 6-phosphogluconate dehydrogenases have been purified, characterized and examined for possible regulatory properties.

INTRODUCTION

*Methylophilus methylotrophus* (*Pseudomonas methylotropha*) is an obligate methylotroph capable of growth on methanol and methylated amines (Large & Haywood, 1981); it is currently being grown on a large scale on methanol by Imperial Chemical Industries Ltd as a source of single-cell protein (Byrom & Ousby, 1975; Windass et al., 1980). This paper describes the characterization, via the enzymic profile, of the modes of carbon assimilation and dissimilation by this organism. The study has involved purification of the following key enzymes: hexulose-6-phosphate synthase, phosphohexulose isomerase, glucose-6-phosphate dehydrogenase and the two 6-phosphogluconate dehydrogenases.

Part of this work has been reported in preliminary form (Beardsmore et al., 1978).

METHODS

Maintenance and growth of the organism. The medium for growth and maintenance contained 0.5% (v/v) methanol as carbon source and the following inorganic salts (g l⁻¹): K₂HPO₄, 1.9; NaH₂PO₄, 1.56; (NH₄)₂SO₄, 1.8; MgSO₄·7H₂O, 0.2; FeSO₄·7H₂O, 5 × 10⁻³; CuSO₄·5H₂O, 1 × 10⁻⁴; MnSO₄·5H₂O, 5 × 10⁻⁴; ZnSO₄·7H₂O, 5 × 10⁻¹; CaCl₂·2H₂O, 1.3 × 10⁻³; CoCl₂, 1 × 10⁻³; H₃BO₃, 7 × 10⁻⁴; Na₂MoO₄·1 × 10⁻⁵.

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Abbreviations: Fru-6-P, fructose-6-phosphate; Glc-6-P, glucose-6-phosphate; 6-P-GlcA, 6-phosphogluconate; Hu-6-P, hexulose-6-phosphate; KDPG, 2-keto-3-deoxy-6-phosphogluconate; Rib-5-P, ribose-5-phosphate; Ru-5-P, ribulose-5-phosphate; GSH, glutathione (reduced); SDS, sodium dodecyl sulphate.
The pH of the medium was adjusted to 6.8. Solid medium was prepared by the addition of 1% (w/v) Oxoid Agar No. 1. Unless stated otherwise, the organism was grown in liquid culture at 37 °C in shake flasks and harvested during mid- to late-exponential phase by centrifugation at 4500 g for 15 min; the cells were washed once with 20 mM KH₂PO₄/NaOH buffer pH 7.0 and either used immediately or stored at −15 °C. For large-scale purification of enzymes, frozen cell paste of *M. methylotrophus*, grown on methanol under carbon limitation at 37 °C, pH 6-8, dilution rate 0-2 h⁻¹, in a chemostat, was supplied by ICI, Billingham.

**Preparation of cell-free extracts.** Cell suspensions containing 1 part (wet weight) of cell paste suspended in 3 vol. buffer (unless otherwise stated the buffer was 20 mM KH₂PO₄/NaOH pH 7.2 containing 5 mM MgCl₂) were disrupted in an MSE sonicator (model 150W) for five 1 min periods, interspersed with periods of cooling in broken ice. The cell debris was removed by centrifugation at 38000 g for 15 min at 2 °C; the resulting supernatant is referred to as ‘crude extract’. In assay systems linked to the oxidation of NADH a further centrifugation at 100000 g for 1 h was necessary to remove NADH oxidase activity.

**Enzyme assays.** Assays were performed at 37 °C unless otherwise stated; a Unicam recording spectrophotometer (SP1800) was used for spectrophotometric assays. One unit of enzyme activity is defined as that amount of enzyme catalysing the transformation of 1 μmol substrate min⁻¹. Specific activities are expressed as units (mg protein)⁻¹. Protein was determined by the Lowry method using bovine serum albumin as standard.

Hexose-6-phosphate synthase (Hu-6-P synthase; α-arabinino-3-hexulose phosphate formaldehyde lyase) was assayed by three methods. (1) Based on Ru-5-P-dependent removal of formaldehyde. The discontinuous method of Ferenci et al. (1974) was used. (2) Based on the Ru-5-P- and formaldehyde-dependent production of hexose phosphate. The continuous enzymically coupled method of van Dijken et al. (1978) was used. (3) Based on the hexose phosphate-dependent production of formaldehyde and Ru-5-P. The continuous, enzymically coupled method of Ferenci et al. (1974) was used.

Phosphohexulose isomerase (Hu-6-P isomerase) was assayed in the forward direction by the continuous, enzymically coupled method of van Dijken et al. (1978) and in the reverse direction by the continuous, enzymically coupled method of Ferenci et al. (1974).

Fructose-bisphosphatase (d-fructose-1,6-bisphosphate 1-phosphohydrolase; EC 3.1.3.11) was assayed using the procedure of Pontremoli (1966) except that 5 mM MgCl₂ replaced 5 mM MnCl₂ and 1 mM EDTA was included.

Fructose-1,6-bisphosphatase aldolase (d-fructose-1,6-bisphosphate d-glyceraldehyde-3-phosphate lyase; EC 4.1.2.13) was assayed in the following assay mixture (1 ml): 50 μmol Tris/HCl pH 7-5, 1 μmol CoCl₂, 5 units triosephosphate isomerase (EC 5.3.1.1), 0.6 unit glycerol-3-phosphate dehydrogenase (EC 1.1.1.8), 0.25 μmol NADH. The assay was started by the addition of 2 μmol fructose-1,6-bisphosphate.

NADP⁺-linked 6-phosphogluconate dehydrogenase [6-phospho-d-glucurate:NAD(P)⁺ oxidoreductase (decarboxylating); EC 1.1.1.44] was assayed in a final volume of 1 ml containing 50 μmol Tris/HCl buffer pH 8-2, 0.25 μmol NADP⁺ and 5 μmol 2-mercaptoethanol. The reaction was started by the addition of 2-5 μmol 6-P-GlcA. The NAD⁺-linked 6-P-GlcA dehydrogenase was assayed in the same volume of assay mixture containing 50 μmol KH₂PO₄/NaOH buffer pH 7-2, 2-5 μmol NAD⁺; the reaction was started by the addition of 2-5 μmol 6-P-GlaC.

NAD⁺-linked formaldehyde dehydrogenase [formaldehyde:NAD⁺ oxidoreductase (glutathione formylating); EC 1.2.1.1] was assayed by a modification of the spectrophotometric method described by Rose & Racker (1966). The reaction mixture in a final volume of 1 ml contained 100 μmol triethanolamine buffer pH 8.5, 3.4 μmol GSH, 10 μmol NAD⁺, 10 μmol formaldehyde (hydroxymine was omitted from the mixture). The reaction was started by the addition of formaldehyde. For assay of this enzyme, cells growing under carbon limitation were harvested and cell-free extracts made immediately. The crude extracts were then dialysed against 250 vol. sonication buffer for 2 h to remove traces of pentose phosphates which, in the presence of Hu-6-P synthase, Hu-6-P isomerase and Glc-6-P dehydrogenase, could give rise to possibly misleading positive results.

