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Propionate metabolism in anaerobic bacteria; determination of carboxylation reactions with ^{13}C-NMR spectroscopy

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The role of carboxylation reactions in propionate metabolism was studied with in vivo high-resolution NMR in two syntrophic propionate oxidizing cocultures and compared with anaerobic propionate forming bacteria with well-established biochemical properties. The inclusion of [^{13}C]propionate and $H^{13}CO_3^-$ gave insight into the process of randomization at the level of propionate in relation to the type of the (de)carboxylating enzyme involved. *Propionibacterium* but not *Veillonella* and *Desulfobulbus* showed a propionate randomization in the absence of substrate. These differences are explained by the type of carboxylation mechanism and the energy state of the cells. Both syntrophic cocultures tested degrade propionate via the succinate pathway involving a transcarboxylase.

Introduction

In many anaerobic propionate forming and propionate degrading bacteria the succinate pathway is involved. In this route pyruvate or phospho*enol*pyruvate, oxaloacetate, malate, fumarate, succinate, succinyl-CoA, methylmalonyl-CoA and propionyl-CoA are intermediates. Fermentation of sugars and lactate with propionate as major reduced product is carried out by several types of organism, both Gram-positive and Gram-negative. In *Propionibacterium* spp. [1,2], *Veillonella alcalescens* [3], *Anaerovibrio lipolytica* [4], *Selenomonas ruminantium* [5], *Arachnia propionica* [6], *Bacteroides fragilis* [7], *Propionispira arboris* [8,9] and in *Pelobacter propionicus* [10] propionate is formed via the succinate pathway. In studies with ^{14}C- and ^{13}C-labelled compounds, evidence was also provided for the operation of the succinate pathway in syntrophic propionate oxidation [11–16]. *Desulfobulbus propionicus* is able to do both, forming and oxidizing propionate via this pathway [17,18].

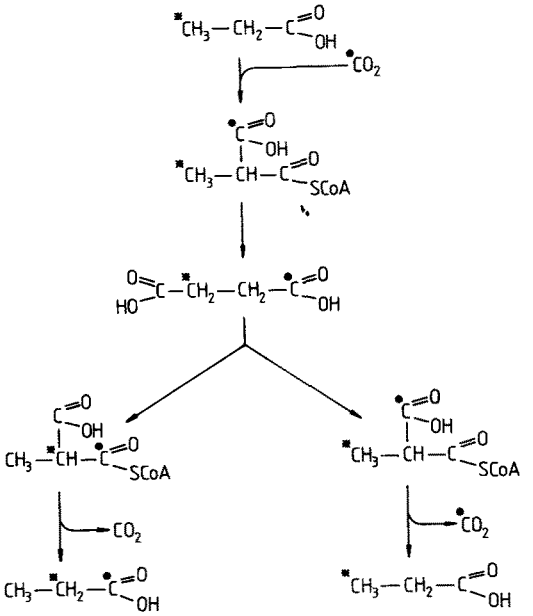

Fig. 1. Distribution of ^{13}C label from [3-^{13}C]propionate and $H^{13}CO_3^-$ during interconversion of propionate and succinate with involvement of a carboxylase and a decarboxylase. Continued interconversion also yields the C_1-C_3 propionate isotopomer. However, propionate labelled at the C-2 and C-3 position (C_2-C_3 isotopomer) is not formed. In case of a transcarboxylase the incorporated CO_2 is expected not to be labelled. In high-resolution NMR the differently labelled molecules can be distinguished.

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In the succinate pathway, (de)carboxylation reactions play an important role. One of the main differences between the different bacteria is the presence or absence of a transcarboxylase. In *Propionibacterium* spp. [19,20], *A. propionica* [6], *D. propionicus* [17,18] and *P. propionicus* [10] the endergonic carboxylation of pyruvate to oxaloacetate is linked to the exergonic decarboxylation of methylmalonyl-CoA via a transcarboxylase. Contrary, in *Veillonella alcalescens* and *Propionigenium modestum*, the decarboxylation energy is conserved in a membrane-potential [21,22]. The membrane-bound decarboxylases translocate Na^+ ions across the cell membrane and the Na^+-gradient formed drives ATP-synthesis via a sodium-ATPase [23].

