Pyruvate-Carbon Dioxide Exchange Reaction of *Desulfovibrio desulfuricans*

BYUNGSE SUH AND J. M. AKAGI

Department of Microbiology, University of Kansas, Lawrence, Kansas

Received for publication 21 January 1966

ABSTRACT

SUH, BYUNGSE (University of Kansas, Lawrence), AND J. M. AKAGI. Pyruvate-carbon dioxide exchange reaction of *Desulfovibrio desulfuricans*. *J. Bacteriol.* 91:2281-2285. 1966.—The pyruvate-CO₂ exchange reaction, catalyzed by *Desulfovibrio desulfuricans*, required the presence of phosphate and coenzyme A. However, the requirement for phosphate disappeared when the concentration of coenzyme A was increased to a level of 3.8×10^{-3} M. Passing crude extracts through a diethylaminoethyl-cellulose column and an Amberlite CG-50 ion-exchange column, to remove ferredoxin and cytochrome *c*₃, resulted in a marked decrease in exchange activity; full activity was restored by the addition of ferredoxin or cytochrome *c*₃. Fe⁺⁺ or Co⁺⁺ stimulated the exchange of CO₂ into pyruvate.

The reversible nature of the phosphoroclastic reaction of *Clostridium butylicum* was first demonstrated by Wilson, Krampitz, and Werkman (20). They found that C¹³O₂ rapidly exchanged with the carboxyl moiety of pyruvic acid. This reaction was subsequently studied by numerous investigators in other bacterial systems. The cofactor requirements and optimal conditions for the exchange reaction were established for *C. butyricum* (8, 21), *C. acidurici* (12), *C. kluyveri* (15), and *Micrococcus lactilyticus* (19).

The sulfate-reducing bacterium, *Desulfovibrio desulfuricans*, is usually grown in a medium containing lactate as an energy source and inorganic sulfate as the electron acceptor. The products of this fermentation are acetate, carbon dioxide, and hydrogen sulfide. Lactate is oxidized to pyruvate, which is degraded to acetyl phosphate and carbon dioxide. The end product, acetate, is formed by a substrate-level phosphorylation of adenosine diphosphate by the acetyl phosphate [see review by Peck (10)]. During this process, the electrons arising from the oxidative steps are transported to the ultimate acceptor, sulfate, which is reduced to sulfide. *D. desulfuricans* grows in a sulfate-free medium if pyruvate is incorporated as the energy source (11). The products of this fermentation are acetate, ethyl alcohol, and carbon dioxide (J. R. Postgate, Abstr. 2nd Intern. Congr. Biochim., Paris, p. 92, 1952). Sadana (13) reported that cell-free extracts of *D. desulfuricans*, strain Hildenborough, converted pyruvate to acetyl phosphate, ethyl alcohol, and carbon dioxide. Conflicting results

were obtained by Millet (7), who demonstrated that cell-free extracts of *D. desulfuricans* oxidized pyruvate to acetyl phosphate, molecular hydrogen, and carbon dioxide. However, the latter experiments were conducted with a different strain (ATCC 7757) of *D. desulfuricans*.

The findings of the above workers reveal that *D. desulfuricans* contains a phosphoroclastic system, which converts pyruvate to acetyl phosphate and carbon dioxide. To determine the reversible nature of this reaction and to elucidate some of the cofactors involved, a study of the pyruvate-carbon dioxide exchange reaction was conducted with cell-free extracts of *D. desulfuricans*.

MATERIALS AND METHODS

Organism. *D. desulfuricans*, strain 8303, was obtained from L. L. Campbell. It was grown at 30 C in medium N described by Saunders, Campbell, and Postgate (14).

Preparation of cell-free extracts. Cells from 50-liter cultures were harvested with a Sharples centrifuge. Cell-free extracts were prepared by passing a 25% suspension of cells in 0.05 M tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride buffer (pH 7.0) through a French pressure cell (20,000 psi). Cell debris was removed by centrifugation at $27,000 \times g$ for 20 min.

Cytochrome *c*₃ was removed from crude extracts by passing the extracts through an Amberlite CG-50, type 2 resin prepared according to Horio and Kamen (3).

The ferredoxin of *D. desulfuricans* (17) was removed from the extracts by the procedure described below for the isolation of this carrier.

