The Role of Pyruvate Ferredoxin Oxidoreductase in Pyruvate Synthesis during Autotrophic Growth by the Wood-Ljungdahl Pathway*

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Pyruvate:ferredoxin oxidoreductase (PFOR) catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA and CO₂. The catalytic proficiency of this enzyme for the reverse reaction, pyruvate synthase, is poorly understood. Conversion of acetyl-CoA to pyruvate links the Wood-Ljungdahl pathway of autotrophic CO₂ fixation to the reductive tricarboxylic acid cycle, which in these autotrophic anaerobes is the stage for biosynthesis of all cellular macromolecules. The results described here demonstrate that the Clostridium thermocaceticum PFOR is a highly efficient pyruvate synthase. The Michaelis-Menten parameters for pyruvate synthesis by PFOR are: V_max = 1.6 unit/mg (k_cat = 3.2 s⁻¹), K_mAcetyl-CoA = 9 μM, and K_mCO₂ = 2 μM. The intracellular concentrations of acetyl-CoA, CoASH, and pyruvate have been measured. The predicted rate of pyruvate synthesis at physiological concentrations of substrates clearly is sufficient to support the role of PFOR as a pyruvate synthase in vivo. Measurements of its k_cat/K_m values demonstrate that ferredoxin is a highly efficient electron carrier in both the oxidative and reductive reactions. On the other hand, rubredoxin is a poor substitute in the oxidative direction and is inept in donating electrons for pyruvate synthesis.

Organisms from all three kingdoms of life (Bacteria, Archaea, and Eukarya) metabolize pyruvate in biosynthetic and catabolic reactions. Mitochondria and aerobic bacteria couple the oxidative decarboxylation of pyruvate to the reduction of NAD⁺ by the pyruvate dehydrogenase multienzyme complex (1). In many anaerobic organisms, pyruvate:ferredoxin oxidoreductase (PFOR) catalyzes the oxidative decarboxylation of pyruvate to CO₂ and acetyl-CoA (Reaction 1) (2–6). In anaerobic acetogenic bacteria, 2 moles of CO₂ generated from the decarboxylation of 2 moles of pyruvate are reduced to another mol of acetyl-CoA in an autotrophic biosynthetic scheme known as the Wood-Ljungdahl pathway (7–9). Thus, PFOR links glycolysis to the Wood-Ljungdahl pathway. The reverse reaction, carboxylation of acetyl-CoA, is an important reaction for anaerobes like methanogens and acetogens that fix CO₂ by the Wood-Ljungdahl pathway (10–13). In this case, PFOR (pyruvate synthase) links the Wood-Ljungdahl pathway to the incomplete reductive tricarboxylic acid cycle, which generates biosynthetic intermediates.

\[
\text{Pyruvate + CoASH} \rightarrow \text{Acetyl-SCoA} + \text{CO}_2 + 2\text{H}^+ + 2e^- \tag{REACTION 1}
\]

\[
\text{CO} + \text{H}_2\text{O} \rightarrow \text{CO}_2 + 2\text{H}^+ + 2e^- \tag{REACTION 2}
\]

PFORs can be homo- (14) or heterodimeric (15) or heterotetrameric (6). It is thought that all PFORs evolved by rearrangements and fusions of four ancestral genes (16, 17). They contain 1–3 iron-sulfur clusters and thiamine pyrophosphate as prosthetic groups (15, 18, 19). The PFOR from Clostridium thermocaceticum is a 240-kDa homodimer, with two [4Fe-4S]²⁺ clusters and one thiamin pyrophosphate/subunit (2, 14). The two electrons generated by the oxidative decarboxylation of pyruvate are transferred to an 8-iron ferredoxin (or possibly another electron carriers, see below) that in turn can reduce a variety of cellular enzymes. This electron pair can also be transferred directly to CODH, which reduces CO₂ to CO, an intermediate in the Wood-Ljungdahl pathway (20).

The PFOR reaction has been most extensively studied in the forward (oxidative decarboxylation) direction beginning with a series of seminal studies published in 1971 (21–24). Rabinozvitz and co-workers (21–24) isolated and characterized pyruvate:ferredoxin oxidoreductase. They also demonstrated that low potential electron donors, like reduced ferredoxin, can drive the reductive carboxylation of acetyl-CoA (24). Subsequently, few studies of the pyruvate synthase activity have been published.