By in vivo ^{13}C-NMR it was shown for a methanogenic coculture that propionate is equilibrated with succinate during propionate degradation [14]. Such a process, which was already recognized in propionate-forming bacteria [20,24,25], leads to the formation of [2-^{13}C]propionate from [3-^{13}C]propionate, and vice versa. In Fig. 1 the distribution of ^{13}C-label from [3-^{13}C]propionate and $H^{13}CO_3^-$ during the interconversion of propionate and succinate is shown [26]. Because with in vivo ^{13}C-NMR it is possibile to distinguish between the differently labelled molecules, the presence of labelled propionate and bicarbonate allowed us to investigate the carboxylation reactions involved in the various propionate metabolisms.

Materials and Methods

Organisms and cultivation

Two propionate-oxidizing cocultures were used: a methanogenic coculture whose properties and cultivation were described before [12,14] and a sulphidogenic biculture (DSM 2805) of *Syntrophobacter wolinii* and a *Desulfovibrio* species [27]. Cultivation was done in a medium containing (in g/l unless otherwise stated): sodium propionate 1.9; Na_2SO_4, 2.9; $Na_2HPO_4 \cdot 2H_2O$, 0.53; KH_2PO_4, 0.41; NH_4Cl, 0.3; NaCl, 0.3; $CaCl_2 \cdot 2H_2O$, 0.11; $MgCl_2 \cdot 6H_2O$, 0.1; $NaHCO_3$, 4; $Na_2S \cdot 9H_2O$, 0.24; yeast extract, 0.2; 1 ml of a 10-fold concentrated trace element solution described by Pfennig and Lippert [28]; 1 ml of the vitamin solution described by Stams et al. [29]; 1 ml of a mixture of Na_2MoO_4, Na_2WoO_4 and Na_2SeO_3 (each 0.1 mM) in 10 mM NaOH. Sodium lactate (1 mM) was added to stimulate the sulfate reducing bacterium. *Desulfobulbus propionicus* (DSM 2032) was the gift of D.R. Kremer, Department of Microbiology, University of Groningen, The Netherlands. The organism was cultivated in a medium containing (in g/l): sodium propionate 1.9; Na_2SO_4, 2.9; $Na_2HPO_4 \cdot 2H_2O$, 0.53; KH_2PO_4, 0.41; NH_4Cl, 0.54; $CaCl_2 \cdot 2H_2O$, 0.15; $MgCl_2 \cdot 6H_2O$, 0.4; KCl, 0.3; $NaHCO_3$, 2.4; $Na_2S \cdot 9H_2O$, 0.48; yeast extract, 0.2. Trace elements and vitamins were the same as described above. *Propionibacterium freudenreichii* (DSM 20270) was cultivated in a medium containing (in g/l): sodium lactate, 5; caseine peptone, tryptic digest, 5; yeast extract, 2,5; $NaHCO_3$, 1.7; sodium propionate, 1.9. The pH was adjusted to 7.0–7.2. The medium for the cultivation of *Veillonella alcalescens* (DSM 1399) contained (in g/l): sodium lactate, 5; yeast extract, 2.5; sodium thioglycollate, 0,5; $NaHCO_3$, 1.7; sodium propionate, 1.9. The pH was adjusted to 7.0. *Megasphaerea elsdenii* (DSM 20460) was kindly provided by M.C. Snoek, Department of Biochemistry, Agricultural University of Wageningen, The Netherlands. The organism was grown in a medium containing (in g/l): sodium lactate, 10; yeast extract, 6; sodium thioglycollate, 1; dissolved in tap water. The pH of the medium was adjusted to 7.4.

The two cocultures and *V. alcalescens* were grown at 37°C; the other organisms were cultivated at 30°C. Except for *M. elsdenii*, which was cultivated under N_2, the gas phase consisted of N_2/CO_2 (80 : 20).

Preparation for NMR

About 1.5 liters of the cultures was centrifuged anaerobically at 21 500 × *g*. All the following procedures were done in an anaerobic glove box with a gas phase of N_2/H_2 (96 : 4). The cell pellets of the methanogenic and sulphidogenic coculture and of *D. propionicus* were resuspended in their respective media without propionate but with $H^{13}CO_3^-$. Since FeS causes significant peak-broadening in the NMR-spectra, the suspensions were allowed to stand for 2 to 7 h, during which the FeS present in the medium could settle. After transfer to an NMR tube of the supernatant containing the cells, 10% (v/v) ^2H_2O was added to provide a lock signal. The NMR tube was closed with an overseal-stopper and the gas phase was changed to N_2 (1.2 atm.). At time zero, 50 mM [3-^{13}C]propionate was added. The final densities of the cultures were $3.6 \cdot 10^{10}$, $4.0 \cdot 10^{10}$ and $2.3 \cdot 10^{10}$ cells/ml, respectively.

