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Observations on the reduction of non-activated carboxylates by *Clostridium formicoaceticum* with carbon monoxide or formate and the influence of various viologens

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Abstract. Crude extracts or supernatants of broken cells of *Clostridium formicoaceticum* reduce unbranched, branched, saturated and unsaturated carboxylates at the expense of carbon monoxide to the corresponding alcohols. The presence of viologens with redox potentials varying from $E'_0 = -295$ to -650 mV decreased the rate of propionate reduction. The more the propionate reduction was diminished the more formate was formed from carbon monoxide. The lowest propionate reduction and highest formate formation was observed with methylviologen. The carbon-carbon double bond of E-2-methyl-butenoate was only hydrogenated when a viologen was present. Formate as electron donor led only in the presence of viologens to the formation of propanol from propionate. The reduction of propionate at the expense of a reduced viologen can be followed in cuvettes. With respect to propionate Michaelis Menten behavior was observed. Experiments are described which lead to the assumption that the carboxylates are reduced in a non-activated form. That would be new type of biological reduction.

Key words: Acylate reduction – *Clostridium formicoaceticum* – Carbon monoxide – Formate – Artificial mediators – Viologens

Recently, we reported on the reduction of carboxylic acids in a non-activated form to alcohols by resting cells of *Clostridium thermoaceticum*. The electron donor for these reductions was carbon monoxide or formate in the presence of artificial mediators such as various viologens or cobalt sepulchrate (Simon et al. 1987). The mediators also caused the formation of methanol (White et al. 1987). Without such mediators the reduction rate of carboxylic acids was less than 20% and not a trace of methanol could be observed.

The reduction of non-activated carboxylic acids or carboxylates seems to be a hitherto not observed enzyme reaction. Also by chemical means a reduction of carboxylates in aqueous solution is not possible. For the reduction of protons to dihydrogen a standard redox potential $E^{\circ\prime}$ of only -420 mV is necessary, wheras, for the reduction of acetate to acetaldehyde a potential of -581 mV is necessary (Loach 1976). This value should be almost the same for most other carboxylates. Since *C. formicoaceticum* carries out a homoacetate fermentation with enzymes similar to that of *C. thermoaceticum* (Andreesen et al. 1970; Diekert and Thauer 1978; Fuchs 1986) we studied the reduction of carboxylates with this clostridium, too. This seemed to be of interest because it turned out that with *C. formicoaceticum* the reduction of carboxylates is faster in the absence of viologens than in their presence. Further differences were observed in the substrate specificity of *C. thermoaceticum* and *C. formicoaceticum* for various types of carboxylic acids, in the pH dependence of the reduction and in the influence of mediators.

Materials and methods

Formate dehydrogenase from yeast (EC 1.2.1.2) was a product from Boehringer, Mannheim, FRG, methylviologen and cobalt sepulchrate were bought from Aldrich, Milwaukee, WI, USA, and benzylviologen from Sigma, St. Louis, MO, USA, the other viologens were a gift of Mr. G. Strobl. The carbon monoxide from Messer-Griesheim, Düsseldorf, FRG, was 99%.

A 5% inoculum was used to grow *Clostridium formicoaceticum* (DSM 92) on a fructose medium according to Diekert and Thauer (1978) starting with pH 8. Growth was followed by measuring the optical density at 578 nm. The cells grown in 20 l and 250 l were harvested at the end of the logarithmic phase with a continuous flow system (Szent-Györgyi and Blum) at $35000 \times g$ and with a Westfalia separator type K.A.2.86.075 at $10000 \times g$, respectively. The cell material was then collected under an atmosphere of CO_2

