Vol. 2, pp 2-34, Academic Press, Orlando, FL.
Harrison, E. H., & Gad, M. Z. (1989) *J. Biol. Chem. 264*, 17142-17147.
Hollander, D., & Muraldihara, K. S. (1977) *Am. J. Physiol. 232*, E471-E477.
Hollander, D., & Ruble, P. E., Jr. (1978) *Am. J. Physiol. 235*, E686-E691.
Huang, H. S., & Goodman, D. S. (1965) *J. Biol. Chem. 240*, 2839-2844.
Lentz, B. R., Barcholz, Y., & Thompson, T. E. (1975) *Chem. Phys. Lipids 15*, 216-221.
Lombardo, G., & Guy, O. (1980) *Biochim. Biophys. Acta 611*, 147-155.
Mason, K. E. (1933) *Am. J. Anat. 52*, 153-163.
Mahadevan, S., Seshardri Sastry, P., & Ganguly, J. (1963a) *Biochem. J. 88*, 531-534.
Mahadevan, S., Seshardri Sastry, P., & Ganguly, J. (1963b) *Biochem. J. 88*, 534-539.
Morton, R. A. (1972) in *Photochemistry of Vision* (Dartnall, H. J. A., Ed.) p 33, Springer-Verlag, New York.
Pind, S., & Kuksis, A. (1988) *Biochim. Biophys. Acta 938*, 211-221.
Pind, S., & Kuksis, A. (1989) *Lipids 24*, 357-362.
Powell, D. W. (1987) in *Physiology of the Gastrointestinal Tract* (Johnson, L., Ed.) Vol. II, p 1293, Raven Press, New York.
Said, H. M., Ghishan, F. K., & Reyadh, R. (1987) *Am. J. Physiol. 15*, G229-G236.
Said, H. M., Ong, D. E., & Reyadh, R. (1988) *Pediatr. Res. 24*, 481-485.
Scharschmidt, B. F., Keefe, E. D. Blankenship, N., & Okner, R. (1979) *J. Lab. Clin. Med. 93*, 790-798.
Vahouny, G. V., & Brecher, A. S. (1968) *Arch. Biochem. Biophys. 123*, 247-254.
Vahouny, G. V., Weersing, S., & Treadwell, C. R. (1965) *Biochim. Biophys. Acta 98*, 607-616.
Wolbach, S. B., & Howe, P. R. (1925) *J. Exp. Med. 43*, 753-777.

Enzymatic Degradation of Cyclic 2,3-Diphosphoglycerate to 2,3-Diphosphoglycerate in *Methanobacterium thermoautotrophicum*†

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ABSTRACT: 2,3-Diphosphoglycerate (2,3-DPG) has been found to be the product of the enzymatic degradation of cyclic 2,3-diphosphoglycerate (cDPG) in the archaebacterium *Methanobacterium thermoautotrophicum* ΔH. Although 2,3-DPG has not previously been detected as a major soluble component of *M. thermoautotrophicum*, large pools accumulated at an incubation temperature of 50 °C (below the optimum growth temperature of 62 °C). Under these conditions, cellular activity was significantly decreased; a return of the culture to the optimum growth temperature restored the 2,3-DPG pool back to original low levels and caused steady-state cDPG levels to increase again. While $^{13}CO_2$-pulse/$^{12}CO_2$-chase experiments at 50 °C showed that the cDPG turned over, the appearance of 2,3-DPG at NMR-visible concentrations required at least 10 h. Production of 2,3-DPG in vivo was prevented by exposure of the cells to O_2. The enzyme responsible for this hydrolysis of cDPG was purified by affinity chromatography and appears to be a 33-kDa protein. Activity was detected in the presence of oxygen and was enhanced by a solution of 1 M KCl, 25 mM $MgCl_2$, and dithiothreitol. Both K_m and V_{max} have been determined at 37 °C; kinetics also indicate that in vitro the product, 2,3-DPG, is an inhibitor of cDPG hydrolysis. These findings are discussed in view of a proposed role for cDPG in methanogens.

Methanogens are archaebacteria with unusual chemistry (Balch et al., 1979; Daniels et al., 1984; Keltjens & van der Drift, 1986; Jones et al., 1987). Most uniquely, they reduce CO_2 with H_2 and use the electron flow from this reaction to drive ATP synthesis. Methanogens have been shown to have a wide variety of unusual compounds. Some, like methanofuran (Leigh et al., 1984), methanopterin (Van Beelen et al., 1984), and coenzyme M (Taylor & Wolfe, 1971), are involved in methanogenesis. Others like cyclic 2,3-diphosphoglycerate, cDPG[1] (Kanodia & Roberts, 1983; Seeley & Fahrney, 1983; Evans et al., 1985), and β-glutamate (Robertson et al., 1989) have functions that are not well understood. Under standard growth conditions, cDPG is found at very high intracellular concentrations (Seely & Fahrney, 1983) and is the major carbon and phosphorus pool in the thermophilic, autotrophic organism *Methanobacterium thermoautotrophicum* ΔH. cDPG has also been detected (Tolman et al., 1986) in several members of Methanobacteriaceae, a phylogenetically coherent group which have a pseudomurein cell wall. More recently, it has been detected at low intracellular levels in *Methanosarcina frisia* (Rudnick et al., 1990), an organism from a non-pseudomurein-containing family. This compound is under unusual and stringent regulation. Its levels have been correlated with cell growth (Seely & Fahrney, 1984); the lower the rate of growth, the lower the cDPG levels. While its

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[1] Abbreviations: cDPG, cyclic 2,3-diphosphoglycerate; 2,3-DPG, 2,3-diphosphoglycerate; 3-PG, 3-phosphoglycerate; 2-PG, 2-phosphoglycerate; 1,3-DPG, 1,3-diphosphoglycerate; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; P_i, inorganic phosphate; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; TEA, triethanolamine.

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steady-state level is kept relatively constant throughout the growth cycle of the cell, its turnover rate varies dramatically. cDPG can turn over rapidly in the cells, considerably faster than the cell doubling time. Under these conditions, the large amounts of ^{13}C label in the cDPG pool end up in polymeric material, presumably the carbohydrate in the cell wall (Evans et al., 1985, 1986). In mid log phase cells the half-life of cDPG is 1–2 h; in stationary phase cultures its turnover slows down dramatically and its synthesis is also inhibited (Evans et al., 1986a). Previous results have been used to suggest that cDPG is involved in carbohydrate synthesis in methanogens which have a pseudomurein cell wall (Evans et al., 1985, 1986a).

Given this background, we have attempted to delineate the products of cDPG enzymatic degradation. The present work shows that in vivo *M. thermoautotrophicum* cells can be induced to accumulate 2,3-DPG by lowering the incubation temperature to 50 °C. The levels of this compound, related by hydrolysis to cDPG, are depleted if the temperature of the culture is shifted back up to the growth temperature, 62 °C. The commercial coupled enzyme assay for 2,3-DPG which follows NADH reduction optically has been adapted to follow 2,3-DPG generation from cDPG in *M. thermoautotrophicum* cell lysates and purified protein fractions. 2,3-DPG production is very low in crude cell extracts. Activity is increased if high KCl and $MgCl_2$ are added to the lysate. Purified cDPG is hydrolyzed to 2,3-DPG by a protein fraction highly enriched in a 33-kDa protein. Optimization of assay parameters is presented and discussed in terms of the role of cDPG in carbohydrate biosynthesis. Such behavior may be relevant to those methanogens found in man, since we show these also contain cDPG.

Materials and Methods

Chemicals. $^{13}CO_2$ (99%)/H_2 (1:4 v/v), [$^{13}C_2$]acetate (99%), and $^{16}O_2$ (99.98%) were acquired from Cambridge Isotopes. Unenriched CO_2/H_2 (1:4 v/v) and CO_2/N_2 were acquired from Air Products. Other chemicals used in media preparation were of reagent grade. Phosphoglycolic acid, ATP, triethanolamine buffer, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, alkaline phosphatase (*Escherichia coli*), TES, NADH, and blue dextran agarose were obtained from Sigma Chemical Co. Sephadex G-150 was obtained from Pharmacia Chemical Co.

Purification of cDPG. cDPG was purified from ethanol extracts by chromatography on QAE-Sephadex (Sigma) as described previously (Kanodia & Roberts, 1983) with additional elution over a Sephadex G-10 column for the highest purity compound. Material was checked by ^{31}P NMR to assess any other phosphorus-containing contaminants; occasionally small amounts of P_i were present, but there were no other carbon-containing species. Total phosphate-containing material was quantified using a colorimetric phosphate assay (Chen & Warner, 1956; Turner & Rouser, 1970). If P_i was detected, the relative amounts of the two species in the NMR spectrum were used to estimate cDPG concentration.

