The Active Species of “CO₂” Formed by Carbon Monoxide Dehydrogenase from *Peptostreptococcus productus*

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*Dedicated to Professor Achim Trebst on the occasion of his 60th birthday*

**Materials and Methods**

**Chemicals and bacteria**

Acetazolamide Na-salt (Diamox; 2-acetylamino-1,2,4-thiadiazole-5-sulfonamide), 5,5-diethylbarbituric acid Na-salt (Veronal sodium), and methyl violoxygen were from Serva (Heidelberg, F.R.G.). Carbamic anhydrase from bovine erythrocytes (carbonate hydro-lyase, EC 4.2.1.1, lyophilized, salt-free, 2000 U/mg) and FMN Na-salt (flavin mononucleotide) were from Boehringer (Mannheim, F.R.G.). TiCl₄ solution in 10% HCl and 0.01 M HCl (Titrisol) were from E. Merck (Darmstadt, F.R.G.). Dye reagent concentrate for protein determination was from Messer Griesheim (Düsseldorf, F.R.G.). *Peptostreptococcus productus* strain Marburg was from G. Diekert (Stuttgart, F.R.G.).

**Preparation of cell extracts**

*P. productus* was grown on carbon monoxide as sole energy source [9]. At the end of the exponential growth phase (O.D.,578 = 1) the cells were harvested anaerobically by centrifugation, washed once with Veronal buffer and then resuspended in the same buffer (per l: 1 mmol Veronal, 200 mmol KCl, 10 μmol resazurin, and 2 ml titanium(III)citrate solution prepared as described previously [12]; adjusted to pH 8.4 with HCl). The cell suspension was passed twice through a French pressure cell at 1 × 10⁸
Pascal. Cell debris and whole cells were removed by centrifugation at 27,000 × g for 30 min. The supernatant (16 mg protein/ml), which is referred to as cell extract, was stored in 5 ml portions under N₂ at −20 °C until use.

**Determination of CO dehydrogenase activity**

CO dehydrogenase activity was assayed at 5 °C in 1.7 ml anaerobic cuvettes closed with rubber stoppers. The cuvettes contained 1 ml assay mixture: 100 mM Veronal/HCl buffer pH 8.4 supplemented with 5 mM FMN. The gas phase was 100% CO at 1.5 × 10⁵ Pascal. Assay mixtures were made anaerobically by the addition of a few µl titanium(III) citrate solution [12]. The reduction of FMN was followed photometrically at 508 nm (at pH 8.4: ε₅₀₈ = 740 cm⁻¹ m⁻¹; ∆ε₅₀₈ (ox minus red) = 700 cm⁻¹ m⁻¹).

**Determination of the active species of “CO₂”**

The assays were performed in a thermostated 50 ml glass vessel with three outlets, which were sealed with rubber stoppers. The vessel, which was covered with aluminium foil in order to prevent photolysis of FMN, contained 20 ml assay mixtures: 1 mM Veronal/HCl pH 8.4; 200 mM KCl, 5 mM FMN; 50 µl titanium(III) citrate solution; 0.1–1.0 ml cell extract; and, where indicated, 12.5 µl carbonic anhydrase solution (200 U/µl). The pH was adjusted to pH 8.4 with 0.1 mM CO₂-free anaerobic KOH. The mixture was continuously stirred at 1,100 rpm with a magnetic bar. The gas phase was 100% N₂. The reaction was started by the addition of 0.1–1.0 ml CO saturated H₂O at 25 °C (0.93 mM CO) [12], or – as a control – of 10–25 µl CO₂ saturated H₂O at 2 °C (70 mM CO₂) [13]. The change in pH was recorded with an electrode (N 5700 A from Schott, Mainz, F.R.G.) which was inserted into the vessel from the top through one of the rubber stoppers. The electrode was connected with a compensation pH meter in combination with a chart recorder. The pH change was calibrated by the addition of 0.05–0.1 ml of 10 mM HCl (Titrisol).