Enzyme assays. The pyruvate- CO_2 exchange reactions were conducted in 15- by 125-mm test tubes. Unless otherwise noted, reaction mixtures contained, in μmoles : potassium phosphate buffer (pH 8.5), 100; sodium pyruvate, 50; coenzyme A (CoA), 0.13; $\text{NaHC}^{14}\text{O}_3$ or $\text{HC}^{14}\text{OONa}$, 50 (containing 1.8×10^4 and 8.0×10^3 counts per min per μmole , respectively); and extract in a total volume of 1.0 ml. Because oxygen completely inhibited the exchange reaction, just before the addition of the extract, the tubes were gassed for 5 min with nitrogen and quickly stoppered with serum bottle caps. After warming the tubes to 30 C, the extract was introduced through the rubber caps with a hypodermic syringe and needle. At the end of the incubation time, the reaction was terminated by removing the serum caps and adding 3.0 ml of 50% trichloroacetic acid to the mixture. After removing the precipitated proteins by centrifugation, 6.0 ml of 0.1% 2,4-dinitrophenylhydrazine in 1 N HCl was added to the supernatant fluid. The phenylhydrazone derivative was washed three times with distilled water, and the washed precipitate was dissolved in 1.0 ml of hot 95% ethyl alcohol. A 0.2-ml sample was removed and counted in a liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Another 0.2-ml sample was dried on a preweighed planchet, and the weight of the phenylhydrazone derivative was determined.

Isolation of ferredoxin. The ferredoxin of *D. desulfuricans* was isolated by passing 50 ml of a crude extract through a 1.5- by 3-cm diethylaminoethyl (DEAE)-cellulose column (chloride form). The column was washed with 0.05 M Tris-HCl buffer (pH 7.0), and then with a solution of 0.1 M KCl in 0.15 M Tris buffer (pH 7.0). The amber-colored ferredoxin remained adsorbed on the column, and it was eluted with a solution of 0.5 M KCl in 0.15 M Tris-HCl buffer (pH 7.0). The ferredoxin fraction was passed through a Sephadex G-25 column (2.8 by 20 cm) equilibrated against 0.05 M Tris-HCl buffer (pH 7.0). The same buffer system was used as in the eluting solvent. The desalted ferredoxin-containing material was lyophilized and stored at -20°C .

A partially purified preparation of ferredoxin was obtained from *C. pasteurianum* by the procedure described above.

CoA was removed from the extracts by the method of Stadtman, Novelli, and Lipmann (16).

Special chemicals. Radioisotopes of C^{14} sodium bicarbonate and sodium formate were obtained from Volk Radiochemical Co., Burbank, Calif. CoA was purchased from P-L Biochemicals, Inc., Milwaukee, Wis. Protein was determined by the method of Lowry et al. (6).

RESULTS

Effect of phosphate and CoA on exchange. The requirement for phosphate in the exchange reaction is shown in Fig. 1. A concentration of approximately 4×10^{-2} M phosphate was optimal, and a slight inhibition was observed at a higher concentration. A similar inhibition by

FIG. 1. Effect of phosphate concentration on exchange activity. The reaction mixture contained, in μmoles : Tris-HCl buffer (pH 8.5), 100; CoA, 0.013; sodium pyruvate, 50; $\text{NaHC}^{14}\text{O}_3$, 50; potassium phosphate buffer (pH 8.5), as indicated; and 2.7 mg of dialyzed extract (see Table 1) in a total volume of 1.0 ml. Incubated under N_2 at 30 C for 15 min.

high phosphate concentration was reported for the pyruvate- CO_2 exchange reaction of *M. lactilyticus* (19).

Figure 2 shows that CoA also was required for the exchange reaction. In the presence of phosphate, the optimal concentration was approximately 8.5×10^{-5} M; higher concentrations were partially inhibitory. The inhibitory effect by higher CoA concentrations also was found with extracts of *C. butyricum* (8) and *M. lactilyticus* (19). However, the requirement of the exchange reaction for phosphate disappeared when the concentration of CoA was increased to a level of 3.8×10^{-4} M. The substitution of CoA for phosphate is similar to that reported by Mortlock, Valentine, and Wolfe (8) on the exchange reaction of *C. butyricum*.

Effect of divalent cations. The exchange reaction was stimulated by the addition of certain divalent cations (Table 1). At the concentrations tested, only Fe^{++} or Co^{++} showed any significant stimulation.