Growth of M. thermoautotrophicum ΔH for Labeling Experiments. Cells of *M. thermoautotrophicum* ΔH (Zeikus & Wolfe, 1972) for NMR studies were grown in pressurized bottles using the following medium (1 L): 984 mL of deionized water, potassium phosphate monobasic (0.42 g), potassium phosphate dibasic (0.22 g), ammonium chloride (0.70 g), sodium chloride (0.60 g), 1.0 mL of magnesium chloride solution (4%), 0.4 mL of calcium chloride solution (8%), 4.0 mL of resazurin (0.025%), and 10 mL of mineral elixir (Daniels et al., 1986). CO_2/N_2 was bubbled through the media while stirring. When the pH stabilized, 1.5 g of sodium carbonate was added. The gas flow was stopped as the pH approached 7.2. A total of 150 mL of medium was distributed in each 500-mL Wheaton bottle. The bottles were then stoppered, crimped, and made anaerobic by evacuating them and flushing with CO_2/H_2 at 5 psi for three cycles, and then injecting 0.9 mL of 200 mM Na_2S. Bottles were autoclaved for 18 min to ensure sterility. A total of 15 mL (10% medium volume) of cells, from a bottle previously grown to OD_{660} ~0.8, was transferred anaerobically and sterilely to the 500-mL Wheaton bottles. The cell suspensions were then cultivated in a shaker bath at 62 °C under 18–20 psi of CO_2/H_2.

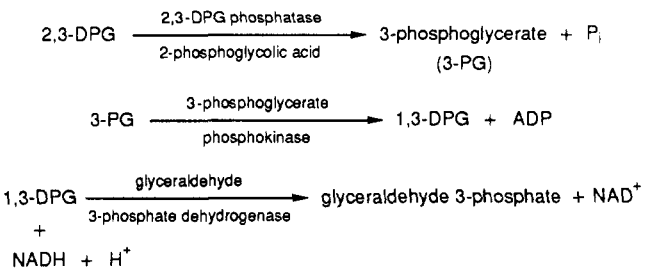
^{13}C Labeling of Cellular Material. For steady-state labeling of cells with [$^{13}C_2$]acetate, cultures at OD_{660} 0.3–0.4 were incubated at 62 °C for ~0.5 generation time (typically 8–10 h) with 10 mM [$^{13}C_2$]acetate. At this point, two control bottles of cells were harvested and extracted with ethanol. The rest of the bottles were incubated at 50 °C for 10–40 h prior to harvesting and extraction. Various additives to the 50 °C incubations include KCN (50 μM), CCCP (55 μM), and O_2. One batch of cells was reincubated at 62 °C after remaining at 50 °C for 24 h. For $^{13}CO_2$-pulse/$^{12}CO_2$-chase labeling studies, cells grown to OD_{660} ~0.5–0.6 (and allowed to use most of the gas in the head-space) were pressurized to 12 psi with $^{13}CO_2/H_2$ and returned to the shaker water bath at 62 or 50 °C. At OD_{660} ~0.65–0.70, the bottles were pressurized with unenriched CO_2/H_2 and incubated at 50 °C unless otherwise indicated. With experiments monitoring the effect of O_2, 30 cm^3 of O_2 was injected into each bottle (except the controls) at the start of the chase. Bottles were harvested and extracted at various times during the chase.

Preparation of Ethanol Extracts. Cells of *M. thermoautotrophicum* ΔH were harvested as described previously (Evans et al., 1985) except that the potassium phosphate wash was omitted. Ethanol extracts were prepared by adding 70% ethanol to the centrifuged cell pellets (approximately 1 mL of ethanol/1 g of wet weight pellet), vortexing the suspension for 4–5 min, and then centrifuging the mixture at 12000*g* for 10 min. The ethanol was decanted off and saved. The remaining cell pellet was reextracted with ethanol. Both supernatants were combined and dried in vacuo. Dried material was redissolved in 0.5 mL of NMR buffer consisting of 10 mM potassium phosphate, 0.1 mM EDTA, and 50% D_2O, pH 7.2.

Preparation of Crude Cell Extracts. Cells for examination of 2,3-DPG generating activity in vitro were grown in a 2-L fermenter as described previously (Evans et al., 1985). Cells grown to an absorbance at 660 nm of 0.6–0.8 were harvested and centrifuged at 9000 rpm for 30 min in a Beckman J2-21 centrifuge. All subsequent operations were carried out at 4 °C. The cell pellet was mixed with 25–30 mL of 75 mM TES/K^+ buffer, pH 7.1, containing 800 μM phenylmethanesulfonyl fluoride and incubated for 15 min on ice prior to passage through a French pressure cell at a minimum of 15 000 psi. In one case, cells were mixed with lysis buffer and 10 mM EDTA prior to treatment with the French pressure cell. This alternate protocol, which should prevent transition metals in the cell from generating oxidizing species which might specifically inactivate enzymes, had no effect on the amount of cDPG hydrolytic activity detected in the crude extract. Cell debris was removed by centrifugation at 16 000 rpm for 75 min. The supernatant was mixed with ammonium sulfate to 100% saturation. The precipitate was collected by centrifugation at 16 000 rpm and then redissolved in the TES buffer (containing 25 mM $MgCl_2$ and 1 M KCl), pH 7.1. It was dialyzed extensively against the same buffer to remove

any endogenous 2,3-DPG or other small phosphate-containing molecules. Total protein content was estimated by the Coomassie blue binding assay of Bradford (1976) using bovine serum albumin as a standard.

In Vitro Assay of 2,3-DPG. The assay for 2,3-DPG, using a kit (35-UV) supplied by Sigma, is characterized by the following coupled reaction scheme:



It is similar to that developed by Lowry et al. (1964), where the reaction is monitored spectrophotometrically instead of fluorometrically [as is done for analyzing 2,3-DPG in blood (Ericson & de Verdier, 1972; Michal, 1974)]. To a 3-mL cuvette were added 2.5 mL of NADH (1 mg/8 mL of TEA buffer, pH 7.9 ± 0.1, containing 10 mM $MgCl_2$), 100 μL of ATP (100 mg/mL of TEA buffer), 250 μL of the cell extract or purified protein which had been incubated with cDPG, and 20 μL of glyceraldehyde phosphate dehydrogenase/phosphoglycerate kinase enzyme mixture (used as supplied in the kit by Sigma). The contents were mixed, and the absorbance (A_i) was recorded against the same buffer devoid of NADH. Upon the addition of 20 μL of phosphoglycerate mutase and 100 μL of phosphoglycolic acid (20 mg/mL in deionized water), the cuvettes were incubated at room temperature and the absorbance at 340 nm was determined, typically after 30 min (A_f). Without the phosphoglycolic acid, 2,3-DPG is not converted to 3-PG (Rose & Liebowitz, 1970). The difference in absorbance ($A_i - A_f$) is proportional to the 2,3-DPG concentration generated by the methanogen cell extract. The amount of 2,3-DPG produced was quantified by comparison of the absorbance change with a standard plot obtained with known concentrations of 2,3-DPG (10–40 μg). Initial rates were calculated from at least three time points and before any nonlinearity appeared in the kinetics. The pH of the assay mixture was measured before and after the reaction to make sure that NADH was not degraded because of a decrease in pH. For the purpose of determining K_m values, the 250-μL sample aliquot was adjusted to maintain a fixed enzyme concentration and the substrate concentration was varied and adjusted with TEA buffer, if necessary, such that the final volume remains unchanged during the assays.

Purification of cDPGase. Purification of the enzyme that hydrolyzes cDPG to 2,3-DPG was achieved by affinity chromatography on blue dextran covalently attached to 4% beaded agarose column (4.1 mg of blue dextran/mL of packed gel). The cell extract obtained as described above was treated with alkaline phosphatase (200 units) at 37 °C for 3 h, and the extract was then loaded on a 10-mL gel column. The breakthrough was reloaded. The column was washed thoroughly with the same TES buffer until the washings showed no significant absorbance at 280 nm against the buffer blank. The column was then eluted with 10–12 mM cDPG in TES buffer until the absorbance at 280 nm was insignificant. The eluted fraction was tested for enzyme activity using the coupled 2,3-DPG assay.

Determination of K_m for cDPG. Assays were carried out in the presence as well as in the absence of phosphoglycerate mutase to study the effect of product inhibition. A comparison of the rates with and without this enzyme will then show the extent of 2,3-DPG product inhibition of cDPGase. cDPGase (typically 30 μg) and substrate were incubated at 37 °C; the mixture was quenched with 0.5% TCA and centrifuged, and the supernatant was assayed for 2,3-DPG.

PAGE. SDS–PAGE was carried out as discussed by Blackshear (1984) on 16 cm × 8 cm slabs using the Tris–glycine system at pH 8.3. An 11% gel was routinely employed for checking the enzyme in SDS–PAGE. After the electrophoresis, the gels were stained with either Coomassie brilliant blue or silver according to Merril et al. (1984).