Unmodified published procedures were used for assaying the following enzymes: glucose-6-phosphate dehydrogenase (d-glucose 6-phosphate:NAD(P)⁺ oxidoreductase; EC 1.1.1.49) (Hohorst, 1963); phosphoglucoisomerase (d-glucose 6-phosphate ketol-isomerase; EC 5.3.1.9) (Hohorst, 1963); phosphofructokinase (ATP:d-fructose-6-phosphate 1-phosphotransferase; EC 2.7.1.11) (Sols & Salas, 1966); hydroxyxpyruvate reductase (d-glycerate:NAD⁺ oxidoreductase; EC 1.1.1.29) (Large & Quayle, 1963); transaldolase (sedoheptulose-7-phosphate: d-glyceraldehyde-3-phosphate dihydroxacetone transferase, EC 2.2.1.2) (Tchola & Horecker, 1966); transketolase (sedoheptulose-7-phosphate: d-glyceraldehyde-3-phosphate glyceraldehydetransferase EC 2.2.1.1) (Ström et al., 1974); ribulose-5-phosphate 3-epimerase (d-ribulose-5-phosphate 3-epimerase; EC 5.1.3.1) (Ström et al., 1974); phosphoribosimutase (d-ribose-5-phosphate ketol-isomerase; EC 5.3.1.6) (Horecker et al., 1958); formate dehydrogenase (formate:NAD⁺ oxidoreductase; EC 1.2.1.2) (Quayle, 1966); sedoheptulose-1,7-bisphosphatase (sedoheptulose-1,7-bisphosphate 1-phosphohydrolase; EC 3.1.3.37) (Racker, 1963); ribulose-5-phosphate carboxylase [3-phospho-d-glyceraldehyde (dimerizing); EC 4.1.1.39] (Racker, 1962); 6-phosphogluconate dehydrogenase (6-phospho-d-gluconate hydratase; EC 4.2.1.12) and 2-keto-3-deoxy-6-phosphogluconate aldolase (KDPG aldolase; 6-phospho-2-keto-3-deoxy-d-gluconate d-glyceraldehyde-3-phosphate lyase; EC 4.1.2.14) were assayed together (Wood, 1971).

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Determination of Michaelis constants. These were determined by the method of Eisenthal & Cornish-Bowden (1974).

Purification of Hu-6-P synthase. The crude extract was prepared from 20 g cell paste suspended in 3 vol. 20 mM-KH₂PO₄/NaOH buffer pH 7.2 containing 5 mM-MgCl₂, prior to sonication. All steps in the purification were performed at 0–4 °C.

Step 1. Protamine sulphate precipitation. A solution of saline sulphate (10%, w/v) was added slowly to 67 ml cell-free extract, to a final concentration of 1 mg saline sulphate to 10 mg protein. After 20 min, the precipitate was removed by centrifugation at 38000 g for 15 min and discarded.

Step 2. (NH₄)₂SO₄ fractionation. Solid (NH₄)₂SO₄ was added to the supernatant from Step 1 to 45% saturation as calculated from the table of Dawson et al. (1969). The resulting precipitate was removed by centrifugation and discarded. (NH₄)₂SO₄ was then added to 65% saturation. The resulting precipitate, containing Hu-6-P synthase, was collected by centrifugation and dissolved in 7.5 ml 20 mM-KH₂PO₄/NaOH buffer pH 7.2 containing 5 mM-MgCl₂.

Step 3. DEAE-cellulose chromatography. The redissolved protein from Step 2 was desalted by passage through a Sephadex G-25 column (33.5 x 2.5 cm), equilibrated with 20 mM-KH₂PO₄/NaOH buffer pH 7.2 containing 5 mM-MgCl₂. The eluted protein was then applied to a DEAE-cellulose (Whatman DE52) column (11.5 x 1.5 cm) equilibrated with the same buffer. The Hu-6-P synthase activity was eluted from the column with a linear NaCl gradient (0–0.1 M-NaCl made up in 400 ml of the column buffer) and 4.5 ml fractions were collected; Hu-6-P synthase activity eluted in fractions 21–40. These fractions were pooled and treated with solid (NH₄)₂SO₄ to 75% saturation. The resulting precipitated protein was collected by centrifugation and dissolved in 3.3 ml 20 mM-KH₂PO₄/NaOH buffer pH 7.2 containing 5 mM-MgCl₂.

Step 4. Sephadex G-200 chromatography. The redissolved protein from Step 3 was applied to a Sephadex G-200 column (38 x 2.5 cm) equilibrated in 20 mM-KH₂PO₄/NaOH buffer pH 7.2 containing 5 mM-MgCl₂. The protein was eluted with the same buffer and fractions (4.6 ml) were collected. Hu-6-P synthase activity appeared in fractions 27–39. Fractions containing activity were combined and concentrated to 6.2 ml by ultrafiltration through a membrane filter (Diaflo PM10; Amicon Corp., Lexington, Mass., U.S.A.).

Step 5. Hydroxylapatite chromatography. The concentrated enzyme from Step 4 was desalted by passage through a Sephadex G-25 column (33.5 x 2.5 cm) equilibrated with 1 mM-KH₂PO₄/NaOH buffer pH 7.2 and 5 mM-MgCl₂. The eluted protein was applied to a hydroxylapatite column (5.5 x 1.5 cm) equilibrated with the same buffer. The Hu-6-P synthase activity was eluted from the column with a linear KH₂PO₄/NaOH gradient (1–50 mM, pH 7.2, made up in 400 ml of the column buffer). Fractions (4.6 ml) were collected and fractions 37–42, which contained enzyme activity, were pooled, concentrated to 6.2 ml by ultrafiltration, and stored at -15 °C.

Purification of Hu-6-P isomerase. The crude extract was prepared from 50 g cell paste suspended in 3 vol. 20 mM-Tris/HCl buffer pH 8.0 containing 5 mM-EDTA, and sonicated in separate 25 ml batches. After sonication the separate extracts were combined for subsequent treatment at 0–4 °C.

Step 1. A solution of saline sulphate (10%, w/v) was added slowly to 151 ml cell-free extract to a final concentration of 1 mg saline sulphate to 10 mg protein. After 20 min, the precipitate was removed by centrifugation at 38000 g for 15 min and discarded.

Step 2. (NH₄)₂SO₄ fractionation. Solid (NH₄)₂SO₄ was added to the supernatant from Step 1 to 60% saturation as calculated from the table of Dawson et al. (1969). The resulting precipitate was removed by centrifugation and discarded. (NH₄)₂SO₄ was then added to 90% saturation. The resulting precipitate, containing Hu-6-P isomerase, was collected by centrifugation and dissolved in 7.5 ml 20 mM-KH₂PO₄/NaOH buffer pH 7.2 containing 5 mM-MgCl₂.

Step 3. DEAE-cellulose chromatography. The redissolved protein was desalted by passage through a Sephadex G-25 column (33.5 x 2.5 cm), equilibrated with 1 mM-KH₂PO₄/NaOH buffer pH 7.2 and 5 mM-MgCl₂. The eluted protein was applied to a DEAE-cellulose (Whatman DE52) column (12.0 x 2.6 cm) equilibrated in the same buffer. The Hu-6-P isomerase activity was eluted from the column with a linear NaCl gradient (0–0.2 M-NaCl made up in 400 ml of the column buffer). Fractions (4.9 ml) were collected; Hu-6-P isomerase was eluted in fractions 40–70. These fractions were pooled and concentrated by ultrafiltration to 7.5 ml as described above.

Step 4. Sephadex G-100 chromatography. The extract from Step 3 was applied to a Sephadex G-100 column (84 x 2.5 cm) equilibrated with 20 mM-Tris/HCl buffer pH 8.0 containing 5 mM-EDTA. The protein was eluted with the same buffer; fractions (4.9 ml) were collected. Hu-6-P isomerase activity eluted in fractions 30–48; these fractions were pooled, concentrated to 7.0 ml by ultrafiltration, and stored at -15 °C.

Purification of Glc-6-P dehydrogenase. The crude extract was prepared from 8 g cell paste suspended in 3 vol. 20 mM-Tris/HCl buffer pH 8.0, prior to sonication. All steps in the purification were performed at 0–4 °C.

Step 1. (NH₄)₂SO₄ fractionation. To the crude extract, solid (NH₄)₂SO₄ was added to 60% saturation. The precipitated protein was removed by centrifugation and discarded. The concentration of (NH₄)₂SO₄ in the supernatant was raised to 80% saturation and the precipitated protein collected by centrifugation and dissolved in 20 mM-Tris/HCl buffer pH 8.0.