Centrifuged cells of *P. freudenreichii*, *V. alcalescens* and *M. elsdenii* were resuspended in their respective media but without lactate, propionate and bicarbonate. The cell suspensions, containing $3.7 \cdot 10^{10}$, $2.5 \cdot 10^{10}$ and $1.1 \cdot 10^{10}$ cells/ml, respectively, were transferred to NMR tubes after addition of ^2H_2O (10% v/v), and the gas phase was changed to N_2 (1.2 atm.). A 10-fold concentrated mixture of $H^{13}CO_3^-$ and sodium lactate was added to obtain concentrations of 20 mM and 2 mM, respectively. The formation of ^{13}C-labelled compounds was followed 45 (3 × 15) min by high-resolution NMR. [3-^{13}C]Propionate was added to a concentration of 20 mM and recording of NMR-spectra was continued for at least 14 h. Then, lactate was added to a concentration of 20 mM and the tubes were incubated further outside the NMR apparatus. After prolonged incubation the cultures were analyzed again by NMR for 1 h.

High-resolution NMR

^{13}C-NMR spectra were obtained at 75.46 MHz on a Bruker CXP-300 NMR spectrometer operating in the Fourier-transform mode, using 10 mm o.d. NMR tubes. The Waltz pulse sequence was employed to decouple protons and the measuring temperature was maintained at about 30°C or 37°C, depending on the organism tested. NMR spectra of cells were collected by sequential storing of 1 h periods (1800 transients) on disc in 8000 data points, using a 45° pulse angle (pulse duration 9 μs) and a pulse interval of 2 s. The chemical shift belonging to the C-3 of propionate (11.1 ppm) was used as internal standard. Since the spectra were recorded under rapid pulsing conditions, they must be corrected for differences in relaxation behaviour and build-up of nuclear Overhauser enhancement (NOE) between the various atomic nuclei, in order to make quantitavive comparison between the labelled compounds possible. The appropiate factors that were assumed to reasonably account for this, were determined from the natural-abundance ^{13}C-NMR spectrum of an equimolar (40 mM) solution of propionate, acetate and succinate in medium as used for the methanogenic coculture, and recorded under identical conditions as used for the cell suspensions [14]. Because CO_2 (and indirectly HCO_3^-) and CH_4 escape partially into the gas phase, their observed areas could not quantitatively be related to the other compounds.

Chemicals

Sodium [3-^{13}C]propionate (90% enriched) and $NaH^{13}CO_3$ (99% enriched) were purchased from Amersham, U.K. Yeast extract and casein tryptic digest were obtained from BBL, Cockeysville, U.S.A. and bioMérieux, Charbonnières-les-Bains, France, respectively. Other chemicals were at least of analytical grade. Gases were obtained from Hoek Loos, Schiedam, The Netherlands.

Results

Propionate oxidizing cultures

Incubation of the methanogenic coculture resulted in a complete degradation of the substrate within 5 h (Fig. 2). The acetate produced was labelled at both carbon atoms. Immediately after addition of [3-^{13}C]propionate, randomization over the C-3 and C-2 carbons of propionate was observed. Succinate, which was previously shown to be an intermediate in propionate oxidation [14], was not detected. Label from preadded bicarbonate was incorporated at the C-1 carbon position of propionate. The observed C-1 resonances were com-