Can PFOR also serve as a pyruvate synthase? A PFOR has been isolated from the methanogenic archaea, Methanosarcina barbieri (18) and Methanobacterium thermautotrophicum (25). These enzymes must function in anabolic reactions, because methanogens cannot grow on substrates with a more complex structure than acetate. The M. barbieri enzyme was shown to catalyze the oxidative decarboxylation of pyruvate to acetyl-CoA and the reductive carboxylation of acetyl-CoA with ferredoxin as an electron carrier (26). The sequences of the methanogenic enzymes are closely related to those of the PFORs from Pyrococcus furiosus and Thermotoga maritima (25, 26), which function in a catabolic direction (27).

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†The abbreviations used are: PFOR, pyruvate:ferredoxin oxidoreductase; CODH, CO dehydrogenase; MOPS, 3(N-morpholino)-propanesulfonic acid; ACS, acetyl-CoA synthase; LDH, lactate dehydrogenase; MES, 4-morpholineethanesulfonic acid; HPLC, high pressure liquid chromatography; Pd, ferredoxin.

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albeit poorly, on pyruvate (28, 29). Yoon et al. (37) have also studied the pyruvate synthase reaction of the PFOR from *Chloribium tepidum*.

The results described above support the hypothesis that PFOR and pyruvate synthase are the same enzyme. However, the reverse reaction of PFOR has been scantily studied and several examples exist of distinct enzymes catalyzing forward or reverse reactions. For example, fumarate reductase and succinate dehydrogenase preferentially catalyze opposing reactions (30, 31) and are separately regulated and distinct gene products (32, 33). In many organisms, H⁺ reduction and H₂ oxidation are catalyzed by separate enzymes with the converse catalytic biases (34, 35). Can PFOR serve as an efficient pyruvate synthase or is it prejudiced toward oxidative decarboxylation? One test of its catalytic bias is to compare the specificity factor (V/Km) for the two opposing reactions. Another test is to determine the relative rates of the opposite reactions at physiological substrate concentrations. A significant catalytic preference in one direction would suggest that PFOR and pyruvate synthase might be separate enzymes. The difficulty in studying pyruvate synthesis from acetyl-CoA and CO₂ is that a sufficiently strong electron donor must be coupled to drive this energetically demanding reaction, with a reduction potential below −540 mV. In the work reported here, we have coupled pyruvate synthase to CO oxidation by CO dehydrogenase (Reaction 2 and Scheme 1), which has a similarly low reduction potential (36).

If PFOR and pyruvate synthase are the same enzyme, one conceivable way to regulate the metabolic direction of pyruvate metabolism is for PFOR/pyruvate synthase to use a high potential electron acceptor in the oxidative direction and a separate low potential donor in the reductive direction. For example, in the fumarate reductase and succinate dehydrogenase systems, the low potential donor menaquinol is a better substrate for fumarate reduction; whereas, ubiquinone, which has a higher midpoint potential, preferentially couples to succinate (32). We do not yet know which electron acceptor(s) are most efficient in the PFOR and pyruvate synthase reactions. Because rubredoxin rapidly accepts electrons from PFOR, it was proposed to be the physiological electron acceptor for the oxidative decarboxylation reaction, whereas reduced ferredoxin was designated as the electron donor for the synthase reaction (37). However, the kinetic parameters for the substrates and electron donors/acceptors in each direction were not rigorously studied leaving some questions about electron carrier specificity unanswered. Furthermore, the electron carrier specificity may be different in different organisms.

*C. thermoacetum* is an interesting model system for studying PFOR because the oxidative decarboxylation and reductive carboxylation reactions are essential for heterotrophic and autotrophic growth, respectively, of this organism. We recently characterized and defined the rate constants for the elementary steps in the C. thermoacetum PFOR reaction (2). At high concentrations of substrates (kcat conditions), the synthase reaction was predicted to be ~10-fold faster than the reverse reaction; however, the synthase reaction was not studied directly. Furthermore, the intracellular concentrations of the substrates and products of the PFOR reaction in C. thermoacetum have not been determined; thus, the relative rates of the opposing reactions at physiological concentrations of substrates are unknown. Whether PFOR is an efficient pyruvate synthase for autotrophic acetogens thus remains an unanswered question.