Step 2. Heat treatment. The extract from Step 1 was heated to 50 °C for 15 min; the resulting precipitate was removed by centrifugation and discarded.
Step 3. DEAE-cellulose chromatography. The supernatant from Step 2 was desalted by passage through a Sephadex G-25 column (33.5 x 2.5 cm) equilibrated with 20 mM-Tris/HCl buffer pH 9-0. Active fractions eluted from this column were pooled and applied to a DEAE-cellulose (Whatman DE52) column (8.0 x 2.5 cm) equilibrated with the same buffer. Glc-6-P dehydrogenase activity was eluted from the column with a linear NaCl gradient (0-0.2 M-NaCl made up in 400 ml of the column buffer). Fractions (4-75 ml) were collected; Glc-6-P dehydrogenase was eluted in fractions 39-49, which were pooled and concentrated by ultrafiltration to 5.6 ml.

Step 4. Sephadex G-200 chromatography. The extract from Step 3 was applied to a Sephadex G-200 column (82.5 x 2.5 cm) equilibrated with 20 mM-Tris/HCl buffer pH 9-0. The protein was eluted with the same buffer and fractions (4-75 ml) were collected. Glc-6-P dehydrogenase activity eluted in fractions 38-45; these fractions were pooled, concentrated by ultrafiltration to 4.6 ml, and stored at -15 °C.

**Purification of NADP+-linked 6-P-GlcA dehydrogenase.** The crude extract was prepared from 10 g cell paste suspended in 3 vol. 20 mM-Tris/HCl buffer pH 8-2 containing 5 mM-2-mercaptoethanol, prior to sonication. All steps in the purification were performed at 0-4 °C.

Step 1. (NH₄)₂SO₄ fractionation. To the crude extract, solid (NH₄)₂SO₄ was added to 40% saturation. The precipitated protein was removed by centrifugation and discarded. The supernatant was further treated with (NH₄)₂SO₄ to 60% saturation and the precipitated protein resuspended in the sonication buffer.

Step 2. First DEAE-cellulose chromatography. The redisolved protein was desalted by passage through a Sephadex G-25 column (34 x 2.5 cm); the active fractions were pooled and applied to a DEAE-cellulose (Whatman DE52) column (24 x 1.5 cm) equilibrated with 20 mM-Tris/HCl buffer pH 8-2 containing 5 mM-2-mercaptoethanol. The NADP+-linked 6-P-GlcA dehydrogenase activity was eluted from the column by a linear NaCl gradient (0-0.2 M-NaCl made up in 160 ml of the column buffer). Fractions (4-1 ml) were collected; enzyme activity appeared in fractions 22-36. The most active fractions (24-27) were pooled, concentrated by ultrafiltration to 4.0 ml, and stored at 4 °C.

**Purification of NADP+-linked 6-P-GlcA dehydrogenase.** The crude extract was prepared from 15 g cell paste suspended in 3 vol. 20 mM-KH₂PO₄/NaOH buffer pH 7-2 containing 5 mM-MgCl₂, prior to sonication. All steps in the purification procedure were performed at 0-4 °C.

Step 1. (NH₄)₂SO₄ fractionation. To the crude extract, solid (NH₄)₂SO₄ was added to 40% saturation. The precipitated protein was removed by centrifugation and discarded. Further (NH₄)₂SO₄ was added to the supernatant to 60% saturation and the precipitated protein was redissolved in the sonication buffer.

Step 2. Heat treatment. The redisolved protein was heated to 50 °C for 10 min. The precipitated protein was removed by centrifugation and discarded.

Step 3. First DEAE-cellulose chromatography. The supernatant from Step 2 was desalted by passage through a Sephadex G-25 column (30 x 2.5 cm) equilibrated with sonication buffer, and applied to a DEAE-cellulose (Whatman DE52) column (9.0 x 1.5 cm) equilibrated with the same buffer. The NADP+-linked 6-P-GlcA dehydrogenase activity bound only weakly to the column and was usually eluted immediately after the void volume of sonication buffer had been passed through the column. In other cases, it was eluted with a linear NaCl gradient (0-0.2 M-NaCl made up in 160 ml of column buffer). The fractions which contained the enzyme were pooled and treated with solid (NH₄)₂SO₄ to 70% saturation; the precipitated protein was redissolved in the sonication buffer. This was desalted in the same way as was the supernatant from the heat treatment step.

Step 4. Second DEAE-cellulose chromatography. The desalted protein solution was applied to a DEAE-cellulose column (9.5 x 1.5 cm) and eluted with a linear NaCl gradient (0-0.2 M-NaCl made up in 400 ml of the column buffer). Fractions (4-7 ml) were collected. Two peaks containing 6-P-GlcA dehydrogenase activity were found: fractions 20-30 contained a small amount of NADP+-linked activity; fractions 30-45 contained a large amount of NADP+-linked activity. Fractions 32-42 were pooled, concentrated by ultrafiltration to 4.0 ml, and stored at 4 °C.

**Molecular weight determination by gel filtration.** The molecular weights of Hu-6-P synthase and Hu-6-P isomerase were determined by chromatography of the enzymes on calibrated columns of Sephadex G-200, by the procedure of Andrews (1965).

**Polyacrylamide gel electrophoresis of proteins.** Discontinuous polyacrylamide gel electrophoresis was done at pH 8.3-8.5 with 7.5% (w/v) polyacrylamide gels as described by Davis (1964). SDS-polyacrylamide gel electrophoresis was done by the method of Zahler (1974); standard proteins were obtained from Boehringer (Combithek electrophoresis calibration kit).
Table 1. Specific activities of enzymes in cell-free extracts of M. methylotrophus

The preparation of crude cell-free extracts from bacteria grown in batch culture, and the assay procedures used, are described in Methods. The assay methods used for Hu-6-P synthase and Hu-6-P isomerase were those of van Dijken et al. (1978).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity [µmol min⁻¹ (mg protein)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexulose-6-phosphate synthase</td>
<td>3.8</td>
</tr>
<tr>
<td>Hexulose-6-phosphate isomerase</td>
<td>15.5</td>
</tr>
<tr>
<td>Glucose-6-phosphate ketol-isomerase</td>
<td>0.39</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase (NADP⁺-linked)</td>
<td>1.58</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase (NAD⁺-linked)</td>
<td>1.25</td>
</tr>
<tr>
<td>6-Phosphogluconate dehydrogenase (NAD⁺-linked)</td>
<td>0.24</td>
</tr>
<tr>
<td>6-Phosphogluconate dehydrogenase (NAD⁺-linked)</td>
<td>0.13</td>
</tr>
<tr>
<td>Transketolase</td>
<td>0.65</td>
</tr>
<tr>
<td>Transaldolase</td>
<td>0.37</td>
</tr>
<tr>
<td>Sedoheptulose-1,7-bisphosphatase</td>
<td>ND</td>
</tr>
<tr>
<td>Phosphoribosylamine</td>
<td>0.74</td>
</tr>
<tr>
<td>Ribulose phosphate 3-epimerase</td>
<td>0.37</td>
</tr>
<tr>
<td>6-Phosphofructokinase</td>
<td>ND</td>
</tr>
<tr>
<td>6-Phosphogluconate dehydrogenase and</td>
<td></td>
</tr>
<tr>
<td>2-keto-3-deoxy 6-phosphogluconate aldolase</td>
<td>0.09</td>
</tr>
<tr>
<td>Fructose-1,6-bisphosphate aldolase</td>
<td>0.07</td>
</tr>
<tr>
<td>Fructose-1,6-bisphosphatephatase</td>
<td>0.03</td>
</tr>
<tr>
<td>Formaldehyde dehydrogenase (NAD⁺-linked)*</td>
<td>0.25</td>
</tr>
<tr>
<td>Formate dehydrogenase</td>
<td>0.015</td>
</tr>
<tr>
<td>Hydroxypyruvate reductase</td>
<td>ND</td>
</tr>
<tr>
<td>Ribulose-1,5-bisphosphate carboxylase</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, None detected.

* Dialysed extracts from freshly harvested cells were used for this assay (see Methods).