Effect of electron acceptors on exchange. When a crude extract of *D. desulfuricans* was passed through an Amberlite CG-50 column to remove

FIG. 2. Effect of CoA on exchange activity. The reaction mixture contained, in μ moles: potassium phosphate buffer (pH 8.5, where added), 100; sodium pyruvate, 50; $\text{NaH}^{14}\text{O}_3$, 50; CoA, as indicated; and 1.9 mg of "CoA-free" extract in a total volume of 1.0 ml. Incubated under N_2 at 30 C for 15 min. Solid line represents results in the presence of phosphate. Broken line represents results in the absence of phosphate.

cytochrome c_3 , and the unadsorbed fraction was passed through a DEAE-cellulose column to remove ferredoxin, the unadsorbed extract showed a marked decrease in exchange activity. Full activity was restored by the addition of either cytochrome c_3 or ferredoxin (Table 2); combining these carriers showed an additive effect. The electron carriers flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), methyl viologen, and *C. pasteurianum* ferredoxin also stimulated the exchange reaction. When the effects of increasing concentrations of ferredoxin and cytochrome c_3 were tested, the results showed a decreased exchange activity with increasing ferredoxin concentration (Table 3). Because this carrier can participate in a number of oxidation-reduction reactions [see review by Valentine, (18)] which can abstract electrons from ferredoxin, at higher concentrations, the exchange reaction was partially inhibited.

In contrast, no inhibition was observed when the concentration of cytochrome c_3 was increased beyond the optimal level. Although it appears that cytochrome c_3 participates as an electron acceptor from pyruvic dehydrogenase, evidence against this hypothesis was obtained (see Discussion).

Effect of pH. Under the conditions of the assay, the pyruvate- CO_2 exchange reaction was not affected by the pH of the reaction mixture, i.e.,

TABLE 1. Effect of divalent cations on the pyruvate- CO_2 exchange reaction of *Desulfovibrio desulfuricans**

Cation	Residual pyruvate (counts per min per μ mole)
None.....	841
Ca^{++}	969
Ba^{++}	771
Fe^{++}	1,238
Co^{++}	1,326
Mg^{++}	755
Mn^{++}	914
Zn^{++}	733

* Assayed as described under Materials and Methods. All reaction mixtures contained a final concentration of 10^{-2} M of the respective divalent cation. Extract used in this experiment was dialyzed for 12 hr at 4 C against 500 volumes of distilled water. Incubation time, 5 min.

TABLE 2. Effect of electron acceptor on the exchange reaction catalyzed by Amberlite-diethylaminoethyl-treated extract of *Desulfovibrio desulfuricans**

Additions†	Residual pyruvate (counts per min per μ mole)
None.....	818
Cytochrome c_3	1,905
<i>D. desulfuricans</i> ferredoxin.....	1,421
<i>D. desulfuricans</i> ferredoxin + cytochrome c_3	3,070
<i>Clostridium pasteurianum</i> ferredoxin.....	2,057
FAD.....	1,934
FMN.....	1,320
Methyl viologen.....	1,680
Crude extract.....	1,336

* Assayed as described under Materials and Methods. Two millimeters of extract were used in all mixtures. Protein concentrations of cytochrome c_3 , *D. desulfuricans* ferredoxin, and *C. pasteurianum* ferredoxin, 0.07, 0.13, and 0.06 mg, respectively. FAD, FMN, and methyl viologen final concentrations were 10^{-3} M. Incubation time, 5 min.

† Abbreviations: FAD = flavin adenine dinucleotide; FMN = flavin mononucleotide.

testing the activity at pH values ranging from 6.5 to 8.5 at intervals of 0.5, no difference in the extent of exchange was observed at each pH interval. Novelli (9) reported that extracts of *C. butylicum* catalyzed the pyruvate- CO_2 exchange equally at pH values of 6.5 and 8.0. He attributed this to a prior formation of a formyl moiety from hydrogen and carbon dioxide by the participation of some unknown carrier; the

formyl moiety was postulated to exchange with the carboxyl group of pyruvate. Extracts of *D. desulfuricans* catalyzed the exchange of formate into pyruvate at all pH values tested. Table 4 shows the CO₂ and formate exchange reactions at the upper and lower pH limits tested. Increasing the incubation time did not alter the results in either the CO₂ or the formate exchange. Although the optimal pH values for the exchange of CO₂ and formate, in other anaerobes examined, are approximately 6.5 and 8.0, respectively, we were not able to demonstrate this with extracts of *D. desulfuricans*. During the formate exchange experiments, it was assumed that the formic hydrogenlyase system was not active during the duration of these studies. This was concluded from manometric studies with the same batch of extract, which showed no H₂ or CO₂ evolution in the presence of formate and a catalytic amount of methyl viologen. If, however, the incubation time was increased to 30 min or more, the formic hydrogenlyase activity became evident.