In the work reported here, we focused on several questions. What are the values of kcat and kcat/Km for the reverse reaction? Are these values consistent with a physiological function for PFOR in pyruvate synthesis? As a low potential electron donor, CO oxidation could conceivably provide sufficient strength to drive pyruvate synthesis. How effectively does the *C. thermoaetum* pyruvate synthase reaction couple to the CO oxidation reaction? What is the most efficient direct electron donor and acceptor for the forward and reverse reactions of PFOR?

**EXPERIMENTAL PROCEDURES**

**Materials**—N₂ (99.8%, from Linweld) was deoxygenated by passage through a heated column containing BASF catalyst. CO (99.99%) was obtained from Linweld. Sodium bicarbonate (minimum 99.5%, acetyl-CoA (96%, sodium salt), β-nicotinamide adenine dinucleotide, reduced form (98%, disodium salt), and l-lactate dehydrogenase (type II; from rabbit muscle) were purchased from Sigma.

**C. thermoacetum Strain and Culture Conditions**—C. thermoacetum (Moorella thermaacetica) strain ATCC 39073 was grown anaerobically at 55 °C in a 14-liter fermentor on glucose and CO₂.

**Enzyme Purification**—CODH, PFOR, and ferredoxin were purified under strictly anaerobic conditions. PFOR was purified as described (20) in anaerobic 50 mM MOPS buffer at pH 7.5. CODH/ACS (38) and ferredoxin (39) were purified under anaerobic conditions at 17 °C in a vacuum atmosphere chamber maintained below 10 ppm of oxygen. Enzyme concentrations were determined by the Rose Bengal method (40) using a bovine serum albumin standard. CODH/ACS activity was assayed by monitoring the CO-dependent reduction of methyl viologen (ε₅₅₀ = 12,900 M⁻¹ cm⁻¹) (38). PFOR activity was measured as described (21) by following pyruvate- and CoA-dependent methyl viologen reduction. Ferredoxin activity was measured by coupling CO oxidation by CODH/ACS to metronidazole reduction (ε₅₃₀ = 9300 M⁻¹ cm⁻¹) (42) in a reaction similar to that described earlier for hydrogenase (43). Rubredoxin was purified from *Clostridium formicoaceticum* (44).

**Steady-state Kinetics of the Pyruvate Synthase Reaction**—Pyruvate synthase activity was determined in a coupled reaction with CO and CO dehydrogenase as the initial electron donor. Pyruvate formation was coupled to NADH oxidation by lactate dehydrogenase (LDH). Stock solutions of 1 mM KHCO₃, 10 mM or 1 mM acetyl-CoA, 22.4 mM NADH, 0.1 mM CODH, 33 μM PFOR, 121 μM ferredoxin, 10 mM methyl viologen, and 22 μM LDH were used. The assay was performed under an atmosphere of CO (1 mmt) in a reaction mixture containing 1 μM CODH, 1 μM ferredoxin, 40 μM methyl viologen, 110 μM LDH, and varying concentrations of acetyl-CoA and CO₂. The CO₂ concentration was estimated by the Henderson-Hasselbalch equation taking into account the pH of the reaction mixture and the KHCO₃ concentration. During the initial velocity time-scale CO₂ produced from CO was too low to affect the CO₂ concentrations in solution. PFOR, CODH, electron carrier (ferredoxin, methyl viologen, or rubredoxin), and LDH concentrations were varied in a coupled reaction to establish the conditions under which pyruvate synthase activity is rate-limiting. In all assays, the buffer was 50 mM MES, pH 6.4, and saturated with CO gas by bubbling for 5 min with CO in a serum-stoppered cuvette. NADH oxidation (ε₅₅₀ = 6400 M⁻¹ cm⁻¹) was followed in an OLIS-modified Cary 14 spectrophotometer. The assays were performed at 25 °C and were initiated by adding PFOR. The Vₐₘₙₐ and Kₗₔₕₐ values were determined by globally fitting the data to the equation for a ping-pong mechanism. PFOR has been shown to follow a ping-pong mechanism (21). The data were analyzed using Sigma Plot 5.0 (Jandel Scientific, San Rafael, CA).