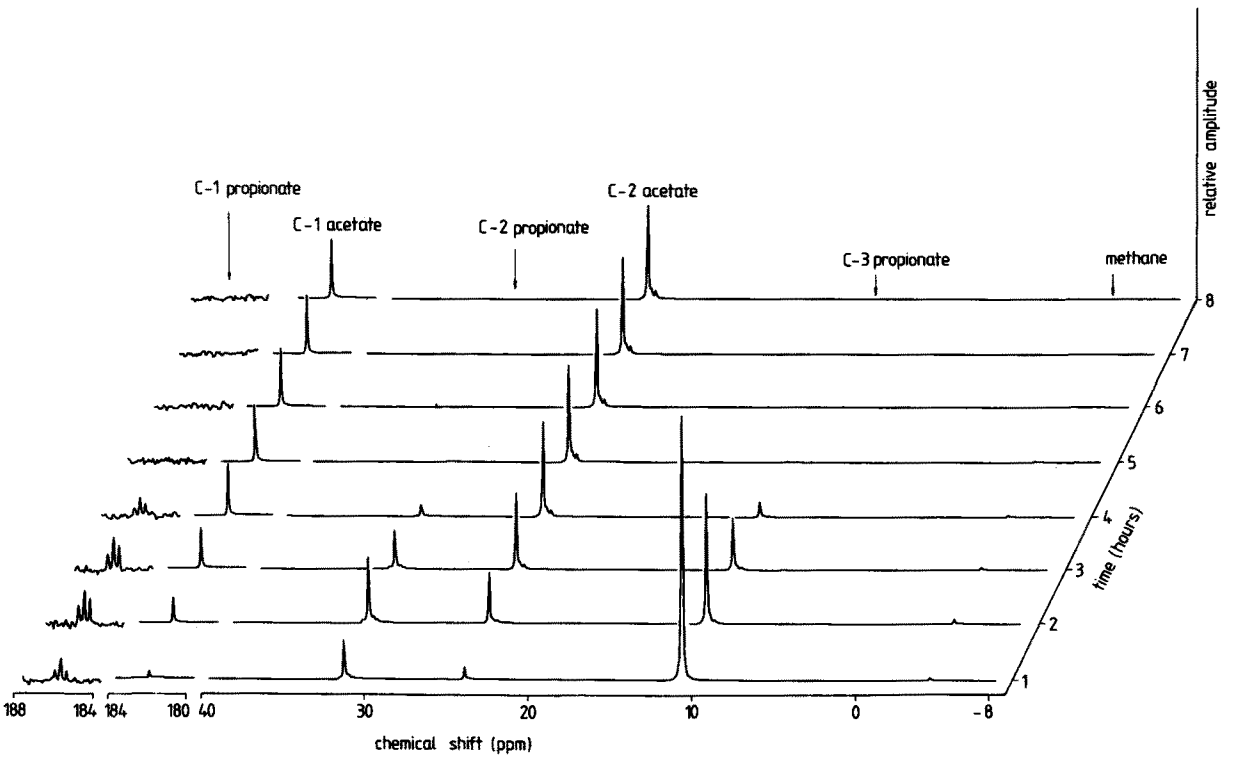

Fig. 2. Time-course of the label incorporation as measured by ^{13}C-NMR during the first 8 h of incubation of the propionate oxidizing methanogenic coculture with [3-^{13}C]propionate and $H^{13}CO_3^-$. The resonances belonging to the C-1 of propionate, composed of a singlet (central) and a doublet, are enlarged by a factor of 20 vertically. $^{13}CO_2$ and $H^{13}CO_3^-$ are not shown. Chemical shifts (ppm): $^{13}CH_3CH_2COO^-$, 11.1, $CH_3^{13}CH_2COO^-$, 31.7; $CH_3CH_2^{13}COO$, 185.6; $^{13}CH_3COO^-$, 24.3; $CH_3^{13}COO$, 182.2; $^{13}CH_4$, −3.7.