**Results**

The experiments were performed at pH 8.4 and 5 °C with FMN as electron acceptor for the oxidation of CO to “CO₂”. At pH 8.4 the reduction of FMN yields FMNH⁺ (FMNH₂ ⇌ FMNH⁺ + H⁺; pKa = 6.7) [14], and H₂CO₃ is almost completely dissociated into HCO₃⁻ + H⁺ (at 5 °C: app K₁ = 6.52; app K₂ = 10.56) [15]. At 5 °C CO₂ is only slowly hydrated to HCO₃⁻ + H⁺. Thus, if CO₂ is the immediate product of CO oxidation, one proton is expected to be generated rapidly at the rate of FMN reduction (reaction (a)) and a second proton more slowly as a result of CO₂ hydration (reaction (b)). The appearance of the second proton should be speeded up in the presence of carbonic anhydrase, which catalyzes reaction (b). If HCO₃⁻ is the immediate product then the oxidation of CO with FMN is directly associated with the formation of two protons (reaction c) and carbonic anhydrase should have no effect.

\[
\begin{align*}
\text{CO} + \text{H}_2\text{O} + \text{FMN} &\rightarrow \text{CO}_2 + \text{FMNH}^+ + \text{H}^+ \quad (a) \\
\text{CO}_2 + \text{H}_2\text{O} &\rightarrow \text{HCO}_3^- + \text{H}^+ \quad (b) \\
\text{CO} + 2 \text{H}_2\text{O} + \text{FMN} &\rightarrow \text{HCO}_3^- + \text{FMNH}^+ + 2\text{H}^+ \quad (c)
\end{align*}
\]

It is therefore possible to discriminate between reaction (a) and (c) by measuring the rate of acidification in the absence and presence of carbonic anhydrase.

Cell extract of *Peptostreptococcus productus* catalyzed the reduction of FMN with carbon monoxide at a specific rate of 40–60 nmol/min·mg protein at 5 °C. The rate increased linearly with protein in the concentration range tested (0.1–1 mg/ml). The apparent \(K_m\) for FMN was found to be 1.5 mM. At 37 °C the specific activity was 0.5 µmol/min·mg. Thus the rate increased with the temperature with a \(Q_{10}\) of approximately 2.2.

The kinetics of acidification during carbon monoxide oxidation with FMN are shown in Fig. 1A. In the experiment the rate of H⁺ formation was approximately 70 nmol H⁺/min·mg and that of FMN reduction 50 nmol/min·mg. Upon addition of carbonic anhydrase, the rate increased to 100 nmol H⁺/min·mg. These results indicate that CO oxidation with FMN proceeded according to reaction (a) rather than to reaction (e).

After start of the reaction by the addition of CO the rate of acidification increased within the first two min when carbonic anhydrase was absent. This can be explained by the fact that the rate of uncatalyzed CO₂ hydration (reaction (b)) increases proportionally with the increasing CO₂ concentration.

The experiment in Fig. 1A was performed at a low carbon monoxide dehydrogenase concentration (0.16 µmol/min). At higher enzyme concentrations (0.8 µmol/min) the rate of acidification was higher.
(Fig. 1B) but also increased in the presence of carbonic anhydrase. Whereas in the absence of carbonic anhydrase several min were required until the acidification process ceased, in its presence the reaction was completed within less than one min. Similar kinetic differences were observed when the acidification upon addition of a CO₂ solution was followed (Fig. 1C).

At higher enzyme concentrations the extent of acidification was proportional to the amount of CO added. Proton to CO stoichiometries of 1.9 (± 0.2) and 2.1 (± 0.2) were determined in the absence and in the presence of carbonic anhydrase, respectively (average values of 10 determinations). The somewhat lower values in the absence of carbonic anhydrase are probably due to the loss of CO₂ into the gas phase of the reaction vessel. This probably also explains why at low protein concentrations (Fig. 1A) the extent of acidification was generally lower than predicted from the amount of carbon monoxide added. When CO₂ rather than CO was added in the presence of carbonic anhydrase the proton to CO₂ stoichiometry was one (Fig. 1C).

The stimulatory effects of carbonic anhydrase as shown in Fig. 1 were not observed when Diamox (1 mM) was added to the assay mixture. This compound inhibits carbonic anhydrase from bovine erythrocytes with a $K_i$ of 0.6 μM [16]. When the experiments were performed at 37 °C, carbonic anhydrase no longer affected the acidification rate. No acidification was observed, when N₂ saturated H₂O rather than CO saturated H₂O was added or when cell extract protein was omitted from the assay mixtures. Traces of O₂ and cyanide (1 mM) inhibited both carbon monoxide oxidation and acidification. Carbon monoxide dehydrogenase from *Peptostreptococcus productus* is known to be rapidly inactivated by O₂ and to be effectively inhibited by cyanide ($K_i = 0.1$ mM) [9, 10].