NMR Spectroscopy. ^1H-decoupled ^{13}C NMR spectra (75.4 MHz) of ethanol extracts of *M. thermoautotrophicum* were obtained in 5-mm tubes with the following parameters: 16 502-Hz sweep width, 54 080 data points, 45° pulse angle, 1.6-s acquisition time, and 4-Hz line broadening. For natural abundance samples, 20 000–40 000 transients were accumulated; for labeled samples, 2000–20 000 transients were accumulated (depending on the sample). All chemical shifts were referenced to dioxane at 67.4 ppm. ^1H-decoupled ^{31}P NMR spectra (121.4 MHz) were obtained on the same spectrometer using WALTZ decoupling, a 7000-Hz sweep width, 11 000 data points, 45° pulse angle, 0.8-s recycle time, 4-Hz line broadening, and 1000–4000 transients.

Results

The small molecule pool in *M. thermoautotrophicum* can be analyzed by ^{13}C NMR spectroscopy of cells incubated with [$^{13}C_2$]acetate (Evans et al., 1985, 1986a,b). Under normal growth conditions (62 °C), very little (if any) 2,3-DPG was found in ethanol extracts of these cells. cDPG (CHOP ~79 ppm and CH_2OP ~70 ppm) was the dominant C-containing as well as the P-containing small molecule (Figure 1A). The splitting patterns have been analyzed before: the ^{13}C–^{31}P doublet in the center of each pattern is the result of some breakdown of the [$^{13}C_2$]acetate and reformation of $^{13}CH_3{}^{12}CO$– prior to incorporation of the label into cellular material, while the doublet of doublets on each side reflect incorporation of an intact [^{13}C]acetate unit (Evans et al., 1986b). The small molecule pool showed a considerably different pattern when cells were subsequently incubated at lower temperatures. After 10 h at 50 °C 2,3-DPG levels increased in cells as detected by the CHOP ~76 ppm and CH_2OP ~67 ppm (Figure 1B). The steady-state levels of both cDPG and 2,3-DPG could be quantified from ^{31}P NMR spectra of these extracts since the cyclic pyrophosphate and phosphate monoester resonances are extremely well separated (Kanodia & Roberts, 1983). When the cells were shifted to 50 °C, the growth rate dropped dramatically (Figure 2A, dotted portion). After about 10 h at the lower temperature 2,3-DPG became visible. Over 36–48 h, the ratio of cDPG to 2,3-DPG increased to greater than 0.8 (Figure 2B). A significant amount of the 2,3-DPG which accumulated at 50 °C could be utilized by the cells if the temperature was shifted back up to the growth temperature (62 °C) for 30 h (Figure 1C). Occasionally in the 50 °C incubations, 2-PG was detectable at half the level of 2,3-DPG (Figure 1D). The sporadic appearance of this metabolite did not correlate with any of the incubation conditions. 2-PG is presumably also involved in the cDPG cycle, its exact position unknown.

Examining the cells incubated in vivo with [$^{13}C_2$]acetate at lower temperatures provides a way of assessing how different metabolic inhibitors affect 2,3-DPG production. For example, there was no significant conversion of cDPG to 2,3-DPG in cells exposed to O_2 and incubated at 50 °C (Figure 3A).

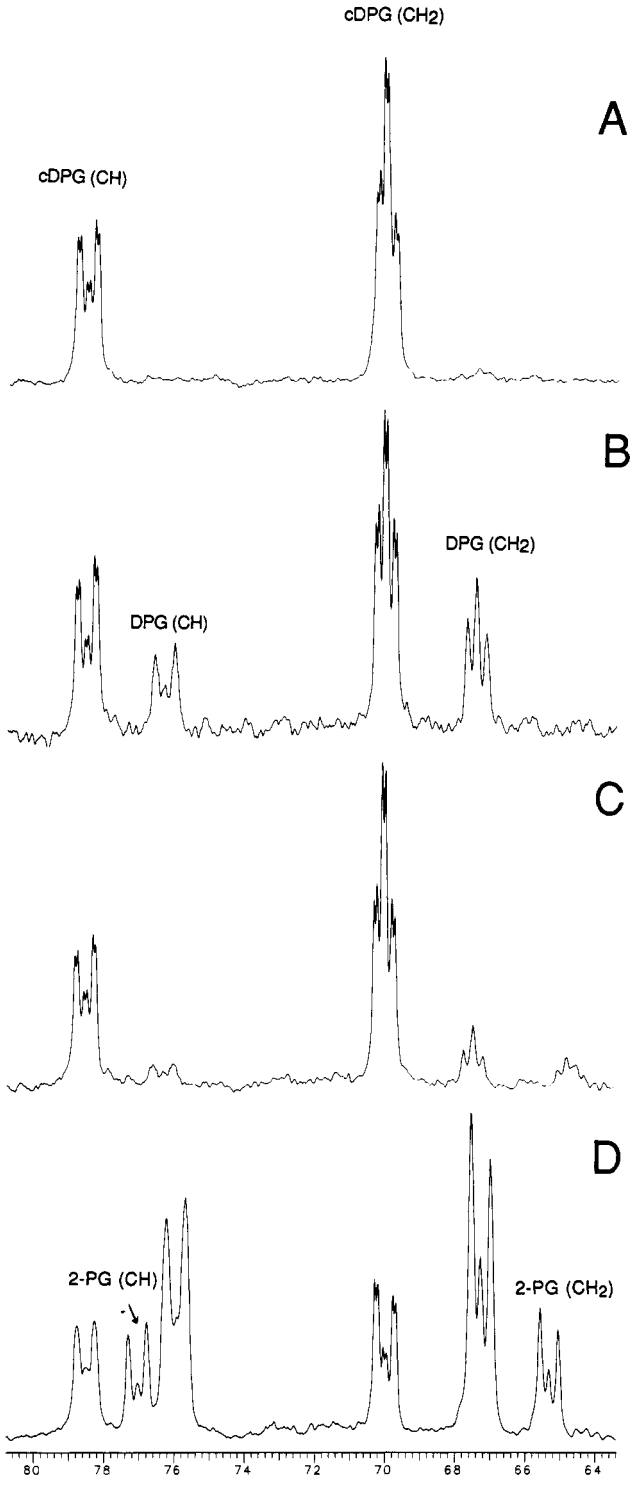


FIGURE 1: ^{13}C NMR spectra (75.4 MHz) of ethanol extracts of *M. thermoautotrophicum* grown for 8 h on [$^{13}C_2$]acetate and (A) harvested immediately, (B) incubated for 48 h at 50 °C prior to extraction, and (C) incubated at 50 °C for 48 h and then raised back to 62 °C for 10 h. Under some circumstances, 2-PG is also visible in samples incubated at 50 °C, as shown in (D). Identities of the carbons are indicated.

Under these conditions no methanogenesis, and hence energy generation, is possible. KCN, an inhibitor of CO dehydrogenase in this organism and hence C assimilation from CO_2, had little effect on the generation of 2,3-DPG at 50 °C (note the resonance ~67 ppm), although it suppressed the exchange of CO_2 into the carboxyl of [$^{13}C_2$]acetate thereby producing [2-^{13}C]acetate to be incorporated into cell carbon (Evans et al., 1986b). This result was indicated by the lack

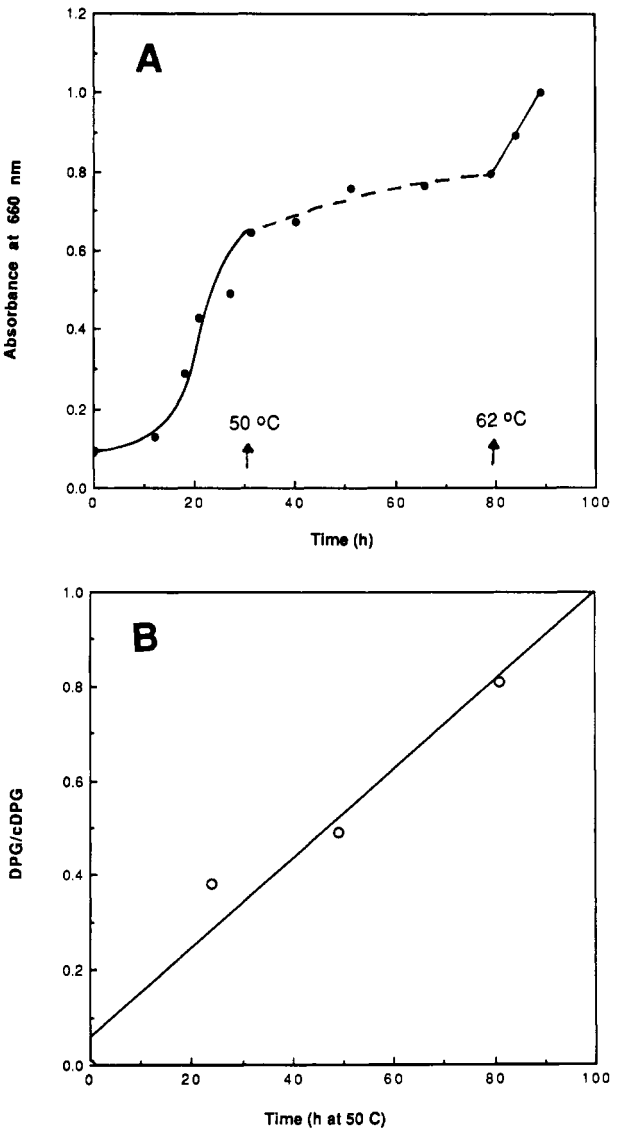


FIGURE 2: (A) Absorbance at 660 nm (monitoring cell growth) as a function of incubation time. Initial growth was at 62 °C with a shift down to 50 °C from t = 31 h to t = 79 h when the culture was raised back up to 62 °C. (B) Ratio of 2,3-DPG to cDPG as measured from integrating ^{31}P NMR spectra of *M. thermoautotrophicum* extracts incubated for various times [corresponding to the dotted line region in (A)] at 50 °C.

