The Active Species of "CO_2" Formed by Carbon Monoxide Dehydrogenase from *Peptostreptococcus productus*

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

CO_2-Fixation, Autotrophy, Carbon Monoxide Dehydrogenase, *Peptostreptococcus productus*

Carbon monoxide dehydrogenase of anaerobic bacteria catalyzes the reversible conversion of CO to "CO_2". With the enzyme from *Peptostreptococcus productus* it is shown that CO_2 rather than HCO_3^- (H_2CO_3) is the active species of "CO_2" formed by this dehydrogenase.

Introduction

Carbon monoxide dehydrogenase (CO dehydrogenase) is found in many strictly anaerobic bacteria, including gram-positive and gram-negative eubacteria as well as archaebacteria. The enzyme, which contains nickel, is involved in several important catabolic and anabolic processes of anaerobic metabolism: (i) acetate synthesis from 2 CO_2 in acetogens, (ii) acetate oxidation to 2 CO_2 in sulfate reducers and acetogens, (iii) acetate disproportionation to CO_2 and CH_4 in methanogens, (iv) autotrophic CO_2 fixation in acetogens, methanogens and sulfate reducers; and (v) assimilation and/or dissimilation of one carbon compounds in many anaerobes. In all these pathways CO (in free or a bound form) is an intermediate or substrate, either formed or utilized by CO dehydrogenase [1–8].

In this communication the active species of "CO_2", *i.e.* CO_2 or HCO_3^-, formed in the CO dehydrogenase reaction was determined and found to be CO_2. The experiments were performed with cell extracts of *Peptostreptococcus productus* which is a mesophilic acetogenic bacterium that grows on carbon monoxide as sole energy source [9, 10]. This organism contains CO dehydrogenase in high specific activity, the enzyme is still partially active at 5 °C, and carbonic anhydrase is lacking [11]. The latter two properties are a prerequisite for the method used to determine whether CO_2 or HCO_3^- is the immediate reactant of CO dehydrogenase.

Abbreviations: When the symbol "CO_2" is used, no distinctions are made between CO_2, H_2CO_3, HCO_3^- and CO_3^{2-}.

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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 viologen were from Serva (Heidelberg, F.R.G.). Carbonic 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.). $TiCl_3$ 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 Bio-Rad Laboratories (München, F.R.G.). Gases (CO, CO_2, N_2) were obtained from Messer Griesheim (Düsseldorf, F.R.G.). *Peptostreptococcus productus* strain Marburg was from G. Diekert (Stuttgart, F.R.G.) [9, 10].

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 1×10^8

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 N_2 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 1.5×10^5 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: $\varepsilon_{508} = 740\ cm^{-1}\ M^{-1}$; $\Delta\ \varepsilon_{508}$ (ox minus red) $= 700\ cm^{-1}\ M^{-1}$).

Determination of the active species of "CO_2"

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 andydrase solution (200 U/µl). The pH was adjusted to pH 8.4 with 0.1 M CO_2-free anaerobic KOH. The mixture was continuously stirred at 1,100 rpm with a magnetic bar. The gas phase was 100% N_2. The reaction was started by the addition of 0.1−1.0 ml CO saturated H_2O at 25 °C (0.93 mM CO) [12], or − as a control − of 10−25 µl CO_2 saturated H_2O at 2 °C (70 mM CO_2) [13]. The change in pH was recorded with an electrode (N 5700A 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 "CO_2". At pH 8.4 the reduction of FMN yields $FMNH^-$ ($FMNH_2 \rightleftharpoons FMNH^- + H^+$; pKa = 6.7) [14], and H_2CO_3 is almost completely dissociated into $HCO_3^- + H^+$ (at 5 °C: app $pK_1 = 6.52$; app $pK_2 = 10.56$) [15]. At 5 °C CO_2 is only slowly hydrated to $HCO_3^- + H^+$. Thus, if CO_2 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 CO_2 hydration (reaction (**b**)). The appearance of the second proton should be speeded up in the presence of carbonic anhydrase, which catalyzes reaction (**b**). If HCO_3^{-1} 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.