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Non-standard abbreviations: glc, Gas liquid chromatography; HPLC, high performance liquid chromatography; RP, reverse phase; Mediators (the figures in parenthesis of the mediators are redox potentials E'_0 in mV); CAV^{2+}: carbamoylmethylviologen, 1,1′-carbamoyl-4,4′-dipyridinium dication ($E'_0 = -296$ mV); BV^{2+}: benzylviologen, 1,1′-dibenzyl-4,4′-dipyridinium dication ($E'_0 = -360$ mV); MV: methylviologen, 1,1′-dimethyl-4,4′-dipyridinium-dication ($E'_0 = -444$ mV); $DMDQ^{2+}$: dimethyldiquat, 4,4′-dimethyl-2,2′-dipyridino-1,1′-ethylendication ($E'_0 = -514$ mV); TMV^{2+}: tetramethylviologen, 1,1′,4,4′-tetramethyl-4,4′-dipyridinium dication ($E'_0 = -550$ mV); PDQ^{2+}: propyldiquat, 2,2′-dipyridino-1,1′-propenyl dication ($E'_0 = -550$ mV); $DMPDQ^{2+}$: dimethylpropyldiquat, 4,4′-dimethyl-2,2′-dipyridino-1,1′-propenyl dication ($E'_0 = -656$ mV); PN, productivity number = mmol product (obtained by the uptake of one pair of electrons) × (biocatalyst (dry weight) kg)$^{-1} \times h^{-1}$

without using additional buffer and again centrifuged at $12{,}000 \times g$ in polycarbonate bottles of 500 ml. The supernatant was decanted, the bottles filled with nitrogen, tightly closed and stored at −16° C.

During all operations the exposure to air was omitted by working under an atmosphere of nitrogen or carbon dioxide.

Enzyme assays

For determining formate (Schaller and Triebig 1984) a suitable aliquot part of the reaction solution was added to a volume of 0.8 ml containing phosphate buffer 0.1 M pH 7.0, 1.25 mM NAD and 0.8 U formate dehydrogenase. The increase of extinction was observed at 334 nm 1 min after the start of the reaction. A calibration curve with formate solutions containing 5−100 mM formate was applied. All other assays were carried out under strict anaerobic conditions. Protein determinations were conducted according to Bradford in a modified form (Read and Northcote 1981).

The formate dehydrogenase activity in supernatants of crude extracts of *C. formicoaceticum* in dependence on the type of mediator was conducted in a total volume of 1.0 ml containing 50 mM triethanolamine maleate buffer pH 7.8, 3 mM dithioerythritol, 20 mM formate, 20 mM electron acceptor and 0.1 mg protein. The extinction increase was observed at 37° C using the following mM extinction coefficients for the cation radicals in concentrations of <0.1 mM: CAV $\varepsilon_{578} = 14.4$; BV $\varepsilon_{578} = 12.0$; MV $\varepsilon_{578} = 9.0$; DMDQ $\varepsilon_{436} = 6.1$; PDQ $\varepsilon_{492} = 3.0$; TMV $\varepsilon_{578} = 8.7$.

Photometric measurements of acylate reductase in crude extracts were conducted at 37° C. A total volume between 0.9 and 1.1 ml contained 0.3 M phosphate buffer pH 6.0, 0.1−5.0 mg protein of the supernatant of a French Press cell extract centrifuged at $40000 \times g$ for 30 min, and 0.01−1.5 mM reduced viologen. Such a system oxidizes some of the reduced viologen prior to the addition of propionate. The acylate reduction was started after 5 min by the addition of 0.5−25 mM propionate. The K_m value for propionate was determined in the presence of 0.7 mM $TMV^{+\cdot}$ at 590 nm. For the reduced form of the various viologens the following mM extinction coefficients were used: CAV $\varepsilon_{705} = 2.2$, MV $\varepsilon_{730} = 2.4$ and TMV $\varepsilon_{715} = 1.2$ and $\varepsilon_{590} = 9.7$.

In the range of 0.1−0.2 mM of $TMV^{+\cdot}$, ε_{590} turned out to be constant. One unit of enzyme activity consumes 4 µmol of reduced viologen. This definition is used because the reduction of the acylate to the aldehyde which is probably an intermediate seems to be slow as compared to the reduction of the aldehyde to the alcohol.