of a ^{13}C–^{31}P doublet in the center of the CH_2OP regions of cDPG (~70 ppm) and 2,3-DPG (~67 ppm) (Figure 3B). CCCP, a weak uncoupler which dissipates the pH gradient across the cell membrane, did inhibit turnover of cDPG and build up of 2,3-DPG (Figure 3C). It also inhibited the exchange of CO_2 into the carboxyl of [$^{13}C_2$]acetate. (The difference in the control $^{13}CH_2$ patterns for panels A and C in Figure 3 occurred because the labeling experiments were done with different concentrations of cells harvested at slightly different OD's on different days. The cells for the experiments in panels A and B in Figure 3 were from the same batch and the experiments were carried out at the same time, hence the controls are nearly identical.) The result of CCCP inhibition of 2,3-DPG production suggested that another factor in the cell, possibly related to energy metabolism, was necessary for maximizing cDPG turnover.

In Vivo Turnover of cDPG at 50 °C. The [$^{13}C_2$]acetate experiments showed that small molecule pools changed in composition when cells were incubated at 50 °C, potentially building up an intermediate in C assimilation. ^{13}C-pulse/

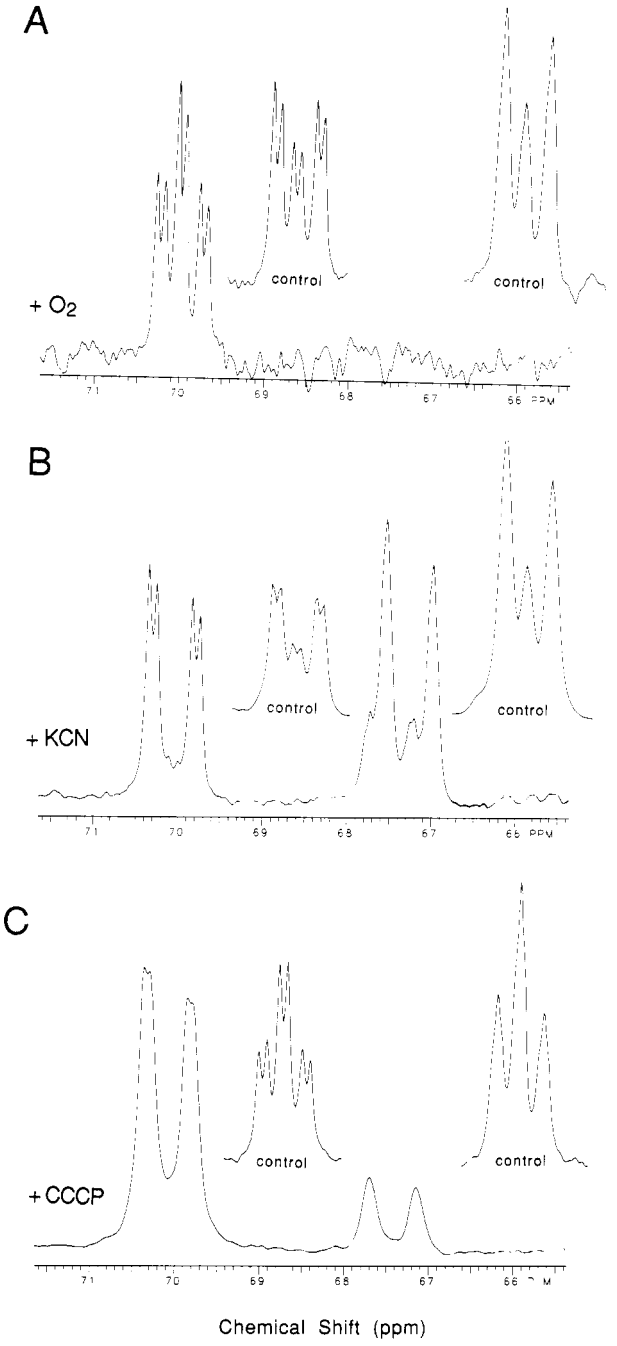


FIGURE 3: ^{13}C NMR spectra (75.4 MHz) of the CH_2OP region in ethanol extracts of *M. thermoautotrophicum* grown at 62 °C for 0.5 generation time on [$^{13}C_2$]acetate then incubated at 50 °C in the presence of a specific metabolic inhibitor for 12–24 h before extraction. The control showing 2,3-DPG production at 50 °C is shown slightly offset above each spectrum. Panels: (A) O_2 added; (B) 50 μM KCN added; (C) 55 μM CCCP added during the incubation at 50 °C.

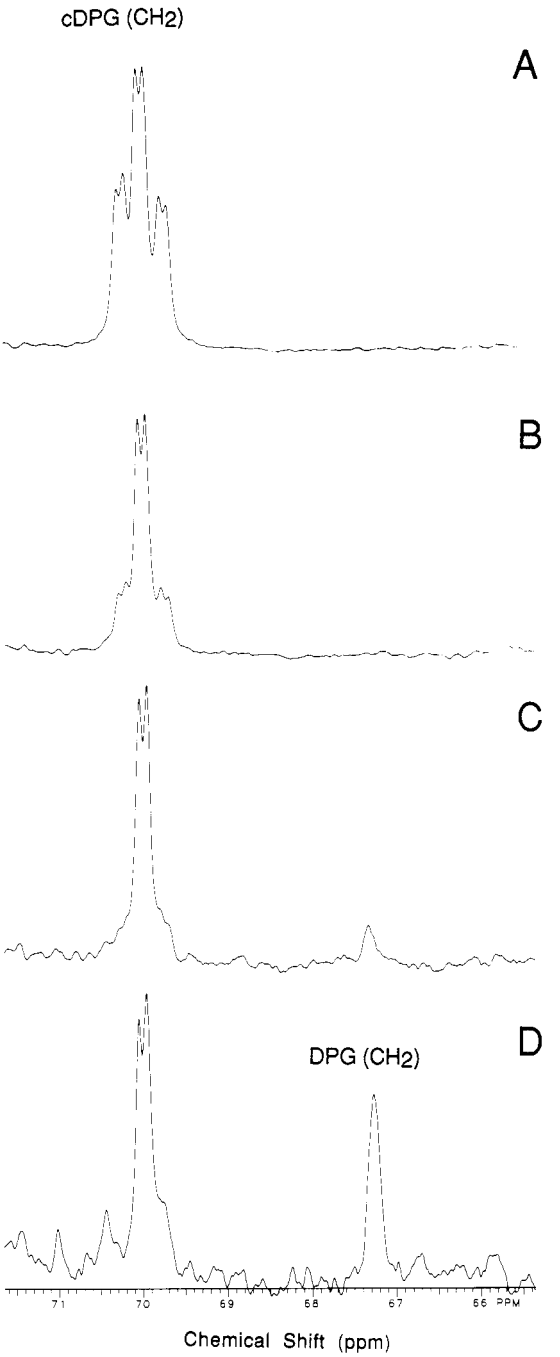


FIGURE 4: CH_2OP region of the ^{13}C NMR spectra (75.4 MHz) of ethanol extracts of *M. thermoautotrophicum* grown at 62 °C for 0.5 generation time with $^{13}CO_2$ and then incubated for (A) 0 h (500 vertical scale), (B) 4 h (900 vertical scale), (C) 13 h (1500 vertical scale), and (D) 40 h (4500 vertical scale at 50 °C in the presence of $^{12}CO_2/H_2$. The resonances corresponding to the CH_2OP or both cDPG and 2,3-DPG are indicated.