Fig. 1. Carbon monoxide oxidation to “CO₂” with FMN as electron acceptor: kinetics of acidification in the absence and presence of carbonic anhydrase. (A) 930 nmol CO and 3.2 mg cell extract protein with 0.16 μmol/min CO dehydrogenase; (B) 750 nmol CO and 16 mg cell extract protein with 0.8 μmol/min CO dehydrogenase; (C) control: 1400 nmol CO₂ rather than CO and 16 mg cell extract protein. The experiments were performed as pH 8.4 and 5 °C as described in the Methods section.
Discussion

Oxidation of carbon monoxide with FMN to \( \text{CO}_2 \) in cell extracts of *Peptostreptococcus productus* was associated with an acidification of the medium. Per mol CO oxidized two mol \( \text{H}^+ \) were formed. One proton is explainable by the property of FMNH at pH 8.4 to dissociate into FMNH\(^+\) + \( \text{H}^+ \) (pK\( \text{a} \) = 6.7) (reaction (a)). The results indicate that the second proton comes from the hydration of \( \text{CO}_2 \) to \( \text{H}_2\text{CO}_3 \) which dissociates into \( \text{HCO}_3^- + \text{H}^+ \) (app. pK\( \text{a} \) = 6.52) (reaction (b)). This is concluded from the finding that at 5 °C and in the absence of carbonic anhydrase the generation of the second proton lagged behind (Fig. 1). This lag was abolished in the presence of carbonic anhydrase or when the reaction was run at 37 °C. If \( \text{HCO}_3^- (\text{H}_2\text{CO}_3) \) were the immediate product formed (reaction (c) no such lag period should occur since at 5 °C the hydration of \( \text{CO}_2 \) to \( \text{H}_2\text{CO}_3 \) rather than the dissociation into \( \text{HCO}_3^- + \text{H}^+ \) is slow.

The carbon monoxide dehydrogenase from anaerobic bacteria is a nickel enzyme which catalyzes both the oxidation of \( \text{CO} \) to \( \text{CO}_2 \) and the reduction to \( \text{CO}_2 \) to \( \text{CO} \) [17, 18]. The latter direction is of importance in acetogenic bacteria and in many autotrophic anaerobic bacteria [2–5]. These organisms synthesize acetyl-CoA from 2 \( \text{CO}_2 \) via reduced \( \text{C}_1 \)-unit intermediates. Two \( \text{CO}_2 \) fixation reactions are involved: (i) the reduction of \( \text{CO}_2 \) to formate which is further reduced to the methyl group of acetyl-CoA [19] and (ii) the reduction of \( \text{CO}_2 \) to \( \text{CO} \) which is the precursor of the carboxyl group of acetyl-CoA [2–5]. The formate dehydrogenase has early been shown to use \( \text{CO}_2 \) rather than \( \text{HCO}_3^- \) [20, 21]. We have now provided evidence that also the second \( \text{CO}_2 \) fixation step in these organisms is specific for \( \text{CO}_2 \).

In acetogenic bacteria most of the acetyl-CoA formed in the carbon monoxide dehydrogenase pathway is converted to acetic acid. In autotrophic anaerobes using this pathway acetyl-CoA is reduc-

