

Life by a new decarboxylation-dependent energy conservation mechanism with Na^+ as coupling ion

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We report here a new mode of ATP synthesis in living cells. The anaerobic bacterium *Propionigenium modestum* gains its total energy for growth from the conversion of succinate to propionate according to: succinate + H_2O → propionate + HCO_3^- ($\Delta G^{0'}$ = -20.6 kJ/mol). The small free energy change of this reaction does not allow a substrate-linked phosphorylation mechanism, and no electron transport phosphorylation takes place. Succinate was degraded by cell-free extracts to propionate and CO_2 via succinyl-CoA, methylmalonyl-CoA and propionyl-CoA. This pathway involves a membrane-bound methylmalonyl-CoA decarboxylase which couples the exergonic decarboxylation with a Na^+ ion transport across the membrane. The organism also contained a membrane-bound ATPase which was specifically activated by Na^+ ions and catalyzed the transport of Na^+ ions into inverted bacterial vesicles upon ATP hydrolysis. The transport was abolished by monensin but not by the uncoupler carbonylcyanide-*p*-trifluoromethoxy phenylhydrazone. Isolated membrane vesicles catalyzed the synthesis of ATP from ADP and inorganic phosphate when malonyl-CoA was decarboxylated and malonyl-CoA synthesis from acetyl-CoA when ATP was hydrolyzed. These syntheses were sensitive to monensin which indicates that Na^+ functions as the coupling ion. We conclude from these results that ATP synthesis in *P. modestum* is driven by a Na^+ ion gradient which is generated upon decarboxylation of methylmalonyl-CoA.

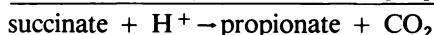
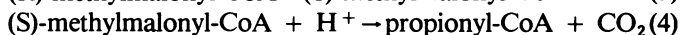
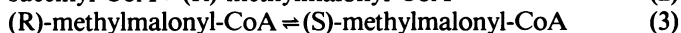
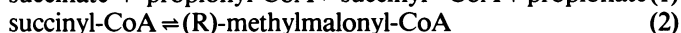
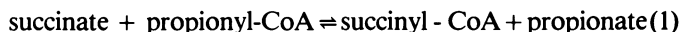
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Introduction

Energy is converted into the biologically useful form of ATP either by phosphoryl group transfer to ADP from a phosphoryl compound with high group transfer potential (substrate level phosphorylation) or by a membrane-bound ATP synthase with concomitant consumption of an electrochemical proton gradient ($\Delta\mu\text{H}^+$) (electron transport phosphorylation) (Mitchell, 1968). Light or chemical redox processes usually provide the energy for $\Delta\mu\text{H}^+$ generation. Neither of these ATP generating systems appears to operate in *Propionigenium modestum* (Schink and Pfennig, 1982), an anaerobic bacterium which grows from the fermentation of succinate to propionate and CO_2 . The small free energy change of this decarboxylation reaction ($\Delta G^{0'}$ = -20.6 kJ/mol) does not allow substrate-linked phosphoryl group transfer (Thauer *et al.*, 1977) and no redox reactions occur which could drive electron transport phosphorylation.

Energy conservation in this organism could therefore be based on the recently discovered conversion of decarboxyl-

ation energy into a Na^+ ion gradient (Dimroth, 1980, 1982a, 1982b). A degradation of succinate to propionate and CO_2 similar to that in *P. modestum* also occurs in the strict anaerobe *Veillonella alcalescens* as part of the fermentation of lactate to acetate and propionate (De Vries *et al.*, 1977; Galivan and Allen, 1967). The reaction sequence of succinate degradation is shown below:



The enzymes participating in this pathway are: succinate propionyl-CoA : CoA transferase (eqn. 1), methylmalonyl-CoA mutase (eqn. 2), methylmalonyl-CoA racemase (eqn. 3), and methylmalonyl-CoA decarboxylase (eqn. 4). Since methylmalonyl-CoA decarboxylase from *V. alcalescens* acts as a Na^+ pump (Hilpert and Dimroth, 1982) energy is conserved during the degradation of succinate to propionate and CO_2 which could explain the growth of *P. modestum* on succinate. We show here that the central energy conserving step in this organism is the conversion of the energy of methylmalonyl-CoA decarboxylation into a Na^+ gradient which in turn drives ATP synthesis via a Na^+ -activated ATPase. This type of decarboxylation-dependent ATP synthesis is new and basically different from all ATP generating phosphorylation mechanisms known so far.