Reduction of carboxylic acids with carbon monoxide or formate

For standard assays wet packed cells were suspended in the 6.5-fold volume of 0.3 M phosphate buffer pH 6.0 and broken by a French Press. Aliquot parts of 3.0 ml were shaken in Warburg flasks at 37° C with 120 strokes per min under an atmosphere of carbon monoxide or with 0.3 M sodium formate under an atmosphere of nitrogen. The Warburg flasks were attached to mercury filled manometers in order to observe the time course of the change of gas pressure. In order to determine the exact consumption of carbon monoxide at the end of an experiment 0.5 ml of a 3 M sodium hydroxide solution was injected into the Warburg flask. After a short time all carbon dioxide was absorbed indicated by a constant manometer reading. One neck of the Warburg flasks was closed with a rubber stopper through which aliquot parts of the suspension were taken for analysis.

Analysis of substrates and products

Volatile alcohols and aldehydes were monitored on poly ethylene glycol 400 (0.4 × 180 cm) or 4000 (0.4 × 120 cm) columns. Volatile acids were analyzed on a column (0.4 × 250 cm) filled with chromosorb PAW 50/80 to which 20% succinate neopentyl glycolpolyester and 2% phosphoric acid was absorbed.

For non volatile substrates or products reverse HPLC on RP-18 columns was used.

Reduction of propionate by the gel filtrate of a crude extract

A crude supernatant was prepared by breaking in a French Press 2.5 g wet packed cells in 2.5 ml 0.3 M phosphate buffer pH 6.0 containing 0.2 M NaCl. After adding 0.25 mg DNase and 2.5 mg lysozyme, the supernatant was incubated 15 min at room temperature and centrifuged for 60 min at $100{,}000 \times g$. All other procedures were carried out at 4° C. In a volume of 5.0 ml 200 mg protein were applied to a column which was prepared as follows: Sephadex G 50 (34 g) was suspended in the above mentioned buffer containing additionally 1 mg/l of resazurin and 40 µM dithionite. The suspension was filled into a 75 × 2.4 cm glass column. For calibration of the column dextran blue, chymotrypsin (25,000 D), cytochrome c (12,500 D), and reduced methylviologen (310 D) were used. The column was eluted with the aforementioned buffer and the protein determined in aliquot fractions. Proteins corresponding to the molecular weight region higher than 30,000 were used in Warburg experiments applying carbon monoxide as electron donor.

Results

Clostridium formicoaceticum is able to reduce propionate and other carboxylates such as (E)-2-methylbutenoate to alcohols at the expense of carbon monoxide or formate. Results with propionate are shown in Table 1. Wheras, the reduction with carbon monoxide is retarded in the presence of artificial mediators such as viologens they are necessary for the reduction of propionate with formate. Formate as such is a poor electron donor as compared with carbon monoxide. The reduction rate observed with formate is only about 1% of that observed with carbon monoxide. In the presence of formate viologens markedly enhance the reduction rate. Depending on their redox potential the rate may be more than 50% of the values with carbon monoxide without a mediator (Table 1).

A control experiment with a crude extract of *C. formicoaceticum* under an atmosphere of nitrogen without an electron donor led to less than 1% of that amount of propanol which was observed with a carbon monoxide atmosphere.

Depending of the redox potential of the mediators the propanol formation shows a minimum at medium redox potentials. Mediators leading to low propanol formation with carbon monoxide cause the highest propanol production with formate (Table 1).

Table 1. Time course of the formation of propanol (μmol) by *Clostridium formicoaceticum* in the absence and presence of various mediators. A total volume of 3.1 ml contained 0.3 M phosphate buffer pH 6.2, crude extract of 400 mg wet packed cells (corresponds to 65 mg dry weight), 60 mM propionate and 5 mM mediator. Reactions were conducted in shaken Warburg vessels at 37°C under an atmosphere of carbon monoxide or in the presence of 0.3 M formate under an atmosphere of nitrogen

Mediator										
Time (h)	Electron-donor	None	CAV	BV	MV	DMDQ	PDQ	TMV[a]	DMPDQ	Co-sepulchrate
1	CO	45	31	27	25	26	21	46	34	37
3	CO	86	57	44	40	39	47	69	66	72
1	$HCOO^-$	0.4	12	11	26	26	16	16	3.0	0.8
3	$HCOO^-$	0.8	26	21	48	51	33	32	6.0	1.9
25	$HCOO^-$	1.4	48	38	63	70	54	—	24	6