$$CO + H_2O + FMN \rightarrow CO_2 + FMNH^- + H^+ \quad \textbf{(a)}$$

$$CO_2 + H_2O \rightarrow HCO_3^- + H^+ \quad \textbf{(b)}$$

$$CO + 2\,H_2O + FMN \rightarrow HCO_3^- + FMNH^- + 2H^+ \quad \textbf{(c)}$$

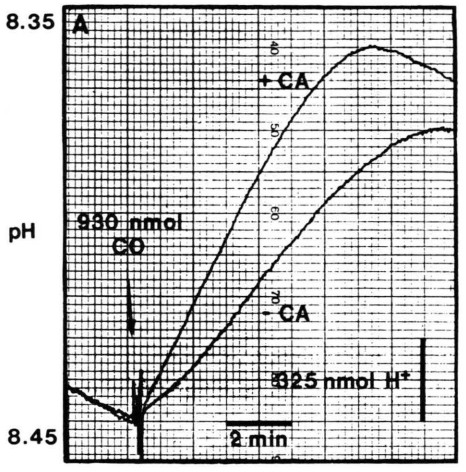
It is therefore possible to discriminiate between reaction (**a**) and (**c**) by measuring the rate of acidification in the absence and presence of carbonic anhydrase.

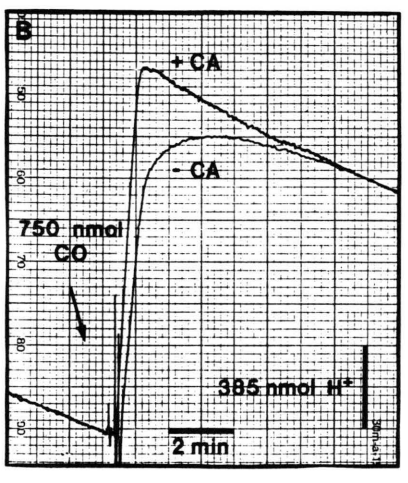
Cell extract of *Peptostreptococcus productus* catalyzed the reduction of FMN with carbon monoxide at a specific rate of 40−60 nmol/min · mg protein at 5 °C. The rate increased linearly with protein in the concentration range tested (0.1−1 mg/ml). The apparent K_m for FMN was found to be 1.5 mM. At 37 °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.

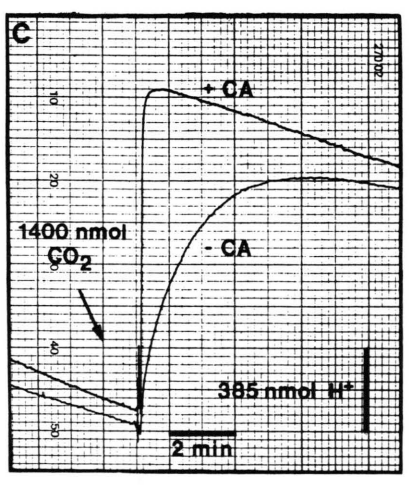
The kinetics of acidification during carbon monoxide oxidation with FMN are shown in Fig. 1 A. In the experiment the rate of H^+ formation was approximately 70 nmol H^+/min · mg and that of FMN reduction 50 nmol/min · mg. Upon addition of carbonic anhydrase, the rate increased to 100 nmol H^+/min · mg.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

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 CO_2 hydration (reaction (**b**)) increases proportionally with the increasing CO_2 concentration.

The experiment in Fig. 1 A 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 CO_2 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 CO_2 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 CO_2 rather than CO was added in the presence of carbonic anhydrase the proton to CO_2 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 N_2 saturated H_2O rather than CO saturated H_2O was added or when cell extract protein was omitted from the assay mixtures. Traces of O_2 and cyanide (1 mM) inhibited both carbon monoxide oxidation and acidification. Carbon monoxide dehydrogenase from *Peptostreptococcus productus* is known to be rapidly inactivated by O_2 and to be effectively inhibited by cyanide (K_i = 0.1 mM) [9, 10].