**Kinetics of Pyruvate Oxidation**—To determine the Michaelis parameters of PFOR for ferredoxin, the reaction mixture contained 10 mM pyruvate, 1 mM acetyl-CoA, 1 mM thiamine pyrophosphate, 2 mM MgCl₂, 0.1 mM metronidazole, and varying concentrations of ferredoxin in 50 mM Tris-HCl buffer, pH 7.1. The reaction was started by adding 2 μl of PFOR (1.3 mg/ml) to a reaction volume of 0.5 ml, and the reduction of metronidazole was followed at 320 nm (ε₃₂₀ = 9300 M⁻¹ cm⁻¹). To determine the kinetic parameters for the reaction with rubredoxin, the reaction was started by adding 1 μl of PFOR (0.42 mg/ml) to a 0.2-mI
FIG. 1. Steady-state kinetics of the pyruvate synthase reaction. The assays were performed at 25 °C and were initiated by adding PFOR. The concentration of CO2 was varied at 5 μM (●), 10 μM (○), and 50 μM (▲) fixed concentrations of acetyl-CoA. The Michaelis parameters Vmax and Km were determined by global fitting of the data to a ping-pong mechanism. The Michaelis parameters determined were Vmax = 1.6 ± 0.1 unit/mg, KmAcetyl-CoA = 9.1 ± 1.4 μM, and KC02 = 2.0 ± 0.3 mM.

Table I

| Substrate       | Kmax | km | kcatal/Km | Expected rate | Measured rate | Ref. |
|-----------------|------|----|-----------|---------------|---------------|-----|
| Acetyl-CoA      | 9 × 10⁻³ | 3.2 | 356       | 3.2           | 1.3           | This work |
| CO2             | 2.0  | 3.2 | 1.6       | 3.2           | 1.3           | This work |
| CoASH           | 4 × 10⁻³ | 28 | 7 × 10³   | 28            | 10.8          | 20 |
| Pyruvate        | 0.30 | 28 | 93.3      | 18.6          | This work     |     |
| Ferredoxin      | 3.2 × 10⁻⁴ | 29.6 | 9.24 × 10⁴ | This work     |               |     |
| Ferredoxin      | 2.7 × 10⁻⁴ | 2.0 | 7.4 × 10⁵ | This work     |               |     |
| Rubredoxin      | 9.4 × 10⁻³ | 19.8 | 2.1 × 10⁶ | This work     |               |     |
| Rubredoxin      | <0.1 | 18.7 | 17        | This work     |               |     |
| Methyl viologen | 1.1  | 18.7 | 17        | This work     |               |     |

- Calculated rate at physiological concentrations of substrates.
- Pyruvate synthase reaction.
- Refers to the PFOR reaction.
- Measured rates at intracellular concentrations of substrates.

RESULTS

Pyruvate Synthesis with PFOR—For convenience, we will refer to the pyruvate synthase or PFOR reactions explicitly but generally refer to the enzyme as PFOR. Pyruvate synthesis from acetyl-CoA and CO₂ requires a strong electron donor. Because CO₂ produced by the pyruvate decarboxylation reaction is the source of the carbonyl group in acetyl-CoA synthesis by clostridial CODH/ACS (20), we coupled pyruvate synthesis to CO oxidation by CODH/ACS. The rate of pyruvate formation was monitored by the lactate dehydrogenase (45) assay. An internal volume of 1 μmol protein (49) and a ratio of 0.6 for the mg of total protein/mg of dry weight (50) were used in calculations of intracellular concentrations of metabolites. So for 0.57 g of dry weight of cells, we calculated 0.342 ml of total internal volume.