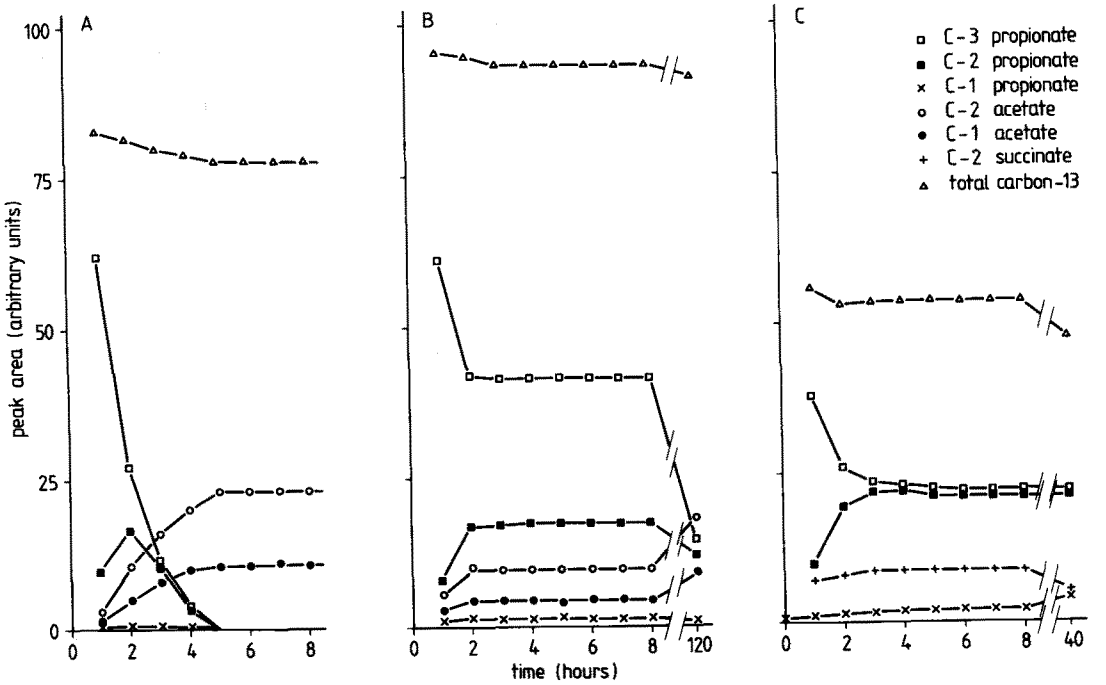

Fig. 3. Time-course during the first 8 h of the various ^{13}C-resonances after addition of [3-^{13}C]propionate and $H^{13}CO_3$ to: (A) the methanogenic coculture; (B) *D. propionicus* in the presence of 20 mM sulphate. After 15 h, extra sulphate (25 mM) was added and incubation was continued for another 5 days; (C) *P. freudenreichii* in the absence of lactate. After 15 h, lactate (20 mM) was added and incubation was continued for another 25 h. The resonances are not corrected for differences in relaxation behaviour and NOE build-up. The upper traces represent the sum of the areas of all nuclei expressed in units of [3-^{13}C]propionate (see text). $^{13}CO_2$, $H^{13}CO_3^-$ and $^{13}CH_4$ are not shown and are not included in the total ^{13}C-label.

posed of a singlet and a doublet, corresponding to the single- and double-labelled propionate isotopomers $^{13}C_1$-$^{12}C_2$ and $^{13}C_1$-$^{13}C_2$, respectively. In the 1st h, the contribution of the doublet was relatively small due to the initially higher concentration of C-3 labelled and unlabelled propionate (10% is not enriched) in contrast to C-2 labelled propionate. The presence of $H^{13}CO_3^-$ also resulted in the formation of $^{13}CH_4$, produced by the hydrogenotrophic methanogens. The decrease of this resonance with time can be accounted for by escape of the methane into the gas phase.

Fig. 3A shows the time course of the various observed ^{13}C-resonances by plotting their areas. After being corrected for differences in relaxation behaviour and NOE build-up as described in Materials and Methods, quantitative comparison between labelled compounds could be made, which was done in a relative rather than an absolute sense.

In the methanogenic coculture propionate was completely randomized over the C-3 and C-2 atoms within the first 3 h. Although the peak area of C-1-labelled propionate is very low, the amount of this compound after 3 h is 17% of the amount of C-2-labelled propionate. In the 4th h this percentage increased to 31%, probably due to the continued interconversion of propionate and succinate. Both carbon atoms in the ultimate breakdown product acetate here labelled to an equal extent.

The sulphidogenic coculture degraded propionate very slowly; after 9 days only 30% (15 mM) of the substrate had been oxidized to acetate. The ^{13}C label in the acetate was equally distributed over both C atoms, indicating that *S. wolinii* also degrades propionate via the succinate pathway. Randomization of label over the C-3 and C-2 position of propionate had occurred to a degree of 80% in the same period. C-1-labelled propionate was present after 1 h and its amount increased only 2-fold upon further incubation. After 9 days, label present at the C-1 carbon of propionate was 8.7% compared to the label at the C-2 carbon of propionate.