Fig. 1. Carbon monoxide oxidation to "CO_2" 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 CO_2 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 CO_2 in cell extracts of *Peptostreptococcus productus* was associated with an acidification of the medium. Per mol CO oxidized two mol H^+ were formed. One proton is explainable by the property of $FMNH_2$ at pH 8.4 to dissociate into $FMNH^- + H^+$ ($pK_a = 6.7$) (reaction **a**). The results indicate that the second proton comes from the hydration of CO_2 to H_2CO_3 which dissociates into $HCO_3^- + H^+$ (app. $pK_1 = 6.52$) (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 HCO_3^- (H_2CO_3) were the immediate product formed (reaction (**c**) no such lag period should occur since at 5 °C the hydration of CO_2 to H_2CO_3 rather than the dissociation into $HCO_3^- + H^+$ is slow.

The carbon monoxide dehydrogenase from anaerobic bacteria is a nickel enzyme which catalyzes both the oxidation of CO to CO_2 and the reduction to CO_2 to 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 CO_2 *via* reduced C_1-unit intermediates. Two "CO_2" fixation reactions are involved: (i) the reduction of CO_2 to formate which is further reduced to the methyl group of acetyl-CoA [19] and (ii) the reduction of CO_2 to CO which is the precursor of the carboxyl group of acetyl-CoA [2–5]. The formate dehydrogenase has early been shown to use CO_2 rather than HCO_3^- [20, 21]. We have now provided evidence that also the second CO_2 fixation step in these organisms is specific for 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 "CO_2" utilized by various enzymes.

Enzymes	Source	Prosthetic group	Substrate or product	Ref.
Carbon monoxide dehydrogenase	*Peptostreptococcus productus*	Ni	CO_2	this paper
Carbon monoxide dehydrogenase	*Pseudomonas carboxydovorans*	Mo	CO_2	[27]
Formate dehydrogenase	*Clostridium pasteurianum*	Mo	CO_2	[20, 21]
	Wolinella succinogenes			[36]
Urease	Jack been	Ni	CO_2	[31]
Pyruvate decarboxylase	Yeast	TPP[b]	CO_2	[31]
Pyruvate synthase[a]	*Clostridium pasteurianum*	TPP	CO_2	[22]
Pyruvate carboxylase	Yeast	Biotin	HCO_3^-	[28, 29, 32]
Propionyl-CoA carboxylase	Pig heart	Biotin	HCO_3^-	[29, 30]
Oxaloacetate decarboxylase	*Klebsiella*	Biotin	CO_2	[37]
Methylmalonyl-CoA decarboxylase	*Veillonella*	Biotin	CO_2	[38]
Phosphoenolpyruvate carboxylase	Plants	Mg^{2+}	HCO_3^-	[28, 29]
Phosphoenolpyruvate carboxykinase	*Rhodospirillum rubrum*	Fe^{2+}	CO_2	[32]
PEP carboxytransphosphorylase	*Propionibacterium shermanii*		CO_2	[32]
Isocitrate dehydrogenase	Ox heart	Mg^{2+}	CO_2	[26, 29]
Phosphogluconate dehydrogenase	Sheep liver	Mn^{2+}	CO_2	[29, 33]
Malic enzyme	Animals and plants		CO_2	[29, 34, 35]
Ribulosebisphosphate carboxylase	Plants		CO_2	[25]

[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 CO_2 as a substrate [22]. Thus the carbon monoxide dehydrogenase pathway for the autotrophic fixation of CO_2 only involves reactions in which CO_2 is the immediate reactant. Interestingly, this is also the case when CO_2 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 CO_2 rather than with HCO_3^- (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 CO_2 as product [27] (Table I).

Enzymes reported to use HCO_3^- rather than CO_2 as immediate reactant are: Phosphoenolpyruvate carboxylase [28, 29], pyruvate carboxylase [28, 29, 32], and propionyl-CoA carboxylase [29, 30] (Table I).

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