Results

Mode of carbon assimilation

No activities of ribulose bisphosphate carboxylase or hydroxypyruvate reductase were detected in cell-free extracts of the organism grown on methanol in batch culture, whereas high activities of Hu-6-P synthase and Hu-6-P isomerase were present (Table 1). This shows that methanol carbon is assimilated via the Ru-5-P cycle of formaldehyde fixation rather than the ribulose bisphosphate cycle of carbon dioxide fixation or a serine pathway (Quayle, 1980a). The Ru-5-P cycle can exist in four possible variants depending on the mode of hexose phosphate cleavage and the mode of rearrangement of hexose phosphate and triose phosphate to pentose phosphate (Quayle & Ferenci, 1978). The lack of 6-phosphofructokinase and sedoheptulose bisphosphatase, coupled with the presence of 6-P-GlcA dehydrase/KDPG aldolase and transaldolase, suggest that the variant of the Ru-5-P cycle that operates in M. methylotrophus involves hexose phosphate cleavage via the Entner–Doudoroff pathway and rearrangement via transaldolase. This would be termed an eda⁺/tal⁺ variant, according to the terminology of Quayle & Ferenci (1978).

The present paper reports the purification and characterization of the Hu-6-P synthase and Hu-6-P isomerase. Studies of the further fate of the 6-P-GlcA carbon skeleton during carbon assimilation by M. methylotrophus will be reported separately.

Mode of carbon dissimilation

Methylophilus methylotrophus, like many other C₁-utilizing bacteria, contains high activities of methanol dehydrogenase, which catalyses the dehydrogenation of methanol to formaldehyde (Ghosh & Quayle, 1978, 1981). This enzyme can also convert formaldehyde to
Table 2. Purification of hexulose-6-phosphate synthase

The crude extract was prepared from 20 g bacteria (chemostat-grown under methanol limitation) suspended in 3 vol. 20 mM-KH$_2$PO$_4$/NaOH buffer pH 7.2 containing 5 mM-MgCl$_2$. Details are described in Methods. Activity is expressed as μmol min$^{-1}$ and specific activity as μmol min$^{-1}$ (mg protein)$^{-1}$.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Purification factor</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>67</td>
<td>7050</td>
<td>1.77</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Protamine sulphate treatment</td>
<td>98.5</td>
<td>6840</td>
<td>5.10</td>
<td>2.9</td>
<td>97</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$ fractionation</td>
<td>7.5</td>
<td>3255</td>
<td>7.6</td>
<td>4.3</td>
<td>46</td>
</tr>
<tr>
<td>DEAE-cellulose chromatography</td>
<td>3.3</td>
<td>2230</td>
<td>50.7</td>
<td>28.6</td>
<td>32</td>
</tr>
<tr>
<td>Sephadex G-200 chromatography</td>
<td>6.2</td>
<td>1645</td>
<td>53.0</td>
<td>30.0</td>
<td>23</td>
</tr>
<tr>
<td>Hydroxylapatite chromatography</td>
<td>6.2</td>
<td>750</td>
<td>97.4</td>
<td>55.0</td>
<td>10.6</td>
</tr>
</tbody>
</table>

formate, although to what extent it catalyses this second oxidation in vivo is uncertain (see Dawson & Jones, 1981). Our initial studies with the bulk-grown cell paste showed little activity that could be unequivocally ascribed to a pyridine nucleotide-linked formaldehyde dehydrogenase, although the presence of high activities of interfering enzymes in crude extracts should be borne in mind. However, later studies (at Billingham) with dialysed extracts of freshly harvested cells grown in the chemostat under carbon limitation gave unequivocal evidence of the presence of an NAD$^+$-linked formaldehyde dehydrogenase, the activity of the enzyme being stimulated approximately 20% by 3.4 mM-GSH.

Low activities of NAD$^+$-linked formate hydrogenase were detected in batch-grown cells although a detailed study of optimal assay conditions was not made.

High activities of pyridine nucleotide-linked Glc-6-P and 6-P-GlcA dehydrogenases were detected, showing the possibility of a major route of formaldehyde dissimilation via a cyclic sequence similar to that suggested by Strøm et al. (1974) in Pseudomonas methanica and Methyllococcus capsulatus and by Colby & Zatman (1975) in other obligate and restricted facultative methylotrophs. Evidence for the operation of such a cycle has been obtained in whole cells and extracts of methanol-grown Pseudomonas C (Ben-Bassat & Goldberg, 1977; Ben-Bassat et al., 1980) and in extracts of Pseudomonas oleovorans (Sokolov & Trotsenko, 1977).

Our overall results for the enzyme profile of M. methylotrophus are in good agreement with those of Large & Haywood (1981), although further work needs to be done to define more precisely the amounts of the NAD$^+$-linked formaldehyde and formate dehydrogenases.

In the following sections we report the purification and characterization of the pyridine nucleotide-linked Glc-6-P and 6-P-GlcA dehydrogenases from bacteria grown under methanol limitation in continuous culture.

Purification and properties of Hu-6-P synthase

Hu-6-P synthase from M. methylotrophus was found to be a soluble enzyme: over 90% of the activity in crude cell-free extracts remained in the supernatant after centrifugation. The enzyme was purified 55-fold with a yield of 10.6% (Table 2). The purified enzyme was free from detectable activities of Hu-6-P isomerase, phosphoribosimutase, phosphoglucoisomerase or Glc-6-P dehydrogenase. Polyacrylamide gel electrophoresis at pH 8.3 showed only one minor protein band in addition to the major band which contained Hu-6-P synthase activity. No enzyme activity could be detected in the minor band, which was estimated to contain approximately 5% of the protein applied to the gel.

pH optimum. The pH optimum of the reaction, assayed in the forward direction, was 7.2. At this pH there was little difference in enzyme activity when assayed in the following different buffers at 20 mM concentration: KH$_2$PO$_4$/NaOH, Tris/HCl, imidazole/HCl.

Substrate specificity. The substrate specificity was tested by the discontinuous assay
Methanol metabolism by M. methylotrophus

Fig. 1. Stability of hexulose-6-phosphate synthase. The purified enzyme was incubated at 60 °C in 20 mM-KH₂PO₄/NaOH buffer containing 5 mM-MgCl₂ and additions as indicated; samples were withdrawn for assay of activity by the procedure of van Dijken et al. (1978). Additions: ■, none; ○, phosphoribosyltransferase (1.75 units); □, 5 mM-formaldehyde; △, 1.5 mM-Ru-5-P; ●, phosphoribosyltransferase (1.75 units) + 5 mM-Rib-5-P.

Table 3. Activation and inhibition of hexulose-6-phosphate synthase by divalent metal ions

Activity of the enzyme in the presence of the cations at a final concentration of 1 mM is expressed as a percentage of the rate measured in the presence of 1 mM-MgCl₂. Inhibition of the enzyme was determined after the addition of the divalent cations at a concentration of 1 mM to the standard assay which included 1 mM-MgCl₂. Inhibition is expressed as a percentage of the rate measured in the presence of 1 mM-MgCl₂ alone. The assay mixture contained 50 mM-imidazole/HCl pH 7.2 as buffer and the reaction was followed by discontinuous measurement of the disappearance of formaldehyde (see Methods).

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>Activity (%)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg²⁺</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Co²⁺</td>
<td>63</td>
<td>20</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>35</td>
<td>28</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>16</td>
<td>3</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>60</td>
<td>37</td>
</tr>
<tr>
<td>Hg²⁺</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

method involving measurement of the substrate-dependent removal of formaldehyde (Ferenci et al., 1974). No activity of the purified enzyme (100 µl) was detected with the following substrates: Rib-5-P (5 mM); Fru-6-P (10 mM); xylulose-5-phosphate (1 mM); erythrose-4-phosphate (1 mM); Glc-6-P (10 mM); fructose-1,6-bisphosphate (5 mM); glyceraldehyde-3-phosphate (1 mM), dihydroxyacetone phosphate (1 mM). Commercially available Ru-5-P (Sigma) at 5 mM concentration gave only 42% of the rate given by an equivalent concentration of Ru-5-P generated in situ from Rib-5-P in the presence of excess phosphoribosyltransferase. Similar behaviour has been noted in the case of the Hu-6-P synthase from Methylococcus capsulatus (Kemp, 1972); this may be due to the presence of an inhibitory impurity in commercial Ru-5-P, which is only claimed to be 80–90% pure.