[a] Value determined in a separate series of experiments. Propanol formation at the expense of carbon monoxide without mediator was 55 μmol after 1 h, at the expense of formate with MV 24 μmol

Nevertheless, a 300 mM phosphate buffer was used the pH did not stay constant during the reduction of propionate with carbon monoxide. In the absence of mediators there was an increase of about 0.2 pH units. In the presence of carbon monoxide and methyl viologen or DMDQ it dropped after 3 h from 6.2 to 5.4. This cannot be explained by the propanol formation since the overall reaction consumes protons:

$$RCOO^- + 2CO + H_2O + H^+ \rightarrow RCH_2OH + 2CO_2. \quad (1)$$

It turned out that by the various mediators different amounts of formate are formed.

However, the reduction of carbon dioxide originating from reaction (1) according to

$$CO_2 + 2e^- + H^+ \rightarrow HCOO^- \quad (2)$$

does consume protons, too. Therefore, the reaction which can be summarized by

$$CO + H_2O \rightarrow HCOO^- + H^+ \quad (3)$$

and which is the sum of the dehydrogenation of carbon monoxide

$$CO + H_2O \rightarrow CO_2 + 2e^- + 2H^+ \quad (4)$$

and reaction 2 must play an important role.

The situation after 18 h of propionate reduction with carbon monoxide can be seen in Fig. 1.

The influence of the viologens on the rate of the acylate reduction depends on their standard redox potential and/or on the capability of the formate dehydrogenase to accept the different viologens for the reduction of carbon dioxide. In reasonable agreement with Table 1 Fig. 1 shows that after 18 h the propanol formation is lowest in the presence of methylviologen or DMDQ. On the other hand with these mediators the formate formation is the highest. The sum of propanol and formate formation in seven cases corresponds in the range of ±7% with the consumed carbon monoxide. Exceptions are the experiments with CAV and TMV. In these cases the products account only for about 70% of the consumed carbon monoxide.

The dependence of the formate dehydrogenase activity on the various mediators tested in crude extract in the direc-

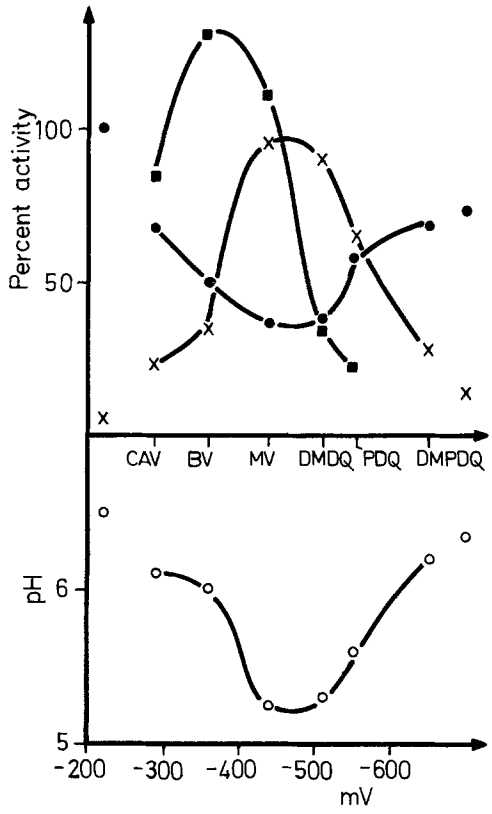

Fig. 1. Formation of propanol and formate as well as the change in pH depending on various mediators after incubation of cells for 18 h under an atmosphere of carbon monoxide as described in Table 1. One hundred percent propanol formation corresponds to 113 μmol, 100% formate formation corresponds to 315 μmol. For comparison the specific activity of formate dehydrogenase in crude extracts of *Clostridium formicoaceticum* tested with various mediators is given. One hundred percent formate dehydrogenase activity tested according to reaction (5) corresponds to 1 unit per mg protein. ●——● Propanol formation, ×——× formate formation, ○——○ change of pH of the 0.3 M phosphate buffer starting at pH 6.2, ■——■ specific activity of formate dehydrogenase tested in cuvettes using the indicated mediators. The symbols ● and × on the left hand side not connected with the curves respresent the propanol and the formate formation as well as the pH in the absence of a mediator. The symbols on the right hand side represent the corresponding values in the presence of cobalt sepulchrate