Results

Generation of a Na^+ gradient during succinate degradation

The fermentation of succinate in *P. modestum* was assumed to proceed by the same reactions as succinate degradation in *V. alcalescens* (eqn. 1–4 in the Introduction). As shown in Figure 1, crude extracts of *P. modestum* were capable of catalyzing the decarboxylation of succinate to propionate. It was essential to add catalytic amounts of propionyl-CoA to initiate succinate degradation in agreement with the anticipated reaction scheme (cf. eqn. 1). The initial activity of propionate formation was 0.26 U/mg protein at 50 mM succinate. Similar activities were found when individual enzymes of the succinate degradation pathway were assayed separately (Table I). These results demonstrate the decarboxylation of succinate in *P. modestum* by the reactions given in eqn. 1–4. The decarboxylation of malonate to acetate was at least two orders of magnitude slower than that of succinate, and malonyl-CoA was a very poor substrate for the CoA transferase in catalyzing propionyl-CoA formation (cf. eqn. 1). These results are in accord with the inability of *P. modestum* to grow on malonate (Schink and Pfennig, 1981).

After separation of membrane particles and cytoplasm by high-speed centrifugation, the CoA transferase and methylmalonyl-CoA mutase were found to be soluble, but most methylmalonyl-CoA decarboxylase was in the particulate

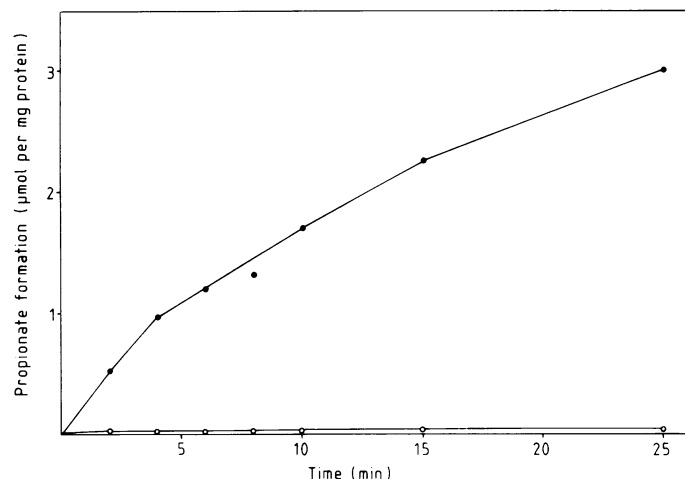


Fig. 1. Kinetics of propionate formation from succinate as catalyzed by a cell-free extract of *P. modestum*. The experiments were performed as described under Materials and methods in the presence (●) and absence (○) of propionyl-CoA.

Table I. Activities of enzymes of the succinate degradation pathway in different cell fractions

Enzyme	Soluble fraction		Particulate fraction	
	U	U/mg	U	U/mg
Methylmalonyl-CoA decarboxylase	0.2	0.03	8.7	0.25
Succinyl-CoA propionate: CoA transferase	27.5	0.47	0	0
Methylmalonyl-CoA mutase	21	0.36	n.d.	

n.d. = not determined.

The extract was prepared from 2 g wet packed cells with buffer C and fractionated by centrifugation as described under Materials and methods.