### Table I. The active species of “\( \text{CO}_2 \)” utilized by various enzymes.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Source</th>
<th>Prosthetic group</th>
<th>Substrate or product</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon monoxide dehydrogenase</td>
<td><em>Peptostreptococcus productus</em></td>
<td>Ni</td>
<td>( \text{CO}_2 )</td>
<td>this paper</td>
</tr>
<tr>
<td>Carbon monoxide dehydrogenase</td>
<td><em>Pseudomonas carboxydoverans</em></td>
<td>Mo</td>
<td>( \text{CO}_2 )</td>
<td>[27]</td>
</tr>
<tr>
<td>Formate dehydrogenase</td>
<td><em>Clostridium pasteurianum</em></td>
<td>Mo</td>
<td>( \text{CO}_2 )</td>
<td>[20, 21]</td>
</tr>
<tr>
<td></td>
<td><em>Wolinella succinogenes</em></td>
<td></td>
<td></td>
<td>[36]</td>
</tr>
<tr>
<td>Urease</td>
<td>Jack been</td>
<td>Ni</td>
<td>( \text{CO}_2 )</td>
<td>[31]</td>
</tr>
<tr>
<td>Pyruvate decarboxylase</td>
<td>Yeast</td>
<td>TPP(^b)</td>
<td>( \text{CO}_2 )</td>
<td>[31]</td>
</tr>
<tr>
<td>Pyruvate synthase(^a)</td>
<td><em>Clostridium pasteurianum</em></td>
<td>TTP</td>
<td>( \text{CO}_2 )</td>
<td>[22]</td>
</tr>
<tr>
<td>Pyruvate carboxylase</td>
<td>Yeast</td>
<td>Biotin</td>
<td>( \text{HCO}_3^- )</td>
<td>[28, 29, 32]</td>
</tr>
<tr>
<td>Propionyl-CoA carboxylase</td>
<td>Pig heart</td>
<td>Biotin</td>
<td>( \text{HCO}_3^- )</td>
<td>[29, 30]</td>
</tr>
<tr>
<td>Oxalacetate decarboxylase</td>
<td>Klebsiella</td>
<td>Biotin</td>
<td>( \text{CO}_2 )</td>
<td>[37]</td>
</tr>
<tr>
<td>Methylmalonyl-CoA decarboxylase</td>
<td>Veillonella</td>
<td>Biotin</td>
<td>( \text{CO}_2 )</td>
<td>[38]</td>
</tr>
<tr>
<td>Phosphoenolpyruvate carboxylase</td>
<td>Plants</td>
<td>Mg(^{2+})</td>
<td>( \text{HCO}_3^- )</td>
<td>[28, 29]</td>
</tr>
<tr>
<td>Phosphoenolpyruvate carboxykinase</td>
<td><em>Rhodospirillum rubrum</em></td>
<td>Fe(^{3+})</td>
<td>( \text{CO}_2 )</td>
<td>[32]</td>
</tr>
<tr>
<td>PEP carboxytransphosphorylase</td>
<td><em>Propionibacterium shermanii</em></td>
<td></td>
<td>( \text{CO}_2 )</td>
<td>[32]</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase</td>
<td>Ox heart</td>
<td>Mg(^{2+})</td>
<td>( \text{CO}_2 )</td>
<td>[26, 29]</td>
</tr>
<tr>
<td>Phosphogluconate dehydrogenase</td>
<td>Sheep liver</td>
<td>Mn(^{2+})</td>
<td>( \text{CO}_2 )</td>
<td>[29, 33]</td>
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<tr>
<td>Malic enzyme</td>
<td>Animals and plants</td>
<td></td>
<td>( \text{CO}_2 )</td>
<td>[29, 34, 35]</td>
</tr>
<tr>
<td>Ribulosebisphosphate carboxylase</td>
<td>Plants</td>
<td></td>
<td>( \text{CO}_2 )</td>
<td>[25]</td>
</tr>
</tbody>
</table>

\(^a\) Pyruvate:ferredoxin oxidoreductase.

\(^b\) Thiamine pyrophosphate.
tively carboxylated to pyruvate which is the biosynthetic precursor of most cellular compounds. The pyruvate synthase, which catalyzes the carboxylation reaction, uses CO₂ as a substrate [22]. Thus the carbon monoxide dehydrogenase pathway for the autotrophic fixation of CO₂ only involves reactions in which CO₂ is the immediate reactant. Interestingly, this is also the case when CO₂ is fixed via the Calvin cycle or the reductive tricarboxylic acid cycle [23, 24]. Ribulosebisphosphate carboxylase [25] (Calvin cycle), isocitrate dehydrogenase [26], 2-oxoglutarate:ferredoxin oxidoreductase [22], and pyruvate synthase [22] (reductive tricarboxylic acid cycle) all react with CO₂ rather than with HCO₃⁻ (Table I).

Aerobic bacteria that can oxidize CO contain a carbon monoxide dehydrogenase which differs from the enzyme in anaerobic bacteria in that it is a molybdo protein rather than a nickel protein and in that it functions only in the direction of CO oxidation. The molybdo enzyme is similar to the nickel enzyme, however, in that it also generates CO₂ as product [27] (Table I).

Enzymes reported to use HCO₃⁻ rather than CO₂ as immediate reactant are: Phosphoenolpyruvate carboxykinase [28, 29], pyruvate carboxylase [28, 29, 32], and propionyl-CoA carboxylase [29, 30] (Table I).

Acknowledgements

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