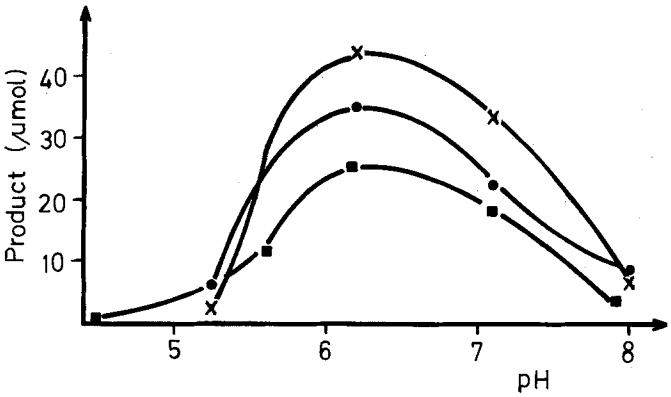

Fig. 2. Dependence of propanol formation on pH with cells of *Clostridium formicoaceticum* broken by freezing and thawing after 1 h with carbon monoxide in the absence and presence of methylviologen (5 mM) or with formate in the presence of methylviologen (1 mM) in a total volume of 3.1 ml at 37°C. In the experiments with carbon monoxide the phosphate buffer was 0.5 M. The experiments with formate (0.3 M) were conducted in 0.3 M buffer. ×——× Carbon monoxide without viologen, ●——● carbon monoxide with viologen, ■——■ formate with viologen

tion of the dehydrogenation of formate is also shown in Fig. 1.

$$HCOO^- + 2V^{2+} \rightarrow 2CO_2 + 2V^{+\cdot} + H^+. \quad (5)$$

Benzylviologen leads to the highest activity for this reaction. In a latter experiment of this kind TMV was tested and compared with MV and PDQ. It showed about the same activity as PDQ.

Also cobalt sepulchrate retards the propanol formation by about 25%. Only small amounts of formate are formed in the presence of cobalt sepulchrate (Fig. 1). With formate as electron donor it causes almost no propanol formation. In this case the redox potential does not seem to be the important factor because under the conditions employed cobalt sepulchrate has a redox potential of about −440 mV which is similar to that of methylviologen.

The various amounts of formate caused by the different mediators leads to a corresponding change of the pH value which decreases more when more formate is formed. This pH drop in turn causes a lower propionate reduction. That can be seen in Fig. 2 which shows a maximum at pH 6.2 for the propanol formation under all three conditions tested. During these measurements the change of the pH was less than 0.1 pH units. Again the reduction of the acylate at the expense of carbon monoxide is faster in the absence of viologen.

The acylate reduction can also be measured in cuvettes, following the decrease of reduced viologens using a 40,000 × *g* supernatant of cells broken in the French Press. By this approach MV, CAV and TMV were compared. A typical recording of the consumption of reduced TMV is shown in Fig. 3. Trace A shows the rate of a reaction by which reduced TMV is reoxidized before propionate was added. The rate of this background reaction is higher at the beginning and declines to a value indicated by trace A. During this time interval the concentration of $TMV^{+\cdot}$ decreases from about 0.7 to 0.2 mM. As soon as propionate is added the rate of the oxidation of reduced TMV increases strongly. By glc it is seen that in such an assay about 65%