TABLE 3. Effect of increasing concentrations of cytochrome *c*₃ and ferredoxin on exchange*

Carrier added	Amt	Residual pyruvate (counts per min per μ mole)
	mg	
None.....	—	482
Cytochrome <i>c</i> ₃	0.08	1,047
Cytochrome <i>c</i> ₃	0.16	1,050
Cytochrome <i>c</i> ₃	0.32	1,155
<i>D. desulfuricans</i> ferredoxin.....	0.13	897
<i>Desulfovibrio desulfuricans</i> ferredoxin.....	0.26	495

* Assayed as described under Materials and Methods. Extract protein, 2 mg. Incubation time, 15 min.

TABLE 4. Effect of pH on C¹⁴O₂ and HC¹⁴OONa exchange into pyruvate on cell-free extracts of *Desulfovibrio desulfuricans**

Additions	pH	Residual pyruvate (counts per min per μ mole)
HC ¹⁴ OONa	6.5	291
C ¹⁴ O ₂	6.5	3,758
HC ¹⁴ OONa	8.5	229
C ¹⁴ O ₂	8.5	3,861

* Assayed as described under Materials and Methods with 3 mg of extract protein. Incubation time, 15 min.

DISCUSSION

The pyruvate-CO₂ exchange reaction, catalyzed by cell-free extracts of *D. desulfuricans*, showed a definite requirement for phosphate and CoA and was stimulated by the addition of ferredoxin, cytochrome *c*₃, and Fe⁺⁺ or Co⁺⁺. Although evidence was not obtained in this study, it can be assumed that thiamine diphosphate (TDP) participates in this reaction, since Millet (7) demonstrated that the phosphoroclastic reaction of *D. desulfuricans* required the presence of TDP.

The ability of CoA, at higher concentrations, to substitute for phosphate suggests that it acts as the primary acceptor for the acetyl moiety from acetyl-TDP, forming acetyl-CoA. This was observed in the case of *C. butyricum* (8) where this cofactor, at higher concentrations, was believed to participate in the oxidation of a C₂ fragment during the degradation of pyruvate to acetate.

The roles of ferredoxin and cytochrome *c*₃, as electron carriers in the exchange reaction, were evident by their stimulatory effects. Evidence was presented by Whiteley and McCormick (19) that ferredoxin stimulates the exchange reaction in *M. lactilyticus*. The role of ferredoxin in the reversal of pyruvate degradation, from CO₂ and acetyl phosphate or acetyl-CoA (1), was definitely established.

The stimulatory effect of cytochrome *c*₃ is, at present, not understood. When we tested extracts of *D. desulfuricans*, depleted of ferredoxin and cytochrome *c*₃, for their ability to catalyze the pyruvic phosphoroclastic reaction, ferredoxin but not cytochrome *c*₃ participated as an electron acceptor (*unpublished data*). Two possible explanations are presented to account for this. (i) The "carrier-free" extract still contained some ferredoxin, and the addition of catalytic amounts of cytochrome *c*₃ resulted in a stimulatory response. (ii) The "carrier-free" extract catalyzed an endogenous degradation of pyruvate to H₂ and CO₂, reducing the residual ferredoxin, which, in turn, reduced cytochrome *c*₃. The reduced cytochrome participated as the unknown electron carrier, postulated by Novelli (9), to form formate. The pyruvate-formate exchange reaction, which is independent of any electron carrier, subsequently formed radioactive pyruvate.

The latter explanation may be acceptable since cytochrome *c*₃ was found to participate as an electron carrier for the formic hydrogenlyase system (5; J. P. Williams and H. D. Peck, Jr., *Bacteriol. Proc.*, p. 102, 1962). A reversal of this reaction, i.e., formation of formate from H₂ and CO₂, may explain the observed effect of cytochrome *c*₃ on the exchange reaction. However,

since the rate of CO₂ exchange into pyruvate was 10 times as rapid as the formate exchange (Table 4), alternative (ii) may not be a valid explanation. A more reasonable interpretation is (i).

Since formate has never been isolated as an intermediate product of *D. desulfuricans* metabolism, the possibility should be considered that the formate exchange may have proceeded via a cytochrome *c*₃-mediated breakdown to H₂ and CO₂, the CO₂ subsequently exchanging with pyruvate. If, however, the formate exchange reaction is catalyzed by *D. desulfuricans*, it would lend support to the hypothesis of Ishimoto et al. (4), who suggested the possibility that formate was an intermediate product during the degradation of pyruvate to acetate and CO₂. Gray and Gest (2) also suggested that *D. desulfuricans* may contain the clostridial-type of a phosphoroclastic reaction and a coli-type of a formic hydrogenlyase system.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service training grant 5 T1 GM 703 and grant AI-04672 from the National Institute of Allergy and Infectious Diseases, and by grant NSF-298 from the National Aeronautics and Space Administration to the University of Kansas.