Table II. Effect of avidin treatment on the activity of methylmalonyl-CoA decarboxylase

Additions	Methylmalonyl-CoA decarboxylase %
Control	100
Avidin	0
Avidin saturated with biotin	108

Vesicles prepared in buffer A (0.36 mg protein, 0.7 U/mg methylmalonyl-CoA decarboxylase) were incubated for 15 min at 0°C with 0.1 mg avidin or with 0.1 mg avidin which had been pre-treated with 0.1 mg biotin. The activities were determined with 1/10 of the incubation mixtures.

fraction. The localization of the decarboxylase is therefore the same as in *V. alcalescens* (Hilpert and Dimroth, 1983). Other analogous properties are the complete inhibition of the enzyme by avidin but not by avidin saturated with biotin (Table II), and the specific activation by Na⁺ ions (Figure 2). The activity increased with increasing Na⁺ concentrations up to ~1 mM, but at concentrations >5 mM the enzyme was significantly inhibited. These results suggested that methylmalonyl-CoA decarboxylase of *P. modestum* was a biotin enzyme acting as a Na⁺ pump. Sodium ion transport was determined from the amount of radioactivity taken up into inverted vesicles of *P. modestum* upon incubation with ²²NaCl. Sodium ions were rapidly accumulated inside these

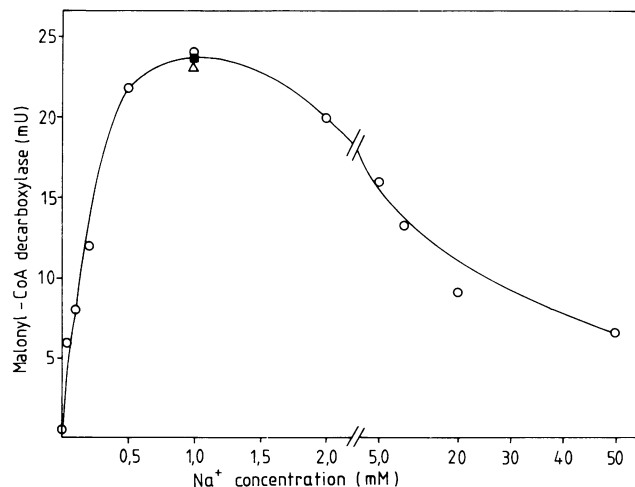


Fig. 2. Activation of methylmalonyl-CoA decarboxylase by Na⁺ ions. Methylmalonyl-CoA decarboxylase was solubilized from the isolated membranes (prepared with buffer C) with 2% octylglucoside and its activity was determined as described under Materials and methods with 0.08 mM malonyl-CoA and the concentrations of Na⁺ indicated. Na⁺ was added as NaCl (○), Na₂SO₄ (△) or Na₂CO₃ (■).

vesicles in the presence of methylmalonyl-CoA or malonyl-CoA, which is an alternative substrate, whereas significantly less Na⁺ was taken up in the absence of substrate or in the presence of the Na⁺-translocating ionophore monensin (Figure 3). The uncoupler carbonyl cyanide-*p*-trifluoromethoxy phenylhydrazone had no significant effect on Na⁺ uptake which indicates that generation of a proton gradient is not the primary event of the Na⁺ transport. Malonyl-CoA was completely decarboxylated in <1 min by the high decarboxylase activity of the vesicles. After cessation of Na⁺ pumping, the accumulated Na⁺ was not retained inside the vesicles, probably due to leakiness of the membranes. These transport kinetics are very similar to the results of Na⁺ transport with membrane vesicles from *V. alcalescens* (Hilpert and Dimroth, 1983).

Na⁺ gradient as driving force for ATP synthesis

The sodium ion gradient established upon decarboxylation of methylmalonyl-CoA is the only form of energy gained during succinate degradation to propionate and therefore must drive ATP synthesis to provide the cells with energy for growth. Crude cell extracts contained a considerable amount of ATPase, part of which was soluble and part bound to the membrane. The membrane-bound ATPase could be solubilized with 2% octylglucoside. Its activity was specifically dependent on the presence of Na⁺ ions (Figure 4) with no difference in the activation profile irrespective of whether the Na⁺ was added as chloride, sulfate or carbonate. Little activity was found at the endogenous Na⁺ concentration of 36 µM, and with 5 mM Na⁺ the activity increased 24-fold to 0.3 U/mg protein which is in the range of the other enzymes of energy metabolism in *P. modestum*. Extrapolation of the curve to zero Na⁺ concentration indicated a small amount of Na⁺-independent ATPase activity. This may be a specific property of this enzyme or may result from the presence of low quantities of Na⁺-independent ATPase. Also noteworthy is the sigmoidal shape of the activation curve which is indicative of cooperativity.