Effect of metal ions. Hu-6-P synthase has an absolute requirement for a divalent metal ion, Mg²⁺ and Mn²⁺ being the most effective (Table 3). Co²⁺, Zn²⁺, Ca²⁺ and Cu²⁺ were less effective and inhibited the reaction in the presence of Mg²⁺ or Mn²⁺. Presence of either of the last two cations was essential for stability of the enzyme: in their absence the enzyme rapidly and irreversibly lost all activity.

Stability. The purified enzyme lost all activity after 30 min at 60 °C in 20 mM-KH₂PO₄/NaOH buffer containing 5 mM-MgCl₂ (Fig. 1). The presence of Ru-5-P dramatically increased the stability, no detectable loss of activity occurring after 60 min at 60 °C. Formaldehyde showed no such stabilizing effect.
Table 4. Purification of hexulose-6-phosphate isomerase

The crude extract was prepared from 50 g bacteria (chemostat-grown under methanol limitation) suspended in 3 vol. 20 mM-Tris/HCl buffer pH 8.0 containing 5 mM-EDTA. Details are described in Methods. Activity is expressed as pmol min\(^{-1}\) and specific activity as pmol min\(^{-1}\) (mg protein\(^{-1}\)).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total activity (pmol)</th>
<th>Specific activity (pmol min(^{-1}) (mg protein(^{-1}))</th>
<th>Purification factor</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>151</td>
<td>91040</td>
<td>15.5</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Protamine sulphate treatment</td>
<td>193</td>
<td>130000</td>
<td>49.0</td>
<td>3.1</td>
<td>143</td>
</tr>
<tr>
<td>(NH(_4))(_2)SO(_4) fractionation</td>
<td>8.5</td>
<td>71740</td>
<td>350</td>
<td>22.7</td>
<td>79</td>
</tr>
<tr>
<td>DEAE-cellulose chromatography</td>
<td>7.5</td>
<td>50600</td>
<td>1406</td>
<td>91</td>
<td>55</td>
</tr>
<tr>
<td>Sephadex G-100 chromatography</td>
<td>7.0</td>
<td>37100</td>
<td>1768</td>
<td>114</td>
<td>41</td>
</tr>
</tbody>
</table>

Similar effects were noted at lower temperatures. In the same buffer mixture as above, the purified enzyme lost 50% of its activity after 2 d storage at 4 °C and all activity was lost within 2 weeks. Its stability was greatly improved by the addition of 1.5 mM-Ru-5-P or 5 mM-Rib-5-P + phosphoriboisomerase.

The purified enzyme in the absence of substrate lost 70% of its activity after storage at -15 °C for 1 month and 90% after storage for 6 months at this temperature. The stability at -15 °C was again greatly improved by the presence of Ru-5-P.

Michaelis constants. For the determination of the \(K_m\) values for Ru-5-P, formaldehyde and Mg\(^{2+}\) the continuous assay method of van Dijken et al. (1978) was used (see Methods); for the determination of the \(K_m\) for Hu-6-P the continuous assay method of Ferenci et al. (1974) was used (see Methods).

At 5 mM-formaldehyde the apparent \(K_m\) and \(V_{\text{max}}\) for Ru-5-P were \(1.36 \times 10^{-4}\) M and 6.15 \(\mu\)mol min\(^{-1}\) (mg protein\(^{-1}\)) respectively. At 1.67 mM-Ru-5-P the apparent \(K_m\) and \(V_{\text{max}}\) for formaldehyde were \(5.25 \times 10^{-4}\) M and 6.4 \(\mu\)mol min\(^{-1}\) (mg protein\(^{-1}\)). The apparent \(K_m\) and \(V_{\text{max}}\) for Hu-6-P were \(4.1 \times 10^{-5}\) M and 3.0 \(\mu\)mol min\(^{-1}\) (mg protein\(^{-1}\)).

For determination of the apparent \(K_m\) for Mg\(^{2+}\) the continuous spectrophotometric assay of van Dijken et al. (1978) was used at concentrations of 1.67 mM-Ru-5-P and 5 mM-formaldehyde. Since Hu-6-P synthase is inactivated in the absence of divalent metal ions, the enzyme solution which was added to the assay mixture itself contained 0.05 \(\mu\)M-MgCl\(_2\). Under these circumstances the apparent \(K_m\) for Mg\(^{2+}\) was \(2.5 \times 10^{-4}\) M.

Modulation of enzyme activity. No appreciable effect on the activity of Hu-6-P synthase was observed on addition of the following compounds at final concentrations of 1 mM to the reaction mixture used in the continuous assay method of van Dijken et al. (1978): AMP, ADP, ATP, NADH, NADPH, NAD\(^{+}\), NADP\(^{+}\), pyruvate or 6-P-GlcA.

Molecular weight. The molecular weight of Hu-6-P synthase was found to be 40000 by gel permeation chromatography on a column of Sephadex G-200 (37.5 x 2.6 cm) equilibrated in 20 mM-KH\(_2\)PO\(_4\)/NaOH buffer pH 7.2 containing 5 mM-MgCl\(_2\), using cytochrome c (mol. wt 13500), ovalbumin (46000), bovine serum albumin (66000), malate dehydrogenase (70000) and lactate dehydrogenase (140000) as reference markers.

SDS-polyacrylamide gel electrophoresis of 5 \(\mu\)g Hu-6-P synthase at pH 8.5 (see Methods) resulted in a single band corresponding to a molecular weight of 22500.

These results suggest that Hu-6-P synthase is a dimeric protein of molecular weight approximately 40000 consisting of two identical subunits.

Purification and properties of Hu-6-P isomerase

Hu-6-P isomerase was purified 114-fold with an apparent yield of 41% (Table 4). As in the purification of the enzyme from Methylococcus capsulatus (Ferenci et al., 1974), there was an apparent increase in activity of the enzyme after treatment of the crude extract with protamine sulphate, probably due to the removal of inhibitors (metal ions?) from the crude extract. The
purified enzyme was free from detectable activities of phosphoriboisomerase, Hu-6-P synthase, Glc-6-P dehydrogenase and 6-P-GlcA dehydrogenase. Phosphoglucoisomerase (0.25 unit ml⁻¹) was present but did not interfere with Hu-6-P isomerase which was present at an activity 10³ times higher. Polyacrylamide gel electrophoresis at pH 8.3 showed five protein bands, enzyme activity was associated with three of these bands.

**pH optimum.** The pH optimum of the reaction in the forward direction in Tris/HCl buffer was 8.6. There was no significant difference in activity in either Tris/HCl or imidazole/HCl buffers.

**Substrate specificity.** The enzyme showed no appreciable isomerase activity towards any substrate tested other than Hu-6-P.

**Effect of metal ions.** A sample of purified enzyme was freed from EDTA by passage through a Sephadex G-25 column equilibrated in 20 mM-Tris/HCl buffer pH 8.6 and the effect of divalent metal ions on the activity of the enzyme measured in the forward direction was tested. Mg²⁺, Ca²⁺ or Mn²⁺ at 5 mM concentration were virtually without effect; 5 mM-Co²⁺ caused 50% inhibition and Cu²⁺ or Hg²⁺ at 2.5 mM concentration completely inhibited the reaction.

**Stability.** Hu-6-P isomerase was stored at −15 °C in 20 mM-Tris/HCl buffer pH 8.0 containing 5 mM-EDTA for 6 months without detectable loss of activity; at 4 °C in the same buffer the enzyme lost 30% of its activity over 6 months; at 40 °C in the same buffer 90% of the enzyme activity was lost in 10 min.

**Michaelis constants.** The apparent $K_m$ for Hu-6-P measured at pH 8.6 was $1.7 \times 10^{-4}$ M and that for Fru-6-P at pH 7.0 was $1.3 \times 10^{-3}$ M.