^{12}C-chase methodology was used to see if the turnover of molecules was also affected by the shift in temperature. As shown in Figure 4, the intensity of the ^{13}C-labeled cDPG CH_2 resonances decreased rapidly during the chase at 50 °C. After 10 h, an NMR detectable amount of 2,3-DPG began to build up (Figure 4C). These data were quantified by integration of carbons for cDPG and 2,3-DPG (Figure 5A). The initial drop in cDPG had a half-life around 1–2 h, not too different from the observed value at 62 °C (1–2 h; Evans et al., 1986a). When 2,3-DPG began to build up, the rate of cDPG turnover decreased dramatically ($\tau_{1/2} \sim$ 19–20 h). The increase in 2,3-DPG correlated with a concomitant decrease in cDPG pool size. The specific ^{13}C content of each pool could be evaluated if the steady-state levels of both cDPG and 2,3-DPG were known. This was quantified (Figure 5B, inset) using ^{31}P spectra of the same extracts where the chemical shifts for cDPG and 2,3-DPG were well resolved (Kanodia & Roberts, 1983; Tolman et al., 1986). Over the time course of 40 h, cDPG decreased and 2,3-DPG increased. The total phosphate content (cDPG plus 2,3-DPG) was constant over this incubation period, again suggesting that 2,3-DPG was directly related to the cDPG. If those phosphate concentrations were used to normalize the ^{13}C turnover, there was still an initial drop in ^{13}C specific activity of cDPG levels within 1–2 h at 50 °C (Figure 5B). The ^{13}C specific activity in 2,3-DPG (filled

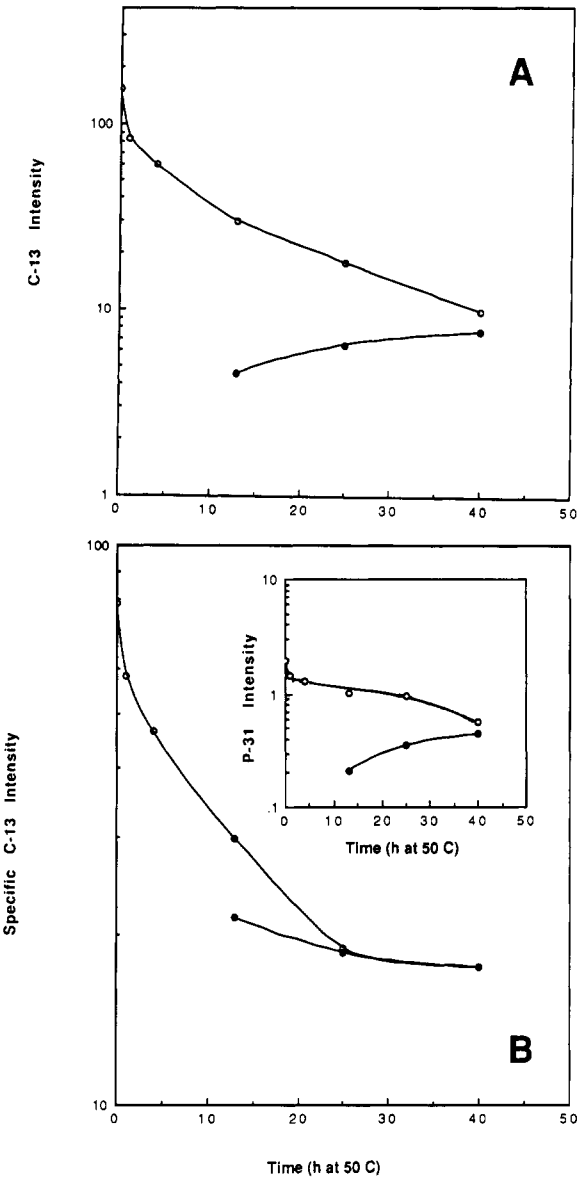


FIGURE 5: (A) Integrated ^{13}C intensity of the CH_2 groups of cDPG (○) and 2,3-DPG (●) as a function of time in the $^{13}CO_2$-pulse (62 °C)/$^{12}CO_2$-chase (50 °C) experiment; (B) Specific ^{13}C intensity calculated by normalizing the values in (A) with the total absolute concentration of cDPG and DPG as determined from ^{31}P-integrated intensities of the same samples (shown in the inset).

circles, Figure 5B) initially was lower than in 2,3-DPG but eventually assumed a value quite close to that in cDPG. This would be the case if 2,3-DPG was derived from cDPG but was metabolized more slowly. The decrease indicated that 2,3-DPG was also turning over, albeit with an extremely slow half-life ($\tau_{1/2} \sim 75$–125 h). For comparison, turnover at 50 °C of free glutamate pools was also biphasic with 1–2-h and 9–10-h half-lives (Table I). These results suggested that cDPG was a storage pool which could be rapidly used even when cells were not growing (i.e., when the temperature dropped to 50 °C). 2,3-DPG production in pulse/chase experiments occurred on a long time scale. Since there was no other source of ^{13}C, this implied that 2,3-DPG must be derived from the cDPG pool.

2,3-DPG Production from cDPG by Cell Extracts. In vivo results suggested that cDPG is hydrolyzed to 2,3-DPG. Therefore, we have examined cell lysates for the production of 2,3-DPG from exogenously added purified cDPG. The assay was carried out at 37 °C since the commercially obtained

Table I: Half-Life of ^{13}C of Turnover in Various Small Molecules in *M. thermoautotrophicum* ΔH

growth condition	$\tau_{1/2}$ (h) cDPG	DPG[a]	glutamate
62 °C, mid log	1–2		3–4
62 °C, stationary	5–6		10–12
50 °C	1–2, 19–20[b]	50	1–2, 9–10[b]
50 °C, O_2	45	25	8

[a] The ^{13}C in DPG actually increases in intensity, so this is the apparent half-life for appearance of label; the data have not been corrected for the specific ^{13}C content by normalizing to total phosphate in each species. [b] The loss of ^{13}C label is biphasic.

Table II: Specific Activity of cDPGase in *M. thermoautotrophicum* ΔH toward 20 mM cDPG

preparation	total protein (mg)	sp act.[a] (μmol min^{-1} mg^{-1}) −PG mutase	+PG mutase[b]	% cDPGase units recovered[c]
1. crude cell extract	62	0.058	0.060	
2. blue dextran Sepharose				
cDPG-eluted[d]	2.5	0.21	1.02	68
2,3-DPG-eluted	0.85	0.01	0.03	1

[a] Determined at 37 °C toward 20 mM cDPG; under these conditions the enzyme is not saturated. [b] 10 units of PG mutase added to each assay mixture. [c] Units of cDPGase recovered as estimated using the specific activities for the blue dextran eluted protein fractions obtained with PG mutase in the assay mixture compared to the total units in the crude protein fraction. [d] Crude dialyzed cell extract was treated with alkaline phosphatase prior to the blue dextran Sepharose column step.

enzymes used in the coupled assay are not stable at 62 °C, the methanogen growth temperature. A reproducible level of cDPG hydrolysis to DPG occurs with this coupled enzyme assay. With crude cell extracts, the blanks were variable depending on extract concentration (the more the extract volume, the larger the blank), suggesting a possible contribution from endogenous 2,3-DPG. Therefore, the protein in the crude cell lysate was precipitated with ammonium sulfate, then dialyzed to remove all soluble small molecules. Under these conditions the blanks had an optical density change of less than 0.01 at 340 nm; protein concentrations in the assay were adjusted so that $\Delta A_{340} > 0.1$ for 2,3-DPG generation. The cytoplasm of *M. thermoautotrophicum* contains unusually high concentrations of K^+. Other soluble proteins when extracted from this organism have been shown to be activated by high salt. The cDPG hydrolysis activity in crude extracts also increased (53%) by the inclusion of 1 M KCl in the assay mix. If the cells were kept anaerobic, lysed in an anaerobic chamber, then assayed for 2,3-DPG production, there was no significant difference, indicating that the enzyme activity detected in lysed cells is not oxygen-sensitive. Activity was also enhanced by the addition of $MgCl_2$; the requirement for this divalent metal ion was not replaced by Ca^{2+} or Zn^{2+}. The activity was optimized with ~25 mM $MgCl_2$ included in the assay mixtures. Under these conditions, the observed specific activity depended on the presence of both cell extract and cDPG. This confirmed that cell extracts, and presumably a specific enzyme, could produce 2,3-DPG from cDPG. With high cDPG (~20 mM) the specific activity of the crude extract was 0.058 μmol of 2,3-DPG min^{-1} mg^{-1} (Table II).