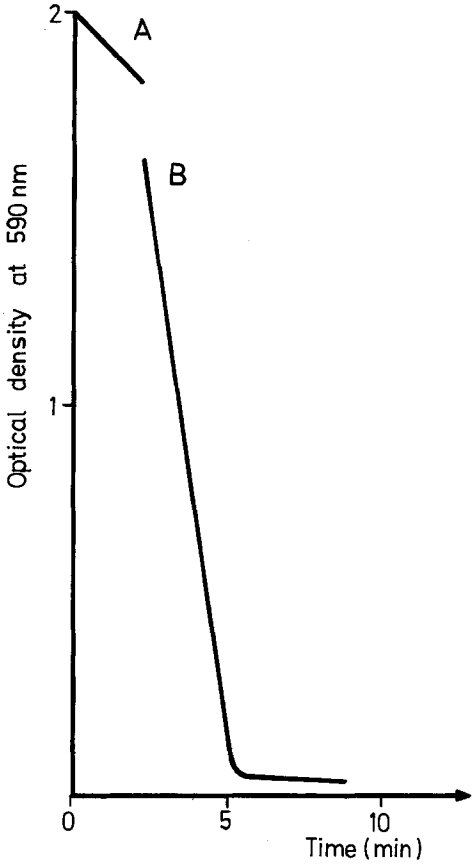

Fig. 3. Time course of the consumption of reduced TMV measured at 590 nm in a total volume of 1.0 ml of 0.3 M phosphate buffer pH 6.0 containing 0.9 mg protein of a crude extract and $TMV^{+\cdot}$ (*trace A*). After 5 min 25 mM propionate was added (*trace B*)

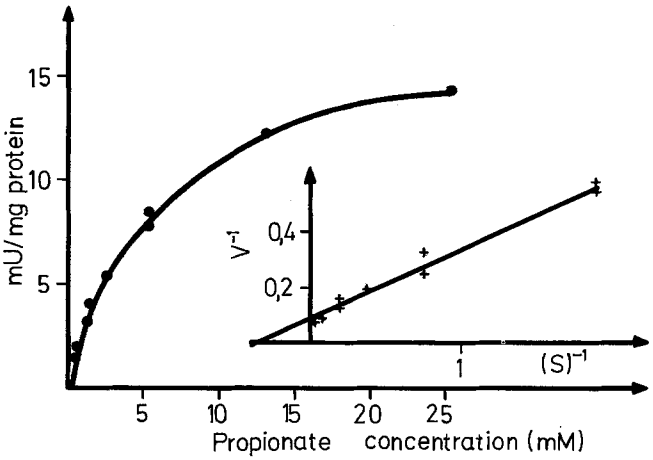

Fig. 4. Reaction rate of the oxidation of $TMV^{+\cdot}$ in dependence on propionate concentration and interrelationship according to Lineweaver and Burk

of the amount of propanol is formed which is expected by the $TMV^{+\cdot}$ consumption. This is calculated according to

$$RCOO^- + 4V^{+\cdot} + 5H^+ \rightarrow RCH_2OH + 4V^{2+} + H_2O. \quad (6)$$

In such experiments no aldehyde could be observed. However, small concentrations of aldehydes are hard to detect because they form Schiff's bases with protein.

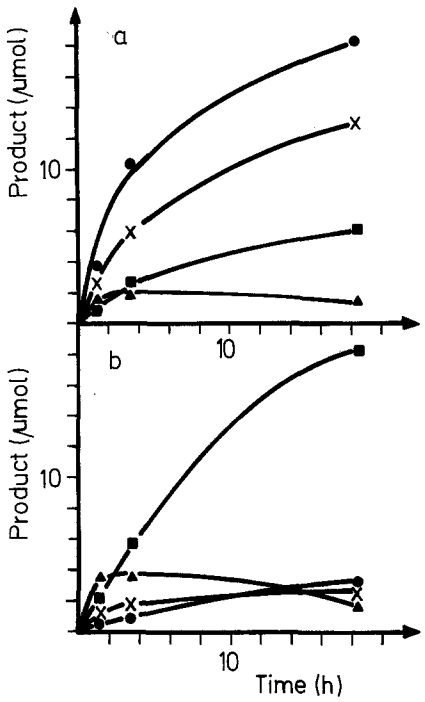

Fig. 5. Time course of the reduction of 33 mM E-2-methylbutenoate by *C. formicoaceticum* under an atmosphere of carbon monoxide with and without methylviologen in 3.1 ml 0.3 M phosphate buffer pH 6.2 at 37°C. **a** In the presence of 1.5 mM methylviologen; **b** in the absence of viologen. ×——× 2-methylbutanoate, △——△ 2-methylbutenal, ■——■ 2-methylbutenol, ●——●

In the range of 0.1 to 0.9 mg protein the rate of viologen oxidation was proportional to the amount of protein in the assay (not shown). Figure 4 presents the dependence of the reaction rate on the concentration of propionate with reduced TMV as a mediator. An apparent K_m value for propionate of about 3.5 mM can be seen.