D. propionicus (Fig. 3B) converted propionate stoichiometrically to acetate with concomitant reduction of sulphate. Net degradation of propionate and randomization of ^{13}C-label over the C-3 and C-2 carbons started immediately after the addition of the substrate. Incorporation at the C-1 position of propionate (singlet + doublet) occurred as well. After 3 h the randomization and the acetate formation stopped. It can be calculated from Fig. 3B that at this time the sulphate must have been depleted. Addition of extra sulphate (25 mM) after 14 h resulted in a further decrease of the total propionate pool, an increase of the acetate pool (labelled equally at both carbon atoms) and an almost complete randomization (96%) at the level of propionate. After 5 days of incubation not all the propionate had disappeared, most likely because of the high sulphide con-

centration. Label at the C-1 position was 19% compared to label at the C-2 carbon of propionate.

Propionate-forming cultures

Incubation of *P. freudenreichii* in the presence of lactate (2 mM) and $H^{13}CO_3^-$ (without labelled propionate) resulted in incorporation of label at the C-1 resonance position of propionate, succinate and to some extent of acetate. All resonances observed were composed exclusively of singlets (data not shown).

Immediately after addition of [3-^{13}C]propionate to the culture, label was incorporated into the C-2 carbon of propionate and also into the C-2/C-3 carbons of succinate (Fig. 3C). The randomization of propionate had been completed within 3 h of incubation. After this time, the degree of incorporation of ^{13}C-label into the C-3 and C-2 carbon position in propionate remained constant. The amount of C-1 propionate (singlet + doublet) increased linearly during the whole experiment. This indicates that the interconversion of propionate and succinate continued even in the absence of the substrate lactate. After 3 h, C-1-labelled propionate was 18% of the amount of C-2 propionate. This percentage increased to 54% after prolonged incubation.

Addition of 20 mM lactate (after 15 h) and further incubation for another 25 h led to a decrease of C-2-labelled succinate; most likely because the label was diluted by unlabelled succinate. Because the level of C-3 and C-2 propionate did not decrease (to the same extent) in this period, this indicates that both compounds were not in equilibrium anymore.

Unfortunately, spectral resolution was not good enough to quantify the integrals belonging to the resonances of the C-1 carbons of succinate and acetate. From the spectra it can be deduced that both compounds were present during the whole experiment and in amounts of less than 50% of the label incorporated in C-1 propionate.

In the experiment with *P. freudenreichii*, only 85% of the ^{13}C-label (originating from [3-^{13}C]propionate) was recovered after prolonged incubation. In all other experiments at least 93% was recovered.

V. alcalescens incorporated $H^{13}CO_3^-$ in the presence of 2 mM lactate, leading to small amounts of C-1 propionate and C-4 oxaloacetate within 15 min. No increase of C-1 propionate was observed in the next 30 min, indicating that no lactate was present anymore. Even 15 h of incubation in the presence of [3-^{13}C]labelled propionate led to a level of C-2 propionate which was hardly detectable. From this it is clear that propionate and succinate are not equilibrated in *V. alcalescens* in the absence of substrate. After the addition of [3-^{13}C]propionate, an increasing amount of C-4 oxaloacetate was formed (Fig. 4), due to carboxylation of pyruvate. From the spectra it was deduced that the amount of this compound did not exceed 3% of the C-3 propionate pool. Incubation for 20 h in the presence of 20 mM lactate resulted in a 7-fold increase of the amount of C-1 propionate. This corresponds to a