**Molecular weight.** During the purification of Hu-6-P isomerase one peak of activity was obtained on passage through a column of Sephadex G-100 (see Methods). A sample of the purified enzyme (60 µg) together with reference proteins [cytochrome c (mol. wt 13 500), ovalbumin (46 000), hexokinase (50 000), bovine serum albumin (66 000) and lactate dehydrogenase (140 000)] was applied to a Sephadex G-200 column (82 × 2.5 cm) equilibrated with 20 mM-Tris/HCl buffer pH 8.6 containing 5 mM-EDTA. The column was eluted with the same buffer and four peaks of enzyme activity were observed (Fig. 2), corresponding to molecular weights of 70 000, 138 000, 200 000 and >200 000; the largest peak of activity corresponded to a molecular weight of 70 000. A sample of crude cell-free extract of methanol-grown *M. methylotrophus* was applied to the same Sephadex G-200 column and only one peak of enzyme activity was observed, at a molecular weight of 70 000. Repetition of chromatography of the purified enzyme through the same Sephadex G-200 column equilibrated with 20 mM-imidazole buffer pH 6.0 containing 5 mM-EDTA resulted in a pattern of four peaks of enzyme activity identical to that observed with Tris/HCl at pH 8.6. It
Table 5. Purification of glucose-6-phosphate dehydrogenase

The crude extract was prepared from 8 g bacteria (chemostat-grown under methanol limitation) suspended in 3 vol. 20 mM-Tris/HCl buffer pH 8.0. Details are described in Methods. Activity is expressed as μmol min⁻¹ and specific activity as μmol min⁻¹ (mg protein)⁻¹.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Purification factor</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>26</td>
<td>1170</td>
<td>1.58</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ fractionation</td>
<td>3.6</td>
<td>954</td>
<td>13.3</td>
<td>8.4</td>
<td>82</td>
</tr>
<tr>
<td>Heat treatment</td>
<td>3.1</td>
<td>971</td>
<td>17.0</td>
<td>11.0</td>
<td>83</td>
</tr>
<tr>
<td>DEAE-cellulose chromatography</td>
<td>5.6</td>
<td>494</td>
<td>25.0</td>
<td>16.0</td>
<td>42</td>
</tr>
<tr>
<td>Sephadex G-200 chromatography</td>
<td>4.6</td>
<td>517</td>
<td>304</td>
<td>192</td>
<td>44</td>
</tr>
</tbody>
</table>

Table 6. Substrate specificity and kinetic parameters of glucose-6-phosphate dehydrogenase

The apparent $K_m$ values for NADP⁺ and NAD⁺ were determined at a fixed concentration of 5.0 mM-Glc-6-P. The apparent $K_m$ for Glc-6-P was determined at a fixed concentration of 0.25 mM-NAD(P)⁺. The assay procedure used was that described by Hohorst (1965).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (m)</th>
<th>$V_{max}$ [μmol min⁻¹ (mg protein)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADP⁺</td>
<td>$1.8 \times 10^{-5}$</td>
<td>262</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>$3.9 \times 10^{-4}$</td>
<td>395</td>
</tr>
<tr>
<td>Glc-6-P (with respect to NADP⁺)</td>
<td>$1.02 \times 10^{-3}$</td>
<td>395</td>
</tr>
<tr>
<td>Glc-6-P (with respect to NAD⁺)</td>
<td>$1.03 \times 10^{-3}$</td>
<td>395</td>
</tr>
</tbody>
</table>

will be recalled that polyacrylamide gel electrophoresis of the enzyme showed five protein bands, three of which had enzyme activity. These results suggest that in vivo the enzyme exists primarily as a single species of molecular weight 70000 but that dimers, trimers and higher oligomers can be formed under certain conditions in vitro. The precise conditions effecting this association were not further investigated.

Purification and properties of Glc-6-P dehydrogenase

Glc-6-P dehydrogenase was purified 192-fold with a yield of 44% (Table 5). The purified enzyme was active with both NADP⁺ and NAD⁺, the ratio of the two activities under the particular assay conditions used being 1.27 in crude extracts and 1.32 for the purified enzyme.

pH optimum. The pH optimum of the reaction as determined in Tris/HCl and glycine/NaOH buffers was 9.0 and the activity was not appreciably different in the two buffers.

Effect of metal ions. Mg²⁺ and Cu²⁺ at concentrations of 5 mM and EDTA at 1 mM concentration did not affect enzyme activity; 1 mM-Hg²⁺ caused complete inhibition.

Stability. The enzyme lost no appreciable activity after 3 months storage at 4°C or −15°C.

Substrate specificity and Michaelis constants. Using the enzyme assay procedure of Hohorst (1963) the apparent Michaelis constants for Glc-6-P, NAD⁺ and NADP⁺ were determined (Table 6). The enzyme has a greater affinity for NADP⁺ than NAD⁺ although at saturating substrate concentrations the $V_{max}$ with NAD⁺ is higher than that with NADP⁺.

Modulation of enzyme activity. In the assay system of Hohorst (1963) no effect of ATP, ADP or AMP was found on the activity of Glc-6-P dehydrogenase in the presence of 0.25 mM-NADP⁺ and 2.5 mM-Glc-6-P. In the presence of suboptimal concentrations of
Table 7. Purification of NADP+-linked 6-phosphogluconate dehydrogenase

The crude extract was prepared from 10 g bacteria (chemostat-grown under methanol limitation) suspended in 3 vol. 20 mM-Tris/Cl buffer pH 8-2 containing 5 mM-2-mercaptoethanol. Details are described in Methods. Activity is expressed as μmol min⁻¹ and specific activity as μmol min⁻¹ (mg protein)⁻¹.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Purification factor</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>32-0</td>
<td>150</td>
<td>0-240</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ fractionation</td>
<td>9-0</td>
<td>137</td>
<td>0-555</td>
<td>2-3</td>
<td>91</td>
</tr>
<tr>
<td>First DEAE-cellulose chromatography</td>
<td>5-0</td>
<td>42-9</td>
<td>2-67</td>
<td>11-1</td>
<td>29</td>
</tr>
<tr>
<td>Second DEAE-cellulose chromatography</td>
<td>4-0</td>
<td>36-8</td>
<td>7-73</td>
<td>32-2</td>
<td>25</td>
</tr>
</tbody>
</table>

NADP⁺, ATP and ADP were found to be competitive inhibitors with respect to NADP⁺. By the method of Dixon (1953) the values for the apparent $K_i$ for ATP and ADP were found to be $7-0 \times 10^{-4}$ M and $2-4 \times 10^{-3}$ M respectively (in the presence of 2.5 mM-Glc-6-P). AMP was only a very weak inhibitor: at 5 mM concentration it caused 8% inhibition in the presence of 0-05 mM-NADP⁺.

In the same assay system no effect of NADPH or NADH was found on the activity of Glc-6-P dehydrogenase in the presence of 0-25 mM-NADP⁺ and 2-5 mM-Glc-6-P. In the presence of suboptimal concentrations of NADP⁺, NADPH (but not NADH) was found to be a competitive inhibitor (apparent $K_i = 3-5 \times 10^{-5}$ M) with respect to NADP⁺.

Molecular weight. Calibration of the Sephadex G-200 column used in Step 4 of the purification procedure showed the molecular weight of the enzyme to be 100000–120000. Polyacrylamide gel electrophoresis at pH 8-3 of the purified enzyme showed two bands, each associated with Glc-6-P dehydrogenase activity towards both NAD⁺ and NADP⁺. When the enzyme was subjected to SDS-polyacrylamide gel electrophoresis, only one protein band could be observed, corresponding to a molecular weight of 52000. It is not presently known whether the two molecular species of the purified enzyme displayed on polyacrylamide gel electrophoresis were present after purification or whether they arose during electrophoresis.

Purification and properties of NADP⁺-linked 6-P-GlcA dehydrogenase

On account of the very different stabilities of the NADP⁺- and NAD⁺-linked 6-P-GlcA dehydrogenases it is important that the respective cell-free extracts are prepared separately in the different buffers as detailed in Methods. Under such conditions the NADP⁺-linked 6-P-GlcA dehydrogenase was purified 32-fold with a yield of 25% (Table 7). The purified enzyme was active with both NADP⁺ and NAD⁺, the ratio of the two activities being 3-7:1 at the pH optimum of 7-8 (Fig. 3).