By similar experiments the rate maximum for $TMV^{+\cdot}$ was observed at about 0.05 mM. Higher concentrations led to a smooth decrease of the rate reaching 60% for 1.5 mM $TMV^{+\cdot}$ (not shown). Surprisingly about 20 – 40 times higher concentrations of $MV^{+\cdot}$ are necessary to reveal the rate maximum. In a concentration up to 0.7 mM $CAV^{+\cdot}$ did not show a reaction.

As revealed in Fig. 5 the reduction of (E)-2-methylbutenoate with carbon monoxide leads to 2-methylbutenol, 2-methylbutanol and 2-methylbutenal as well as 2-methylbutanoate. (E)-2-Methylbutenal should be the precursor of 2-methylbutenol. The saturated aldehyde could not be observed. It may be reduced to the corresponding alcohol with a rate higher than that of the unsaturated aldehyde. Again methylviologen exerts a marked influence (Fig. 5). Without methylviologen almost no saturated acid is formed. This seems to be the reason why in this case only a small amount of 2-methylbutanol could be observed whereas, the unsaturated alcohol is the main product.

Table 2 shows that unbranched carboxylates up to a carbon number of ten are reduced. Benzoate and 2-phenylpropionate are substrates for the reduction, too. In contrast to *C. thermoaceticum* (Simon et al. 1987) dicarboxylic acids such as succinate or glutarate are not reduced by *C. formicoaceticum*.

Table 2. Rates of alcohol formation from different acylates in 1 h under standard conditions in the presence of carbon monoxide without mediator

Substrate	Product	µmol	PN
Acetate	Ethanol	49	1480
Propionate	1-Propanol	55	1670
Butyrate	1-Butanol	17	515
Valerate	1-Pentanol	10	300
Caproate	1-Hexanol	6	180
Caprylate	1-Octanol	7	212
Caprinate	1-Decanol	2.5	76
Benzoate	benzylalcohol	15	454
2-Phenylpropionate	2-Phenylpropanol	3	90
E-2-Methylbutenoate	2-Methylbutanol	3	90
E-2-Methylbutenoate	2-Methylbutenol	7	212

In order to see how much propanol can be formed by resting cells at the expense of carbon monoxide a 500 mM solution of propionate was shaken with carbon monoxide. In about 80 h 65 mg (dry weight) of cells are able to reduce 250 µmol of propionate to propanol and form an additional 25 µmol of ethanol from acetate. In 20 h already 200 µmol propanol were formed. Such experiments were carried out at 20°C. At 37°C the system does not show this long stability. The amount of acetate observable after the formation of 250 µmol of propanol and 25 µmol ethanol was about 15 µmol. On the other hand the amount of cells applied in such an experiment already contain about 22 µmol of acetate at the beginning. That means that during the reduction of altogether 275 µmol acylate to alcohol less than 20 µmol acetate are formed from endogenous material.

From the following experiment it can be concluded that membranes are not necessary for the reduction of acylates. The supernatant of a crude extract obtained after centrifugation at 100,000 × *g* was passed through a size exclusion chromatography. By this measure all molecules of a molecular weight of less than 1500 and all membranes were eliminated. The protein fraction of the molecular weight above 30,000 conducted the reduction of propionate, however, only in the presence of methylviologen. Based on protein such a fraction shows 50% of the activity of the supernatant before it was applied to the column and stored at 4°C for the length of time necessary to run the gel filtration experiment at this temperature. Compared with a freshly prepared extract the protein of the gel filtration reveals 10% activity.