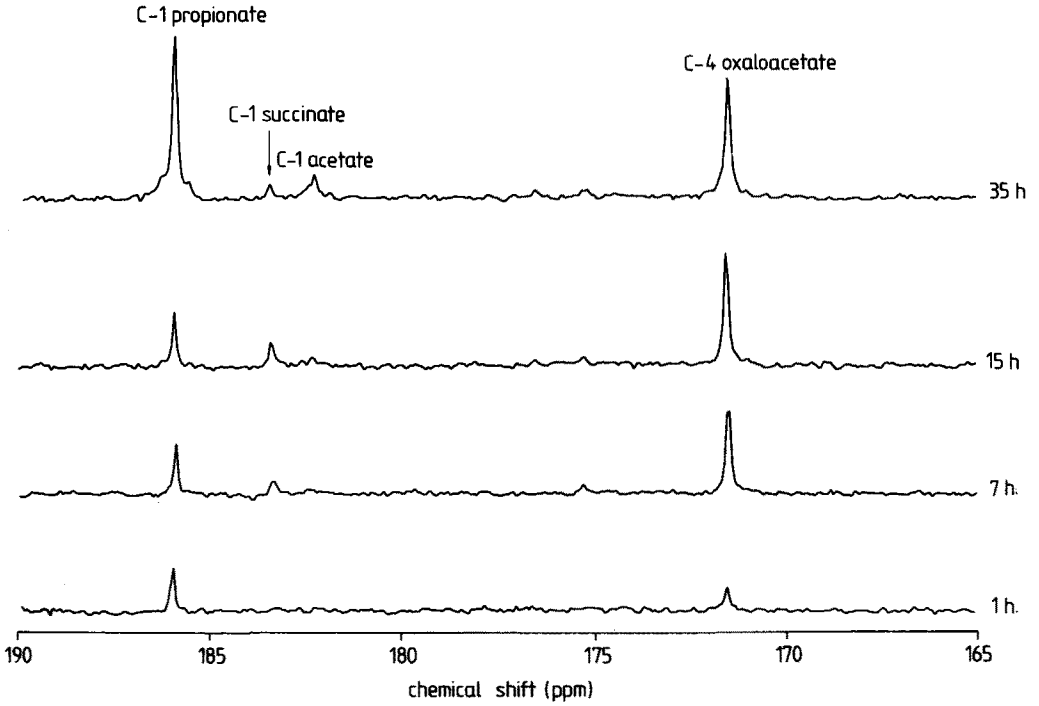

Fig. 4. Time-course of the incorporation of ^{13}C-label in carboxyl groups as measured in NMR-spectra during incubation of *V. alcalescens* in the presence of $H^{13}CO_3^-$ and [3-^{13}C]propionate. Between 15 and 35 h of incubation lactate was present as substrate (see text). Chemical shifts (ppm): $^-OO^{13}CCH_2COCOO^-$, 171.9; $CH_3^{13}COO^-$, 182.2; $^-OOCCH_2CH_2^{13}COO^-$, 183.2; $CH_3CH_2^{13}COO^-$, 185.6.

conversion of 14 mM lactate to propionate. Randomization of label in propionate was still only minor; 10% of the label was found at the C-2 carbon of propionate. Some label was found in the C-1 and C-2 carbon atoms of succinate and acetate. However, their amounts were always less than 50% of the label incorporated in C-1 propionate.

M. elsdenii did not incorporate any ^{13}C label at the C-2 position of propionate during 4 days of incubation. This is not unexpected, because this organism uses the non-randomizing acrylate pathway for the conversion of lactate to propionate [30].

Discussion

In this study, the involvement of the (de)carboxylation reactions in propionate metabolism was investigated. The inclusion of $H^{13}CO_3^-$ in the in vivo NMR experiments gave insight into the process of randomization at the level of propionate in relation to the type of (de)carboxylating enzyme involved.

In *D. propionicus* and *P. freudenreichii* the decarboxylation of methylmalonyl-CoA is coupled to the carboxylation of pyruvate via a transcarboxylase [17,19], and therefore incorporation of label at the C-1 position of propionate is not expected (Fig. 1). However, during interconversion of propionate and succinate in these organisms, ^{13}C-label was incorporated at the carboxyl group of propionate (and succinate). This may be explained in three different ways: (1) The transcarboxylases exchange to some extent CO_2 with the environment. (2) Label from $H^{13}CO_3^-/^{13}CO_2$ is first incorporated into the C-4 carbon of oxaloacetate by anaplerotic enzymes and then transferred to propionyl-CoA. *P. freudenreichii* contains phospho*enol*pyruvate carboxytransphosphorylase [24,31], whereas malic enzyme activity was found in *D. propionicus* [17]. (3) The C-4 labelled oxaloacetate formed by anaplerotic enzymes is reduced to C-4(= C-1)-labelled succinate. Which of these possibilities contribute(s) significantly to the incorporation of label into the C-1 of propionate is not clear from our experiments.

At the time at which complete randomization over the C-3 and C-2 atoms in propionate was achieved, the label at the C-1 position of propionate was 19% and 18% for *D. propionicus* and *P. freudenrichii*, respectively, compared to the other carbon atoms of propionate. This indicates that comparable reactions are involved in this process.