Effect of metal ions. Mg²⁺ at 5 mM concentration stimulated the NAD⁺-linked activity 2-4-fold but had no effect on the NADP⁺-linked activity.

Stability. The purified enzyme lost all activity when stored at −15 °C for 24 h. When the enzyme was stored at 4 °C, activity diminished by 70% after 24 h and by 90% after 4 d. The losses in activity were the same with either NADP⁺ or NAD⁺ as coenzyme.

Michaelis constants. At 2-5 mM-6-P-GlcA, the apparent $K_m$ and $V_{max}$ for NADP⁺ were 2-5 × 10⁻⁵ M and 5-0 μmol min⁻¹ (mg protein)⁻¹ respectively. At 0-25 mM-NADP⁺, the apparent $K_m$ and $V_{max}$ for 6-P-GlcA were 7-25 × 10⁻⁵ M and 3-9 μmol min⁻¹ (mg protein)⁻¹ respectively. For the NAD⁺-linked activity with 2-5 mM-6-P-GlcA the apparent $K_m$ and $V_{max}$ for NAD⁺ were 9-05 mM and 2-51 μmol min⁻¹ (mg protein)⁻¹ respectively.

Modulation of enzyme activity. Certain nucleotides were found to inhibit the activity of NADP⁺-linked 6-P-GlcA dehydrogenase. By the procedure of Dixon (1953), the following apparent $K_i$ values were determined: NADPH, 3-75 × 10⁻⁵ M; NADH, 1-625 × 10⁻⁴ M; ATP, 3-15 × 10⁻⁴ M; ADP, 1-1 × 10⁻³ M; and AMP, 1-825 × 10⁻³ M.
Table 8. Purification of NAD\textsuperscript{+}-linked 6-phosphogluconate dehydrogenase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total activity (pmol)</th>
<th>Specific activity (pmol min(^{-1}) (mg protein(^{-1}))</th>
<th>Purification factor</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>33</td>
<td>159</td>
<td>0.123</td>
<td>1</td>
<td>100</td>
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<td>(NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} fractionation</td>
<td>8.0</td>
<td>160</td>
<td>0.253</td>
<td>2.0</td>
<td>101</td>
</tr>
<tr>
<td>Heat treatment</td>
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<td>135</td>
<td>0.459</td>
<td>3.7</td>
<td>85</td>
</tr>
<tr>
<td>First DEAE-cellulose chromatography</td>
<td>2.8</td>
<td>67</td>
<td>1.24</td>
<td>10</td>
<td>42</td>
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<tr>
<td>Second DEAE-cellulose chromatography</td>
<td>4.0</td>
<td>27</td>
<td>6.15</td>
<td>50</td>
<td>17</td>
</tr>
</tbody>
</table>

Purification and properties of NAD\textsuperscript{+}-linked 6-P-GlcA dehydrogenase

NAD\textsuperscript{+}-linked 6-P-GlcA dehydrogenase was purified 50-fold with a yield of 17\% (Table 8). The purified enzyme did not contain detectable activities of NADP\textsuperscript{+}-linked 6-P-GlcA dehydrogenase, phosphoglucoisomerase or KDPG aldolase/6-P-GlcA dehydrase. The enzyme was strongly inhibited by NADH to the extent that the rate of reaction during spectrophotometric assay rapidly decreased. The enzyme showed no detectable activity with NADP\textsuperscript{+}.

**pH optimum.** When the activity was assayed with 2.5 mM-NAD\textsuperscript{+} the maximum activity was observed at pH 7.5; at higher pH values the initial rate of reaction decreased so rapidly due to NADH formation that it became impossible to measure. With 20 mM-NAD\textsuperscript{+} the maximum activity was observed at pH 8.0, although it should be noted that at pH values below 7.6 the actual activity observed then became less than that with 2.5 mM-NAD\textsuperscript{+} (Fig. 4). Assay of this enzyme is therefore critically dependent on both pH and coenzyme concentration.

**Effect of Mg\textsuperscript{2+}.** Mg\textsuperscript{2+} at a concentration of 5 mM had no effect on enzyme activity.

**Stability.** There was a loss in activity of 27\% on storage of the enzyme at -15 °C for 20 h and of 35\% on storage at 4 °C for 2 weeks.

**Michaelis constants.** At 2.5 mM-6-P-GlcA the apparent \(K_m\) and \(V_{max}\) for NAD\textsuperscript{+} were 1.05 \(\times 10^{-4}\) M and 2.72 \(\mu\)mol min\(^{-1}\) (mg protein\(^{-1}\)) respectively. At 2.5 mM-NADP\textsuperscript{+} the apparent
Methanol metabolism by M. methylotrophus

Kₘ and Vₘₐₓ for 6-P-GlcA were 1.75 × 10⁻⁴ M and 2.70 µmol min⁻¹ (mg protein)⁻¹ respectively.

Modulation of enzyme activity. ATP and NADH showed strong competitive inhibition of the activity of NAD⁺-linked 6-P-GlcA dehydrogenase, with respect to NAD⁺. By the method of Dixon (1953) the values for the apparent Kᵢ for ATP and NADH were found to be 6.1 × 10⁻⁴ M and 2.2 × 10⁻⁵ M respectively. NADPH was only a relatively weak inhibitor.

DISCUSSION

Hu-6-P synthase has previously been purified from the following organisms: methane-grown Methylococcus capsulatus (obligate methane or methanol utilizer) (Ferenci et al., 1974); Methylomonas M15 (obligate methanol utilizer) (Sahm et al., 1976); Methylomonas aminofaciens 77a (obligate methanol utilizer) (Kato et al., 1977, 1978); methanol-grown Pseudomonas oleovorans (facultative) (Sokolov & Trotsenko, 1978; Müller & Sokolov, 1979); methanol-grown Bacterium MB58 (facultative) (Müller & Babel, 1978). Comparison of the properties of these different enzymes reveals many similarities. As far as has been tested they are all completely specific with respect to Ru-5-P, formaldehyde and Hu-6-P as substrates; the specific activities of the final preparations lie between 53 and 97 µmol min⁻¹ (mg protein)⁻¹ except for that from Pseudomonas oleovorans, whose specific activity is reported as 196 µmol min⁻¹ (mg protein)⁻¹ (Sokolov & Trotsenko, 1978). The pH optima for the enzymes occur in the range 7–8 and they are absolutely dependent on Mg²⁺ or Mn²⁺ for activity and stability. They are all soluble enzymes except for that from Methylococcus capsulatus, which has to be solubilized from the membrane system by extraction with 1 M-NaCl. So far, they appear to fall into three groups on the basis of their molecular weights, as follows. (a) The enzymes from Methylophilus methylotrophus, Methylomonas aminofaciens, Methylomonas M15 and Pseudomonas oleovorans have molecular weights of 40000–47000; in the case of the first three organisms the enzymes are dimeric proteins consisting of two apparently identical subunits, but whether that from Pseudomonas oleovorans is dimeric was not stated. (b) The enzyme from Bacterium MB58 has a molecular weight of 80000 and consists of four apparently identical subunits. (c) The enzyme from Methylococcus capsulatus has a molecular weight of 310000 and consists of six apparently identical subunits. This enzyme readily dissociates into subunits under conditions of low pH or ionic strength. It would be interesting to know whether high molecular weight, particle-bound enzymes are characteristic of methanotrophs and connected with possession by these organisms of intracellular membrane systems.

Apart from the dissociation into subunits effected by SDS treatment, no evidence was obtained in the present study for multiple, interconvertible forms of the Hu-6-P synthase of Methylophilus methylotrophus as reported for the enzymes from Pseudomonas oleovorans (Müller & Sokolov, 1979) and Bacterium MB58 (Müller & Babel, 1980).