Intercellular Concentrations of Pyruvate, CoA, and Acetyl-CoA—C. thermoacetica cells (2 g, 0.57 g of dry weight) in 5 ml of sonication buffer were sonicated for 5 min (15 s on, 45 s off). The sonication buffer contained 0.002 ng/ml DNase, 0.017 mg/ml phenylmethylsulfonyl fluoride, and 1 mg/ml lysozyme in 50 mM Tris-HCl, pH 7.6. After sonication, the proteins were precipitated with 2 M perchloric acid at 4 °C. After centrifugation at 14,000 rpm for 10 min, the pH was increased to pH 5.5 with 1 M NaOH, the solution was again centrifuged, and the concentrations of metabolites were measured in the supernatant. Radioactive acetyl-CoA (1 μl of a 850 μM stock solution with 96,950 dpm/nmol) was added to the supernatant as an internal control to determine recovery from the extraction procedure. More than 99% of the acetyl-CoA added was recovered after the final extraction step. The amounts of CoASH and acetyl-CoA were determined by reversed-phase high performance liquid chromatography on a Waters 616/626 LC System equipped with a Waters 996 PDA detector (Waters Inc.). We obtained the best separation of the cell extract components by eluting with a gradient from 5 to 50% methanol in 100 mM phosphate buffer, pH 5, over a 30-min time period. We used a C-18 Bondapack chromatographic column. Under these conditions, the retention times for CoASH and for acetyl-CoA were 10.3 and 13.3 min, respectively. The concentrations of CoASH and acetyl-CoA in the cell extract were estimated based on a standard curve (nmol CoASH, acetyl-CoA versus peak area). The concentration of pyruvate was determined by the lactate dehydrogenase assay. An internal volume of 1 μmol protein was used. A ratio of 0.6 for the mg of total protein/mg of dry weight (50) were used in calculations of intracellular concentrations of metabolites. So for 0.57 g of dry weight of cells, we calculated 0.342 ml of total internal volume.

The concentrations of metabolites were measured in the supernatant. Radioactive counts were determined in a liquid scintillation spectrometer (Beckman LS 6500). The concentrations of metabolites were determined by high performance liquid chromatography with a Waters 996 PDA detector (Waters Inc.)

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concentration. Michaelis parameters: $V_{\text{max}}$, the rate-limiting enzyme and at saturating concentrations of the other substrates. $A$, dependence of the PFOR reaction rate on ferredoxin concentration. Michaelis parameters: $V_{\text{max}} = 14.8 \text{ units/mg}$, $K_m = 0.32 \mu M$, and $k_{\text{cat}}/K_m = 9.25 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. $B$, dependence of the pyruvate synthase reaction rate on ferredoxin concentration. Michaelis parameters are $V_{\text{max}} = 1.0 \text{ unit/mg}$, $K_m = 0.27 \mu M$, and $k_{\text{cat}}/K_m = 7.4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$.

LDH, 1 mM CO. At concentrations of PFOR at or below 16.5 nM, the reaction rate was strictly dependent upon PFOR. Different concentrations of CO$_2$ (0.56–22.4 mM) and acetyl-CoA (0.005–0.05 mM) were used to define the kinetic parameters for the pyruvate synthase reaction (Fig. 1).

The kinetic data did not fit a ternary complex mechanism; the kinetic parameters $K_{\text{in}}$ and $K_{\text{out}}$, and $k_{\text{cat}}/K_m$, had unreasonably large standard deviations (over 200% error). On the other hand, the data fit a ping-pong mechanism quite well (<15% standard deviations). The kinetic parameters were determined by globally fitting the data to a ping-pong mechanism. The Michaelis parameters for the pyruvate synthase are given in Table I along with those for the PFOR reaction, which we also determined. With CO and CODH as the electron donor, the pyruvate synthase activity is high. At saturating CO$_2$ (22.4 mM) and acetyl-CoA (100 $\mu M$), the specific activity is 1.6 unit/mg, which translates to a $k_{\text{cat}}$ of 3.2 $\text{ s}^{-1}$. This is only 9-fold lower than the $k_{\text{cat}}$ for the PFOR reaction (28 s$^{-1}$), which agrees well with predictions based on kinetic simulations (2).

When methyl viologen was replaced by ferredoxin (1 $\mu M$) at saturating concentrations of pyruvate and CoA, there was no difference in the specific activity, indicating that the second half reaction of pyruvate synthesis, not electron donation to the enzyme, is rate-limiting under these conditions.