LITERATURE CITED

1. BUCHANAN, B. B., R. BACHOFEN, AND D. I. ARNON. 1964. Role of ferredoxin in the reductive assimilation of CO₂ and acetate by extracts of the photosynthetic bacterium, *Chromatium*. Proc. Natl. Acad. Sci. U.S. 52:839-847.
2. GRAY, C. T., AND H. GEST. 1965. Biological formation of molecular hydrogen. Science 148:186-192.
3. HORIO, T., AND M. D. KAMEN. 1961. Preparation and properties of three pure crystalline bacterial heme proteins. Biochim. Biophys. Acta 48:266-286.
4. ISHIMOTO, M., J. KOYAMA, T. OMURA, AND Y. NAGAI. 1954. Biochemical studies on sulfate-reducing bacteria. III. Sulfate reduction by cell suspension. J. Biochem. (Tokyo) 41:537-546.
5. ISHIMOTO, M., T. YAGI, AND M. SHIRAKI. 1957. Biochemical studies on sulfate-reducing bacteria. VIII. The function of cytochrome of sulfate-reducing bacteria in decomposition of formate and reduction of sulfur and hydroxylamine. J. Biochem. (Tokyo) 44:707-714.
6. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
7. MILLET, J. 1954. Degradation anaerobie du pyruvate par un extrait enzymatique de *Desulfovibrio desulfuricans*. Compt. Rend. 238:408-411.
8. MORTLOCK, R. P., R. C. VALENTINE, AND R. S. WOLFE. 1959. Carbon dioxide activation in the pyruvate clastic system of *Clostridium butyricum*. J. Biol. Chem. 234:1653-1656.
9. NOVELLI, G. D. 1955. The exchange of H¹⁴ COOH with the carboxyl group of pyruvate by *Clostridium butylicum* and *Micrococcus lactolyticus*. Biochim. Biophys. Acta 18:594-596.
10. PECK, H. D. 1962. Symposium on metabolism of inorganic compounds. V. Comparative metabolism of inorganic sulfur compounds in microorganisms. Bacteriol. Rev. 26:67-94.
11. POSTGATE, J. R. 1952. Growth of sulfate-reducing bacteria in sulfate-free media. Research (London) 5:189-190.
12. RABINOWITZ, J. C. 1960. Factor B and other compounds related to vitamin B₁₂ in pyruvic acid-CO₂ exchange. J. Biol. Chem. 235:PC50-51.
13. SADANA, J. C. 1954. Pyruvate oxidation in *Desulphovibrio desulphuricans*. J. Bacteriol. 67:547-553.
14. SAUNDERS, G. F., L. L. CAMPBELL, AND J. R. POSTGATE. 1964. Base composition of deoxyribonucleic acid of sulfate-reducing bacteria deduced from buoyant density measurements in cesium chloride. J. Bacteriol. 87:1073-1078.
15. SCHUSTER, C. W., AND F. LYNEN. 1960. The role of cofactors and enzyme-bound biotin in the CO₂-pyruvate exchange reactions. Biochem. Biophys. Res. Commun. 3:350-355.
16. STADTMAN, E. R., G. D. NOVELLI, AND F. LIPMANN. 1951. Coenzyme A function in and acetyl transfer by the phosphotransacetylase system. J. Biol. Chem. 191:365-376.
17. TAGAWA, K., AND D. I. ARNON. 1962. Ferredoxins as electron carriers in photosynthesis and in the biological production and consumption of hydrogen gas. Nature 195:537-543.
18. VALENTINE, R. C. 1964. Bacterial ferredoxin. Bacteriol. Rev. 28:497-517.
19. WHITELEY, H. R., AND N. G. MCCORMICK. 1963. Degradation of pyruvate by *Micrococcus lactilyticus*. III. Properties and cofactor requirements of the carbon dioxide-exchange reaction. J. Bacteriol. 85:382-393.
20. WILSON, J., L. O. KRAMPITZ, AND C. H. WERKMAN. 1948. Reversibility of a phosphoroclastic reaction. Biochem. J. 42:598-600.
21. WOLFE, R. S., AND D. J. O'KANE. 1955. Cofactors of the carbon dioxide exchange reaction of *Clostridium butyricum*. J. Biol. Chem. 215:637-643.