Other enzymatic activities are present in crude cell extracts which under the right conditions can degrade the 2,3-DPG to 3-PG. DPG phosphatase (phosphoglycerate mutase) activity in other systems is activated by phosphoglycolate (Rose & Liebowitz, 1970). Addition of this compound to reaction mixtures with 2,3-DPG activated a corresponding phosphatase

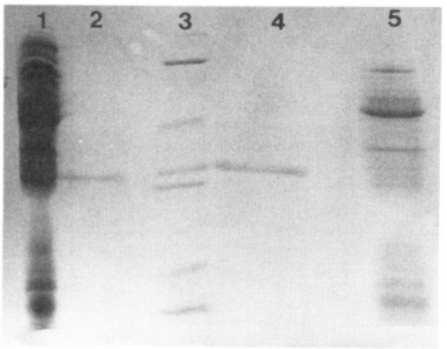


FIGURE 6: SDS–PAGE patterns of (lane 1) crude methanogen extract, (lanes 2 and 4) purified protein (3 μg and 3.5 μg) eluted from the blue dextran Sepharose column by cDPG, (lane 3) purified protein mixed with Bio-Rad molecular weight markers including lysozyme (14 400), soybean trypsin inhibitor (21 500), carbonic anhydrase (31 000), hen egg white ovalbumin (45 000), bovine serum albumin (66 200), and phosphorylase B (97 400) and (lane 5) column breakthrough after loading the crude enzyme on the blue dextran Sepharose column. The gels were stained with Coomassie blue.

activity in *M. thermoautotrophicum* cell extracts as monitored by the detection of 3-PG (1.8 ppm) in the ^{31}P NMR spectrum of a crude cell extract incubated with 20 mM 2,3-DPG for 24 h. The resonance for 3-PG only appeared in crude cell extracts incubated with the phosphoglycolate and not in ethanol extracts of cells (Kanodia & Roberts, 1983). Another activity that could be observed by ^{31}P NMR in crude extracts was the conversion of 20 mM 2-PG (chemical shift at 1.1 ppm) to 3-PG in the presence of 2,3-DPG (20 mM) and crude cell extract for 24 h. If the extract was incubated with 3-PG, a much smaller amount of the 2-PG was produced.

Purification of cDPGase Activity. The protein extract from *M. thermoautotrophicum* with detectable cDPG hydrolytic activity was applied to a blue dextran agarose column. SDS–PAGE of this material prior to elution from the column showed a large variety of protein bands (Figure 6, lane 1). After being washed to remove nonspecifically bound proteins (a gel of this wash fraction is shown in Figure 6, lane 5), the column was eluted with cDPG (7–10 mM). Clearly, a single major protein fraction was detected by SDS–PAGE (Figure 6, lanes 2 and 4). Comparison to Bio-Rad molecular weight markers indicated the molecular weight of this cDPG-eluted protein was 33 000. Lane 3 in Figure 6 shows the markers with a similar amount of the purified enzyme added. If the extract was treated with alkaline phosphatase prior to chromatography on blue dextran, the amount of cDPGase (milligrams of protein) recovered from the column increased ~10-fold. The specific activity was the same for column-eluted material with or without alkaline phosphatase treatment. Therefore, this step was incorporated into the purification scheme. In a typical purification, 62 mg of crude protein was applied to the blue dextran agarose column yielding 2.5 mg of protein specifically eluted by cDPG. If another small phosphate-containing molecule (e.g., up to 75 mM sodium pyrophosphate) was used to elute the column, the 33-kDa protein did not elute or eluted only to a very small extent. If 2,3-DPG was used to elute the blue dextran agarose column, a major band with a distinctly different molecular mass (~40 kDa) was detected along with a much smaller amount of the 33-kDa protein. It was anticipated that the protein eluted specifically with cDPG should display the hydrolysis activity detected in vitro in crude extracts. The cDPGase specific activity (as determined without exogenous PG mutase in the assay mixture) associated with this band was ~0.21 μmol min^{-1} mg^{-1} for 20 mM cDPG (Table II). For comparison, the protein eluted by 2,3-DPG did not show any appreciable cDPG hydrolytic activity. Thus, the 33 000-Da protein appeared to be responsible for the hydrolysis of cDPG to 2,3-DPG. Blanks in these assays, samples with just enzyme or cDPG, were extremely low (<0.01 absorbance change at 340 nm) and stable for several hours.

At this juncture it is interesting to note that even higher amounts of 2,3-DPG were detected when commerically available phosphoglycerate mutase and its activator 2-phosphoglycolic acid were present in the assay mixture. When it was added in place of the methanogen cell extract, the phosphoglycerate mutase had no effect on production of 2,3-DPG from cDPG. This enzyme, a critical part of the coupled enzyme assay for 2,3-DPG detection, functions in the direction of 2,3-DPG hydrolysis when phosphoglycolate is present (Rose & Liebowitz, 1970). With the phosphoglycerate mutase (and phosphoglycolate) added, the specific activity of purified cDPGase toward 20 mM substrate became ~1 μmol min^{-1} mg^{-1}. An explanation for the higher activity of cDPGase in the presence of the added mutase is the removal of 2,3-DPG as it is formed. This suggests that 2,3-DPG, the product of cDPG hydrolysis, also inhibits cDPGase activity. In contrast, adding the mutase to the assay mix with the crude cell extract had no effect on enzyme specific activity (Table II). This suggested that an endogenous mutase/phosphatase activity was already present in the crude extract. It accounted for the low apparent purification factor when crude cell extracts and purified cDPGase were compared (specific activities of 0.06 vs 0.2 μmol min^{-1} mg^{-1}). A better estimate of the purification is to compare the specific activities determined in the assay with the mutase present. From these values, the cDPGase has been purified ~17–18-fold. In Table II, one also sees that 68% of the cDPGase units in the crude extract have been recovered in the 33-kDa protein fraction eluted in cDPG, while less than 1% was eluted by 2,3-DPG. The purified cDPGase represented ~4% of the protein in the crude cell extract. Keeping in mind that the cDPGase specific activities were obtained at 37 °C and not at the higher methanogen growth, this amount of protein in the cells would be adequate to account for the turnover of cDPG in vivo.

K_m and V_{max} Determination. The pure enzyme from the blue dextran agarose column was assayed for its dependence on the concentration of cDPG. The assay mix contained variable cDPG, 1 M KCl, 25 mM $MgCl_2$, DTT, buffer, and purified enzyme. 2,3-DPG production was compared in the absence and presence of phosphoglycerate mutase (and its activator phosphoglycolate). Typical results showing the change in absorbance at 340 nm as a function of time are presented in Figure 7A. These data were for the assay conducted in the presence of phosphoglycerate mutase. Results for the system without the mutase added were similar, but 2,3-DPG production was considerably lower. A V_{max} of 0.335 μmol of 2,3-DPG min^{-1} mg^{-1} and a K_m of 11.8 ± 1.2 mM were obtained without the exogenous mutase added (Figure 7B); a higher V_{max} (1.62 μmol of 2,3-DPG min^{-1} mg^{-1}) but the same K_m within experimental error (13.9 ± 1.5 mM) were obtained when the mutase was present (Figure 7B). These kinetic results strongly suggested that 2,3-DPG inhibition is not competitive; presumably the product binds at a site distinct from the active site. This type of product inhibition may be involved in in vivo regulation of the enzyme.

Occurrence of cDPG in Other Pseudomurein-Containing Methanogens. Originally we noted cDPG in three other methanobacteria (*M. thermoautotrophicum* Marburg, *Methanobacterium bryantii*, and *Methanobacterium formi-*

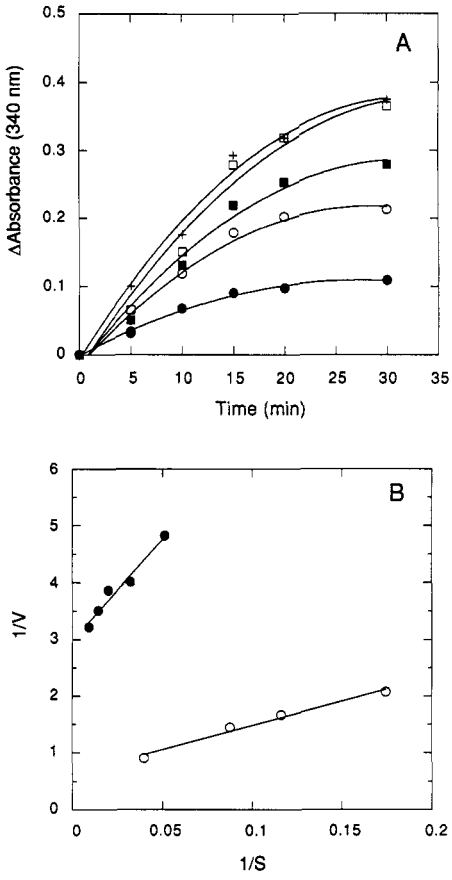


FIGURE 7: (A) Change in absorbance at 340 nm as a function of time after the addition of purified enzyme (30 μg): (●) 2.9, (○) 5.7, (■) 8.6, (□) 11.4, and (+) 25 mM cDPG. (B) Reciprocal of cDPGase specific activity as a function of the reciprocal of the concentration of cDPG (at 25 mM $MgCl_2$, 1 M KCl, pH 7.9): (●) reaction allowed to proceed in assay buffer for fixed times, quenched, and then mixed with PG mutase, phosphoglycolate, NADH, etc.; (○) all components of the 2,3-DPG assay (including PG mutase) mixed together with the cDPG, cDPGase, and other buffer components.

cicum) and *Methanobrevibacter ruminantium* (Tolman et al., 1986) and not in several other genera tested. Hensel and Konig (1988) showed it occurred at high levels in *Methanothermus fervidus*. Two other methanogens which have pseudomurein walls have now been examined by ^{13}C NMR for the occurrence of this compound. cDPG can be easily detected in ^{13}C NMR spectra of ethanol extracts of cells incubated with [$^{13}C_2$]acetate for labeling cellular material and $^{12}CO_2$ and H_2 for methane generation (Figure 8). Where the compound is a major constituent of the cytoplasm, the ^{13}C-labeled doublets for cDPG (each showing a small splitting by phosphorus) were easily observed, as in Figure 8A with extracts of *Methanothermus fervidus*. Because the splitting pattern and chemical shifts are unique to cDPG, it can also be detected in cells where it is not the dominant species. cDPG occurs in *Methanobrevibacter smithii* (Figure 8B), the organism found in human intestines and dental caries, and *Methanosphaera stadtmanae* (Figure 8C), another organism, found to a lesser extent in intestines, which uses only methanol and H_2 for generating methane. In *Methanosphaera stadtmanae*, it is a minor component compared to all the other labeled species.