Physiological Electron Carriers(s) for Pyruvate Synthase and PFOR—Initial velocity experiments were used to determine the physiological electron carriers(s) for PFOR (Fig. 2A) and pyruvate synthase (Fig. 2B). With ferredoxin as the mediator, the $k_{\text{cat}}/K_m$ for ferredoxin for the synthase reaction is only ~10-fold lower than for the oxidative decarboxylation. The 8-iron ferredoxin from the archaeon, *Methanosarcina thermophila*, also was as good an electron mediator as the *C. thermoaceticum* ferredoxin (Fig. 3), suggesting that these proteins are homologous enough to interact in a similar way with both PFOR and CODH/ACS.

Recently, it was proposed for the PFOR of *C. tepidum* that rubredoxin is the electron acceptor, whereas ferredoxin is the electron donor for the pyruvate synthase reaction (37). This hypothesis is reasonable because rubredoxin has a midpoint potential near 0 mV (in *C. tepidum* it is ~87 mV); whereas, ferredoxins have much more negative midpoint potentials, generally below ~400 mV (46). However, the values of $V/K$, the so-called specificity factors, for rubredoxin and ferredoxin in the pyruvate synthase or PFOR reactions have never been compared. The value of $k_{\text{cat}}/K_m$ for rubredoxin in the oxidative decarboxylation reaction is $2.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (Fig. 4), which is 50-fold lower than that for ferredoxin. Thus, although the driving force for reducing rubredoxin with pyruvate is much stronger in *C. thermoaceticum*, there is a strong preference of PFOR for ferredoxin in the oxidative decarboxylation reaction.

Rubredoxin could not couple CO oxidation to the pyruvate synthase reaction (Table I and Fig. 3). Because rubredoxin is a highly active electron acceptor for CODH/ACS, the bottleneck is in the pyruvate synthase step. This is reasonable because the standard reduction potential for the Fe$^{3+}$/Fe$^{2+}$ couple of rubredoxin is much more positive than that of the acetyl-CoA + CO$_2$/pyruvate couple.

Physiological Concentration of Pyruvate, Acetyl-CoA, and CoA—To assess the physiological relevance of the pyruvate synthase reaction of PFOR, we determined the pyruvate, acetyl-CoA, and CoASH concentrations in growing *C. ther-
The concentration of components other than rubredoxin are items in Table I. These predicted values, we ran the forward and reverse reaction rates, slower than oxidative decarboxylation of pyruvate. To verify carboxylation of acetyl-CoA is predicted to occur about 8-fold at physiological concentrations of pyruvate and acetyl-CoA, the concentration of acetyl-CoA in the cell. The consequence is that, particularly, the pyruvate synthase reaction would be limited by the concentration of pyruvate. Similarly, the pyruvate synthase reaction would be limited by the concentration of acetyl-CoA in the cell. The consequence is that, at physiological concentrations of pyruvate and acetyl-CoA, the carboxylation of acetyl-CoA is predicted to occur about 8-fold slower than oxidative decarboxylation of pyruvate. To verify these predicted values, we ran the forward and reverse reactions in the presence of the measured intracellular concentrations of pyruvate, coenzyme A, acetyl-CoA, and CO₂ (Table I). The results confirmed the prediction with a ratio of oxidative decarboxylation to reductive carboxylation of 8.3.

**DISCUSSION**

The oxidative decarboxylation of pyruvate by PFOR generates low potential electrons that can be coupled to important reactions in the C, N, S, and H cycles, e.g. dinitrogen reduction, proton reduction to H₂, sulfate reduction, and CO₂ reduction. PFOR also generates the key metabolic intermediate, acetyl-CoA. Thus, this important reaction has been studied rather extensively. The reverse reaction, reduction of acetyl-CoA to pyruvate, is equally important for autotrophic anaerobes because it links the Wood-Ljungdahl pathway of acetyl-CoA for-

![FIG. 4. PFOR reaction with rubredoxin as external electron acceptor.](http://www.jbc.org/)

**TABLE II**

| Metabolites        | Intracellular concentration | Method used      |
|--------------------|-----------------------------|------------------|
| Pyruvate           | 180 ± 40 (n = 3)             | Lactate dehydrogenase assay |
| Coenzyme A         | 280 ± 90 (n = 3)             | HPLC             |
| Acetyl-Coenzyme A  | 10.4 ± 3.5 (n = 3)           | HPLC             |

moacetum cells (Table I). The amounts of these metabolites in the cell extract, determined by HPLC, were related to the intracellular volume (0.57 g of cell dry weight = 0.342 ml of cell internal volume) (Table II). We estimate the intracellular concentrations of CoASH and acetyl-CoA to be 0.28 and 0.01 mM, respectively. The intracellular pyruvate concentration is 0.2 mM, whereas the CO₂ concentration is estimated to be 33 mM, because cells are continually sparged with 100% CO₂ during growth.