Müller & Babel (1980) have reported that if rate measurements are performed with the Hu-6-P synthases from a variety of methylotrophic bacteria at a sufficiently large number of closely spaced substrate concentrations, small deviations from Michaelis–Menten kinetics are observed. They ascribe this behaviour to the existence of multiple, interconvertible forms of the enzymes, imparting properties of possible regulatory significance to the enzymes. Such detailed measurements were not made in the present study of the Hu-6-P synthase from Methylophilus methylotrophus and no appreciable departure from Michaelis–Menten kinetics was observed. No appreciable rate modulation by adenine or pyridine nucleotides was observed and hence it remains an open question whether regulation of the Hu-6-P synthase from Methylophilus methylotrophus occurs as with the enzymes from other methylotrophs.

The Hu-6-P synthase from M. methylotrophus is dramatically stabilized by the presence of Ru-5-P; this is a property that may not have been tested in the case of the other enzymes which have been purified.
Hu-6-P isomerase has previously been purified from methane-grown Methylococcus capsulatus (Ferenci et al., 1974) and methanol-grown Methylophilus methylotrophus (Kato et al., 1977). The specific activities of the enzyme in crude extracts of Methylococcus capsulatus and Methylophilus methylotrophus were very high, viz. 10 and 15.5 units (mg protein)$^{-1}$ respectively; after purifications of 150- and 114-fold their final specific activities were $1.56 \times 10^3$ and $1.77 \times 10^3$ units (mg protein)$^{-1}$ respectively. Polyacrylamide gel electrophoresis and gel permeation chromatography of the enzyme from Methylococcus capsulatus gave one major protein band, associated with enzyme activity, of molecular weight corresponding to 67000. Application of the same techniques to the Hu-6-P isomerase from Methylophilus methylotrophus indicated that in crude extracts one molecular species of molecular weight 70000 was present, but the purified enzyme associated to some extent into polymeric forms. In other respects the properties of these very stable, highly active enzymes were similar. No detailed comparison can be made with the enzyme partially purified from Methylomonas aminofaciens (Kato et al., 1977), but its final specific activity of 20 units (mg protein)$^{-1}$ appears low in comparison with the two enzymes discussed above.

The Glc-6-P dehydrogenase of Methylophilus methylotrophus exhibits dual substrate specificity with respect to NADP$^+$ and NAD$^+$, with the former coenzyme showing the lower $K_m$ value. Glc-6-P dehydrogenases exhibiting dual coenzyme specificity have been found in various methylotrophic bacteria, e.g. Pseudomonas W6 (Miethe & Babel, 1976), Methylomonas M15 (Steinbach et al., 1978), Pseudomonas C (Ben-Bassat & Goldberg, 1980) and Pseudomonas oleovorans (Sokolov et al., 1980). The molecular weights and subunit structures are closely similar for the enzymes from Methylophilus methylotrophus, Methylomonas M15 and Pseudomonas oleovorans. Although strictly comparable data for all the individual enzymes are not available, inhibition by reduced pyridine nucleotides and nucleoside triphosphates is a common feature.

It may be noted that NADP$^+$-specific Glc-6-P dehydrogenase activity has been detected in crude extracts of the facultative methylotrophs Arthrobacter P1 (methylamine-grown) (Levering et al., 1981) and strains S2A1 and PM6 (trimethylamine-grown) (Colby & Zatman, 1975). Glc-6-P dehydrogenase activities exhibiting dual substrate specificities with respect to NAD$^+$ and NADP$^+$ have been detected in crude extracts of both obligate and restricted facultative methylotrophs grown on trimethylamine e.g. strains 4B6, C2A1, W3A1 and W6A (Colby & Zatman, 1975). Without purification of the enzymes concerned, it is not known whether such activities are due to multiple enzymes or single enzymes possessing dual substrate specificity.

Methylophilus methylotrophus has two 6-P-GlcA dehydrogenases, one of which is relatively unstable and is active with NADP$^+ >$ NAD$^+$, whereas the other, more stable enzyme is specific for NAD$^+$. This is in contrast to methanol-grown Pseudomonas C which has only a single enzyme of dual coenzyme specificity (Ben Bassat & Goldberg, 1980). A 6-P-GlcA dehydrogenase that is active with NADP$^+$ has been purified from methylamine-grown Pseudomonas oleovorans (Sokolov et al., 1980); although crude extracts show 6-P-GlcA dehydrogenase activity with NADP$^+ \Rightarrow$ NAD$^+$, it is not stated whether the purified enzyme itself is active with NAD$^+$. A single enzyme showing activity only towards NADP$^+$ has been isolated from methanol-grown Candida boidinii (Kato et al., 1979). Enzyme multiplicity is encountered with 6-P-GlcA dehydrogenases in non-methylotrophic bacteria, e.g. Streptococcus faecalis (Brown & Whittenberger, 1972) and Pseudomonas multivorans (Lee & Lessie, 1974). With all these enzymes, including those from Methylophilus methylotrophus, inhibition by reduced pyridine nucleotides and nucleoside phosphates (particularly triphosphates) is a common feature.

NADP$^+$-specific 6-P-GlcA dehydrogenase activity has been detected in crude extracts of methylamine-grown Arthrobacter P1 (Levering et al., 1981), while both NAD$^+$ and NADP$^+$ display activity towards 6-P-GlcA in crude extracts of several methylotrophic bacteria grown
Methanol metabolism by M. methylotrophus

Fig. 5. Scheme for cyclic oxidation of formaldehyde in M. methylotrophus (→X signifies inhibition).

on trimethylamine (Colby & Zatman, 1975). In the latter case, the possibility of enzyme multiplicity cannot be ruled out.

In those organisms such as Methylophilus methylotrophus which utilize Entner–Doudoroff cleavage in their formaldehyde assimilation sequence and cyclic oxidation for one of their dissimilation sequences, a metabolic segment of economical enzyme profile and control can be envisaged (Fig. 5; see Quayle, 1980a, b). Owing to the different pH optima of the Glc-6-P and 6-P-GlcA dehydrogenases, a figure for their relative activities in crude extracts of Methylophilus methylotrophus is dependent on the assay pH. Nevertheless, over the pH range 7–8, the activity of Glc-6-P dehydrogenase is apparently several-fold higher than the combined activities of the two 6-P-GlcA dehydrogenases; its $V_{max}$ values are approximately 100-fold greater than for either of the last two enzymes. The activity of the Glc-6-P dehydrogenase towards NADP$^+$ is competitively inhibited by NADPH and ATP>ADP, and thus the overall flow of formaldehyde carbon through the initial stage of combined assimilation and dissimilation might be diminished under conditions of high energy status in the cell. The product of dehydrogenation of Glc-6-P, viz. 6-P-GlcA, stands at a metabolic cross-road between assimilation via Entner–Doudoroff cleavage and further dehydrogenation by the two separate 6-P-GlcA dehydrogenases, both of comparable relative activity. One of these enzymes is much more active towards NADP$^+$ than NAD$^+$ and is inhibited by NADPH>NADH, ATP>ADP>AMP; the other is specific for NAD$^+$ and is inhibited by NADH>NADPH, ATP>ADP>AMP. Thus, under conditions of high energy status, more of the 6-P-GlcA could be diverted towards Entner–Doudoroff cleavage (and hence assimilation) and less towards dissimilation, and vice versa. Furthermore, the multiplicity of the 6-P-GlcA dehydrogenases offers a mechanism for fine tuning of the NADPH/NADH ratio. Clearly measurements of intracellular pool sizes of substrates and coenzymes are needed before the significance in vivo of the above properties of these enzymes can be fully assessed. In addition, the significance of independent control of NADPH/NADP$^+$ and NADH/NAD$^+$ ratios when NADPH and NADH are probably energetically equivalent [the organism contains only a soluble, energy-independent transhydrogenase (Dawson & Jones, 1981)] needs further study.
It should be emphasized that the present study only concerns the possible regulation of assimilation of formaldehyde versus its dissimilation through the cyclic oxidation pathway. The role and regulation of the linear oxidation sequence via formate must await study of the kinetic parameters of the enzymes concerned.

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