Discussion

The working hypothesis in understanding the function of the unique cyclic pyrophosphate molecule, cDPG, in methanobacteria and methanobrevibacter, is that this compound serves as an intermediate pool in carbohydrate biosynthesis.

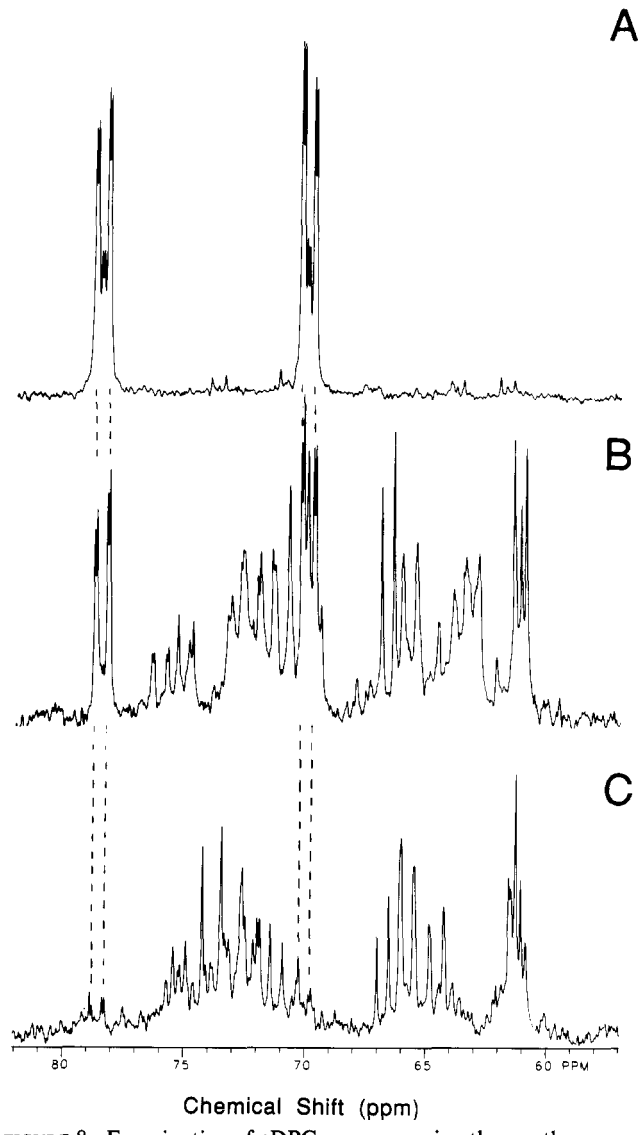
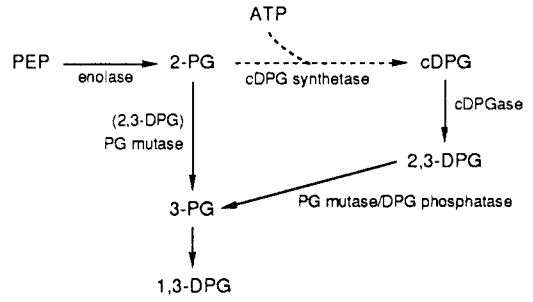


FIGURE 8: Examination of cDPG occurrence in other methanogens with pseudomurein cell walls. ^{13}C NMR spectra (75.4 MHz) of ethanol extracts of (A) *Methanothermus fervidus* grown on $^{12}CO_2$, H_2, and [$^{13}C_2$]acetate; (B) *Methanobrevibacter smithii* grown on $^{12}CO_2$, H_2, and [$^{13}C_2$]acetate; and (C) *Methanosphaera stadmanae* grown on $^{12}CH_3OH$, H_2, and [$^{13}C_2$]acetate. The carbon resonances for cDPG are indicated by the dotted lines.

While it has also been speculated to have a role in lipid metabolism (Seeley & Fahrney, 1984) or phosphate accumulation (Seeley & Fahrney, 1983), no data exist to support these alternative explanations. Hensel and Konig (1988) showed that 300 mM cDPG stabilized glyceraldehyde 3-phosphate dehydrogenase and malate dehydrogenase activities in extracts of *Methanothermus fervidus*, although whether this was specific to that anion or could be achieved by potassium salts of other polyanions at such high concentrations was unclear. cDPG occurs in species of Methanobacteriaceae, including the methanogens found in human intestines and dental caries. The correlation of this compound with the presence of a pseudomurein cell wall originally suggested a connection with that particular structure (Tolman et al., 1986), although finding cDPG in *Methanosarcina frisia* (Rudnick et al., 1990) may suggest that it occurs at NMR-undetectable levels in a wider range of methanogens. Solid-state ^{13}C NMR experiments (Evans et al., 1986a) and the isolation of an enzyme which hydrolyzes cDPG to 2,3-DPG have placed the compound in the sequence of reactions which traditionally led to carbohy-

drates. The steps proposed for the synthesis and further metabolism of cDPG include the following: (1) PEP is converted to 2-PG via enolase; (2) 2-PG (with ATP or another high-energy phosphate donor as a possible cosubstrate) is converted to cDPG, although there is no direct proof for this reaction yet; (3) cDPG is hydrolyzed to 2,3-DPG via the 33-kDa cDPGase activity characterized in the present work; and (4) the 2,3-DPG is then converted to 3-PG via phosphoglycerate mutase/DPG phosphatase (with activation by a species that can be substituted by phosphoglycolate in in vitro assays):



Previous studies by Fuchs and co-workers (Jansen et al., 1982) have demonstrated enolase and phosphoglycerate mutase activity in crude cell extracts and phosphate- and nucleotide-free extracts of *M. thermoautotrophicum*. An interesting observation in that work (Jansen et al., 1982) was that in crude cell extracts the phosphoglycerate mutase reaction (measured in the direction of 2-PG synthesis) appeared to be 2,3-DPG-independent. In dialyzed extracts no activity for the 2-PG/3-PG interconversion was observed unless 2,3-DPG was added. This is understandable in light of the present work with cDPG and cDPGase. In crude extracts cDPG hydrolysis to 2,3-DPG by cDPGase provides the cosubstrate for the endogenous phosphoglycerate mutase. Since there is so much cDPG in the cells, amounts of 2,3-DPG in the micromolar range will easily be present in the assay system. In dialyzed extracts, cDPG is removed. Therefore, there is no source of 2,3-DPG. Whether or not the phosphoglycerate mutase in these organisms is distinct from the DPG phosphatase is unclear.

Fixing carbon and phosphorus in cDPG provides a pool which can be rapidly expanded or contracted depending on the cell's metabolic needs. If cDPG is not an effector of gluconeogenesis enzymes, then it serves as a temporary storage reservoir. Regulation of carbohydrate biosynthesis in *M. thermoautotrophicum* is then achieved by changes in the concentration of other intermediates (e.g., 2,3-DPG) which alter specific enzyme activities. For example, when little 2,3-DPG is present, the mutase reaction (2-PG → 3-PG) is inefficient and carbon flow is through cDPG which is then hydrolyzed to 3-PG and continues into carbohydrate precursors. If 2,3-DPG builds up (e.g., in stationary phase cells or cells incubated at 50 °C), the cDPGase is inhibited and the mutase activity is comparatively increased. Carbon flow now can bypass cDPG, and 2-PG is converted directly to 3-PG. In essence, cDPG provides a side cycle to control cell carbon flow into carbohydrate. Which path is operational depends on cellular conditions. The major conclusion of the present work is that cDPG is transformed by a specific 33-kDa protein into 2,3-DPG, and not the other way around, and that this is a metabolically significant reaction. The K_m of this enzyme, cDPGase, for the substrate cDPG is relatively high (~12 mM) in keeping with the normal intracellular concentrations of substrate present in the cells.