**Rates of Pyruvate Formation and Oxidation at Physiological Substrate Concentrations**—Because the intracellular pyruvate concentration is below its $K_m$ value (0.3 mM), the PFOR reaction would be limited by the concentration of pyruvate. Similarly, the pyruvate synthase reaction would be limited by the concentration of acetyl-CoA in the cell. The consequence is that, at physiological concentrations of pyruvate and acetyl-CoA, the carboxylation of acetyl-CoA is predicted to occur about 8-fold slower than oxidative decarboxylation of pyruvate. To verify these predicted values, we ran the forward and reverse reactions in the presence of the measured intracellular concentrations of pyruvate, coenzyme A, acetyl-CoA, and CO₂ (Table I). The results confirmed the prediction with a ratio of oxidative decarboxylation to reductive carboxylation of 8.3.

**What is the physiological electron carrier(s) for pyruvate synthase and PFOR?** With a midpoint potential for the acetyl-CoA + CO₂/pyruvate couple of −520 mV, pyruvate is a strong reductant. Correspondingly, it requires a very strong reductant to carboxylate acetyl-CoA. Tabita (37) discovered that rubredoxin is a strong electron acceptor for the PFOR from the green sulfur bacterium *C. tepidum*, indicating that it could be the physiological reductase mediator in this direction. The *C. thermoacetica* CODH is a highly promiscuous electron donor and can rapidly reduce rubredoxin. Several roles of rubredoxin in anaerobic energy metabolism have been proposed. These have been recently discussed in relation to a new proposed role for rubredoxin in protecting cells from oxygen (41, 48). Rubredoxin is expected to be a very good electron acceptor from pyruvate, because its Fe⁵⁺/²⁺ couple is near 0 mV. Therefore, on the basis of driving force alone, rubredoxin could accept electrons six orders of magnitude faster than ferredoxin (midpoint potential of about −350 to −400 mV). However, the $k_{cat}/K_m$ for rubredoxin⁳ in pyruvate oxidation is 50-fold lower than that for ferredoxin. Thus, although the driving force for ferredoxin reduction is much less relative to rubredoxin, ferredoxin is a much more efficient acceptor of electrons from PFOR.

An apparently more serious problem with rubredoxin as an electron acceptor for PFOR is that it is a very poor electron donor for subsequent reactions. Therefore, it seems unreasonable to expect rubredoxin to couple pyruvate oxidation to proton, CO₂, or dinitrogen reduction. The CO₂/CO and the CO₂ +

³ We used rubredoxin from *C. formicoaceticum* not *C. thermoacetica* to couple to the *C. thermoacetica* PFOR, because this protein is relatively abundant in iron-limited cultures of *C. formicoaceticum*. These small electron transfer mediators can generally be used fairly interchangeably among related microbes. We do not expect this to significantly alter our conclusions, because the *C. formicoaceticum* rubredoxin couples equally well to the *C. formicoaceticum* and *C. thermoacetica* CODH. Furthermore, the *M. thermophila* ferredoxin is nearly as good an electron acceptor as the *C. thermoacetica* protein.
PFOR as Pyruvate Synthase

acetyl-CoA/pyruvate couples have similar standard reduction potentials. Accordingly, we found that rubredoxin is unable to couple CO oxidation to the pyruvate synthase reaction. On the other hand, ferredoxin is an excellent mediator in this reaction. Although, rubredoxin is inept as an electron donor for pyruvate synthase, the value of $k_{\text{red}}/K_m$ for ferredoxin in the synthase reaction is only ~10-fold lower than in the oxidative decarboxylation. Thus, our results indicate that PFOR is an efficient pyruvate synthase and that ferredoxin serves as the electron acceptor for pyruvate oxidation as well as the electron donor for pyruvate synthase.

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