In *V. alcalescens* the amount of label at the C-1 position of propionate was during the whole experiment 1.5–2-times higher than at the C-2 position. Because in this organism $^{13}CO_2$ is incorporated into oxaloacetate by means of pyruvate carboxylase [32], all succinate formed will be labelled at the C-1 carbon. Half of the subsequent conversions to propionate yields C-1-labelled propionate (Fig. 1). C-2-labelled propionate is formed during interconversion of [3-^{13}C]propionate and succinate. However, because of the involvement of a decarboxylase and a (different) carboxylase during this interconversion [33], equal amounts of C-1-labelled propionate are formed at the same time (Fig. 1). Therefore, labelling at the C-1 carbon always will be higher than labelling at the C-2 carbon of propionate if the organism is incubated in the presence of [3-^{13}C]propionate and $H^{13}CO_3^-$.

In the methanogenic coculture, after complete randomization, label at the C-1 position was 17% compared to the C-2 position of propionate. In the sulphidogenic coculture this percentage was 8.7% at the point where 80% randomization had occurred. Because of the similarity with *D. propionicus* and *P. freudenreichii* and the discrepancy with *V. alcalescens*, we conclude that both syntrophic propionate-oxidizing organisms degrade propionate via the succinate pathway using a transcarboxylase. This conclusion was recently supported by enzyme activity measurements in cell-free extracts [34]. Similar percentages as described above for incorporation or loss of label at or from the C-1 position of propionate were previously found for *Propionibacterium* sp. [20,24,25], in an anaerobic digestor and a continuous culture fermenter [35].

From the incorporation of label at the C-1 carbon of propionate a possible involvement of the randomizing α-OH-glutarate pathway, as discussed by Wegener et al. [36], clearly can be excluded. Theoretically, succinate might also be formed by a condensation of propionyl-CoA and glyoxylate with subsequent oxidation to succinic-semialdehyde-CoA [37]. Such a pathway could lead to label at the C-2 carbon of propionate, but not to label incorporation at the C-1 of propionate. Instead, glyoxylate labelled at the carboxyl group would be produced. In the five cultures with the succinate pathway, double (C-2 + C-1)-labelled propionate was formed. This is a strong indication that methylmalonyl-CoA is an intermediate in all of them.

The interconversion of propionate and succinate gives to some extent information about the mechanism of energy conservation. In the propionate-oxidizing organism in the methanogenic coculture, *S. wolinii*, *D. propionicus* and *P. freudenreichii*, a transcarboxylase is present. In these organisms incorporation at the C-2 position of propionate occurred to a great extent. *D. propionicus* did not degrade propionate when sulphate was depleted. Moreover, the interconversion of propionate and succinate stopped. Although no net energy is involved in transcarboxylation [19], activation of propionate and succinate requires catalytic amounts of ATP in this organism [17]. Thus, because of other ATP-requiring reactions in the cell, interconversion will terminate if no energy is generated. In *P. freudenreichii* a succinyl-CoA : propionate transferase is present [2],

therefore the activation of both compounds is not dependent on the ATP pool. This explains the continued incorporation of ^{13}C label at the C-1 carbon of propionate in the absence of substrate. *V. alcalescens* showed only minor formation of C-2-labelled propionate even in the presence of an energy substrate. This indicates that the Na^+-gradient across the cell membrane is easily dissipated and is in this respect less efficient than a transcarboxylase. Because only a few organisms were tested, it remains unknown whether significant interconversion of propionate and succinate is restricted to propionate degrading and propionate forming bacteria having a transcarboxylase.

In *P. freudenreichii* only 85% of the label (originating from [3-^{13}C]propionate) was recovered after incubation in the presence of substrate. This was due exclusively to a decrease in the amount of C-2-labelled succinate (Fig. 3C). Formation of $^{13}CO_2$ from [^{13}C]succinate via the citrate cycle in conjunction with the transcarboxylase cycle and/or a pathway involving the glyoxylate enzymes, as well as the citrate and transcarboxylase cycle enzymes, would explain the loss of visible label [25,38]. Another possibility is the incorporation of label into relatively immobile compounds which are beyond detection in high-resolution NMR. The fact that the amount of labelled propionate (C-3 + C-2) did not decrease to the same extent, indicates that a labelled and an unlabelled propionate pool existed and that the former was not in equilibrium with succinate. Different pools of propionate and succinate were previously discussed for *Propionibacterium arabinosum* [39].

This study clearly shows the great value of the application of in vivo NMR in biological systems. Besides information about biochemical pathways involved in propionate metabolism, the use of a second labelled compound gave strong evidence for the presence of some important enzymes. Even in complex systems (mixed cultures) this technique offers the possibility to obtain detailed information about biochemical pathways which are operative.

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