2,3-DPG accumulates in vivo when the cells are incubated 12 °C below the optimum growth temperature. From the ^{13}C NMR pulse/chase work, we know that cDPG and 2,3-DPG still turn over, although the latter does so much more slowly under these conditions. These results show that cDPG is metabolized even when the cells barely grow. The NMR work together with the isolation of the purified enzyme indicate that build up of 2,3-DPG is a direct result of cDPG turnover and suggests that build up of 2,3-DPG may be related to the inhibition of cell growth. In human erythrocytes, 2,3-DPG levels affect a wide variety of enzyme activities: the compound is an inhibitor of AMP deaminase (Askari & Rao, 1968), PRPP synthetase (Hershko et al., 1969), and some of the enzymes of carbohydrate metabolism (Dische, 1964). In animal tissues, 2,3-DPG is a cofactor for the glycolytic enzyme phosphoglycerate mutase (Rose, 1968). Occasionally, we observed cultures at 62 °C with initially higher 2,3-DPG levels (~5% cDPG). These invariably had much slower doubling times. Also from the ^{13}C NMR work, we have seen that when 2,3-DPG starts to accumulate cDPG turnover slows down dramatically.

Purified cDPGase, which is a soluble enzyme, appears insensitive to O_2, while in intact cells exposed to O_2 virtually no 2,3-DPG is produced by the enzyme. Other carbon fixation reactions do occur in cells exposed to oxygen. For example, glutamate is utilized, and aspartate levels increase in the pulse/chase experiments. Clearly, O_2 exposure modifies some cellular component which affects cDPGase in vivo but which can be removed during the purification scheme. The identity of this component is unknown at present.

In summary, a novel compound, cDPG, whose position in carbon assimilation in methanobacteria has been the subject of considerable speculation (Kanodia & Roberts, 1983; Seely & Fahrney, 1983, 1984; Evans et al., 1985, 1986; Hensel & Konig, 1988), has been shown to be degraded to 2,3-DPG both in vivo and in vitro, the latter of which case is by a purified 33-kDa protein. Furthermore, the product of this reaction, 2,3-DPG, affects the activity of the cDPGase and may be a key factor in cell growth.

Registry No. 2,3-DPG, 138-81-8; cDPG, 88280-54-0; cDPGase, 132421-58-0.

References

Askari, A., & Rao, S. N. (1968) *Biochim. Biophys. Acta 151*, 198–203.

Balch, W. E., Fox, G. E., Magrum, L. J., Woese, C. R., & Wolfe, R. S. (1979) *Microbiol. Rev. 43*, 260–296.

Blackshear, P. J. (1984) *Methods Enzymol. 104*, 237–256.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–253.

Daniels, L., Sparling, R., & Sprott, G. D. (1984) *Biochim. Biophys. Acta 768*, 113–163.

Daniels, L., Belay, N., & Rajagopal, B. S. (1976) *Appl Environ. Microbiol. 51*, 703–709.

Dische, Z. (1964) in *The Red Blood Cell* (Bishop, C., & D. M. Surgener, D. M., Eds.) p 208, Academic Press, New York.

Ericson, A., & de Verdier, C. H. (1972) *Scand. J. Clin. Lab. Invest. 29*, 85–90.

Evans, J. N. S., Tolman, C. J., Kanodia, S., & Roberts, M. F. (1985) *Biochemistry 24*, 5693–5698.

Evans, J. N. S., Tolman, C. J., Raleigh, D., & Roberts, M. F. (1986a) *J. Biol. Chem. 261*, 16323–16331.

Evans, J. N. S., Tolman, C. J., & Roberts, M. F. (1986b) *Science 231*, 488–491.

Hensel, R., & Konig, H. (1988) *FEMS Microbiol. Lett. 49*, 75–79.

Hershko, A., Razin, A., & Mager, J. (1969) *Biochim. Biophys. Acta 184*, 64–76.

Jansen, K., Stupperich, E., & Fuchs, G. (1982) *Arch. Microbiol. 132*, 355–364.
Jones, W. J., Nagle, D. P., & Whitman, W. B. (1987) *Microbiol. Rev. 51*, 135–177.
Kanodia, S., & Roberts, M. F. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 5217–5221.
Keltjens, J. T., & van der Drift, C. (1986) *FEMS Microbiol. Rev. 39*, 259–303.
Lowry, O. H., Passonneau, J. V., Hasselberger, F. X., & Schulz, D. W. (1964) *J. Biol. Chem. 239*, 18–25.
Michal, G. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.) pp 1433–1438, Verlag Chemie, Weinheim, and Academic Press, New York.
Miller, T. L., & Wolin, M. J. (1983) *J. Bacteriol. 153*, 1051–1055.
Robertson, D., Lesage, S., & Roberts, M. F. (1989) *Biochim. Biophys. Acta 992*, 320–326.
Rose, Z. (1968) *J. Biol. Chem. 243*, 4810–4820.
Rose, Z., & Liebowitz, J. (1970) *J. Biol. Chem. 245*, 3232–3241.
Rudnick, H., Hendrich, S., Pilatus, U., & Blotevogel, K.-H. (1990) *Arch. Microbiol. 154*, 584–588.
Seely, R. J., & Fahrney, D. A. (1983) *J. Biol. Chem. 258*, 10835–10838.
Seely, R. J., & Fahrney, D. A. (1984) *J. Bacteriol. 160*, 50–54.
Tolman, C. J., Kanodia, S., Daniels, L., & Roberts, M. F. (1986) *Biochim. Biophys. Acta 886*, 345–352.
Zeikus, J. G., & Wolfe, R. S. (1972) *J. Bacteriol. 109*, 707–713.

Characterization of the Multiple EPR Line Shapes of Iron–Semiquinones in Photosystem 2†

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ABSTRACT: We have compared the temperature-dependence characteristics of the EPR signals of Qa and Qb iron–semiquinones from both purple bacterial and plant photosystems. The data obtained were analyzed and estimates of the splitting parameters of the non-heme Fe^{2+} spin sublevels obtained. The study confirms the similarities of the $g = 1.8$ Qa iron–semiquinone signal ($D/k = 15.6$ K, $E/k = 3.3$ K) formed in formate-treated plant photosystem 2 to the signal found in purple bacteria. However, the $g = 1.9$ Qa iron–semiquinone signal ($D/k = 7.1$ K, $E/k = \ll 1$ K), formed in photosystem 2 when bicarbonate remains bound, has a unique temperature behavior. A series of spectral features associated with the iron–semiquinone in bicarbonate-bound photosystem 2 appear as the temperature is lowered, and the analysis of these data requires that some of these features be assigned to the higher spin states. The results are discussed in terms of the requirement for bicarbonate to be a ligand of the non-heme iron.

Photosystem 2 (PS2)[1] is a membrane–protein complex in algae, cyanobacteria, and plants which catalyzes the light-induced transfer of electrons from water to plastoquinone. The reaction center core complex of polypeptides and cofactors shows homology with that of purple photosynthetic bacteria. The purple bacterial reaction center has been studied extensively both structurally, from analysis of X-ray data, and functionally, by many biochemical and biophysical techniques (Michel et al., 1986; Michel & Deisenhofer, 1988; Feher et al., 1989).

In purple bacteria, the quinone electron acceptors Qa and Qb have binding sites on the core reaction center polypeptides L and M. Similar binding sites have been proposed for Qa and Qb of PS2 on the polypeptides D1(Qb) and D2(Qa), which show homology to the L and M polypeptides (Michel & Deisenhofer, 1988). In both PS2 and purple bacteria, a non-heme iron atom is located between the two quinone binding sites. Neither quinone provides a ligand to the non-heme iron. The function of the non-heme iron appears to be either electrostatic, influencing the properties of the bound quinones, or to maintain the structural integrity of the reaction center core.

The semiquinones of Qa and Qb can be detected by electron paramagnetic resonance (EPR) spectrometry. The line shape and g value of the signal obtained is influenced by the nearby non-heme iron, and the resulting "iron–semiquinone" spectra of both Qa and Qb in purple bacteria have a first-derivative peak near $g = 1.8$. The interaction between the non-heme iron and the semiquinone has been studied and the line shape simulated (Butler et al., 1984; Dismukes et al., 1984). Spin sublevels of the high-spin Fe^{2+} are split by a combination of spin–orbit and ligand field interactions. There is an exchange interaction between the semiquinone and the Fe^{2+}, perturbing the semiquinone EPR spectrum. The nature of the pertur-

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[1] Abbreviations: TBTQ, 2,3,5-tribromo-6-methyl-1,4-benzoquinone (tribromotoluquinone); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; EPR, electron paramagnetic resonance; OP, 1,10-phenanthroline; PS2, photosystem 2.

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