Discussion

Clostridium formicoaceticum seems to catalyze the reduction of non-activated carboxylates. This hitherto only for *C. thermoaceticum* described reaction (Simon et al. 1987) is interesting because it needs probably a redox potential more negative than necessary for the reduction of protons. The question arises whether under certain conditions this reaction is of biological significance and whether a known or unknown enzyme is responsible for it. Under the applied growth conditions only 3.6 mM ethanol were found in the fermentation broth. Whereas, in agreement with Andreesen et al. (1970) an acetate concentration of 46 mM was observed. The fact that activation of the carboxylate seems not a prerequisite for its reduction seems to be well documented:

(i) Resting cells without a substrate reduce large amounts of propionate at the expense of carbon monoxide. If an endogenous substrate would be necessary to deliver an amount of energy equivalent to 1 mol of ATP per mol of reduced carboxylate 65 mg cells must contain about 13 mg of a hexose or a corresponding amount of another energy delivering compound. Thirteen milligrams hexose would form 275 µmol ATP if one assumes that 1 mol of hexose is converted to 3 mol of acetic acid leading to 4 mol ATP per mol of hexose. The carbohydrates or intermediates of the glycolytic pathway delivering this amount of ATP should lead to about 210 µmol of acetic acid if their metabolism would deliver the energy for the reduction of acylate via acyl-CoA. Less than 10% of that amount of acetate could be observed. (ii) Energy production in a membrane-dependent process driven by the oxidation of carbon monoxide is also unlikely because the supernatant of a crude extract after ultracentrifugation shows activity. Even more convincing is the experiment in which a $100{,}000 \times g$ supernatant was applied to a gel filtration column in which all low molecular weight compounds and membrane particles should be eliminated. The fraction of compounds with a molecular weight $>30{,}000$ is able to reduce propionate at the expense of carbon monoxide but only in the presence of viologen. This may be indicative for a low molecular weight factor which was eliminated but is necessary for the transfer of electrons from carbon monoxide dehydrogenase to the unknown enzyme system. Reconstitution experiments were not conducted yet because the fraction of the low molecular weight compounds were rather diluted. That carboxylate reducing enzymes exist has been shown with *C. thermoaceticum* (Simon et al. 1987). From this organism we enriched a protein almost 100-fold to a purity greater 80% catalyzing the reduction of propionate (unpublished). However, it turned out that the enzyme activities in *C. formicoaceticum* and *C. thermoaceticum* behave rather differently. Also the substrate specificity reveals marked variations. *C. formicoaceticum* does not reduce dicarboxylates whereas *C. thermoaceticum* reduces them with about the same activity as monocarboxylates (Simon et al. 1987).

The difference between both microorganisms with respect to the influence of viologens on the reduction may depend to a large extent on the different affinity of the formate dehydrogenase of *C. formicoaceticum* for the various viologens. Figure 1 shows clearly, that low formate formation leads to high propanol contents and vice versa. For the use of formate as electron donor MV and DMDQ are the best mediators (Table 1). The mediators most effective in formate formation do not show the highest rates in the dehydrogenation of formate (Fig. 1). For instance BV and DMPDQ give rise to similar formate concentrations but are rather different for the dehydrogenation of formate. The redox potential of BV may be too positive for an efficient reduction of carbon dioxide to formate. The redox potentials of DMDQ and PDQ are probably too negative for an efficient reduction by formate. The opposite interrelation between formate and propanol formation and the fact that the more negative viologens lead to a rather low formate formation may cause the effect that the amount of propanol is the same in the presence of CAV and DMPDQ, even though their redox potentials show a difference of about 350 mV. Methylviologen increases the reduction rate of propionate at the expense of carbon monoxide by *C. thermoaceticum* depending on growth conditions 2.5–10 times. The same mediator retards this reaction with *C. formicoaceticum*. However, this influence may be indirect. Reduced methylviologen may channel most of the electrons into the formation of formate.

When propionate is reduced by crude extracts of *C. formicoaceticum* at the expense of reduced viologens only i.e., without the regeneration of the viologens by carbon monoxide or formate, the reaction shows a Michaelis-Menten behavior with respect to propionate. Surprising is the big difference of the apparent K_m values for $MV^{+\cdot}$ and $TMV^{+\cdot}$. In a crude extract in which the reduced viologen is not regenerated there may be two ways by which electrons reach the acylate reductase. One via reduced viologen → natural mediator → enzyme and another leading directly from the reduced viologen to the enzyme. In this respect the two viologens could be rather different leading to rather different apparent K_m-values.

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