The results shown in Figure 5 indicate that addition of ATP to the vesicles resulted in a rapid accumulation of Na⁺

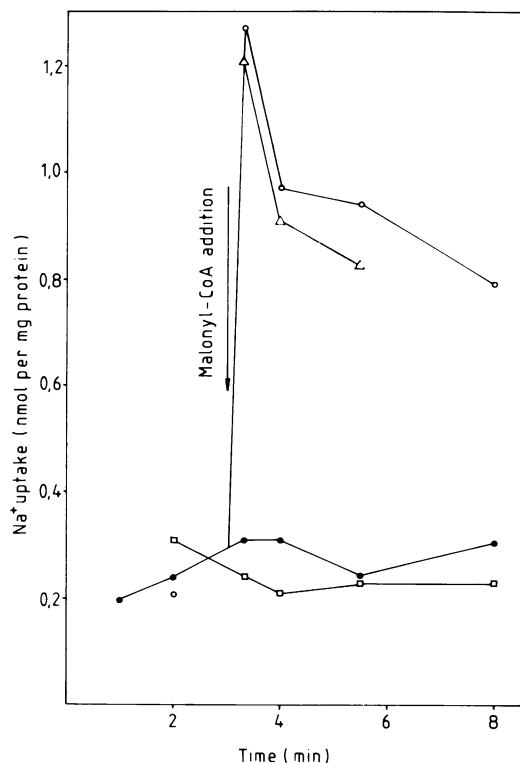


Fig. 3. Kinetics of $^{22}\text{Na}^+$ uptake into membrane vesicles of *P. modestum*. The incubation mixture is described under Materials and methods. The transport was initiated by adding 1.2 mM malonyl-CoA (lithium salt) at the arrow (○). The other experiments were performed without malonyl-CoA addition (●), with 30 μM monensin (□), and with 40 μM carbonylcyanide-*p*-trifluoromethoxy phenylhydrazone (△).

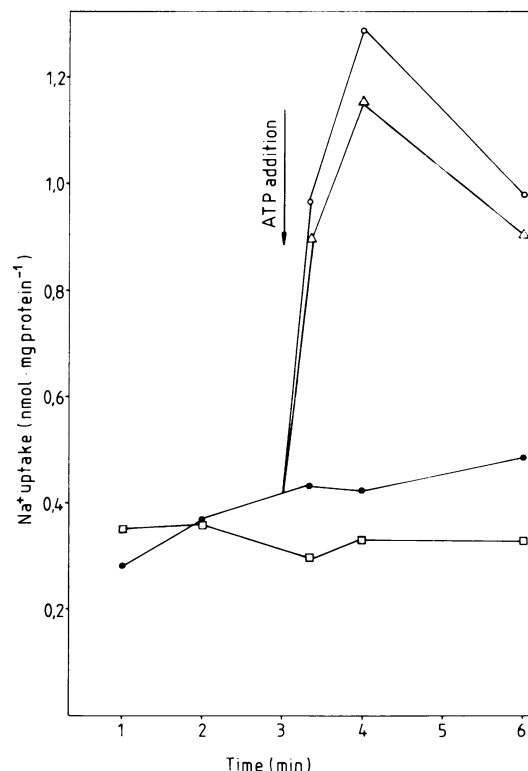


Fig. 5. ATP-dependent uptake of $^{22}\text{Na}^+$ into vesicles of *P. modestum*. The transport was determined as described under Materials and methods. ATP was added at the arrow (○). The other experiments contained 50 μM carbonylcyanide-*p*-trifluoromethoxy phenylhydrazone (△), 50 μM monensin (□), no ATP (●).

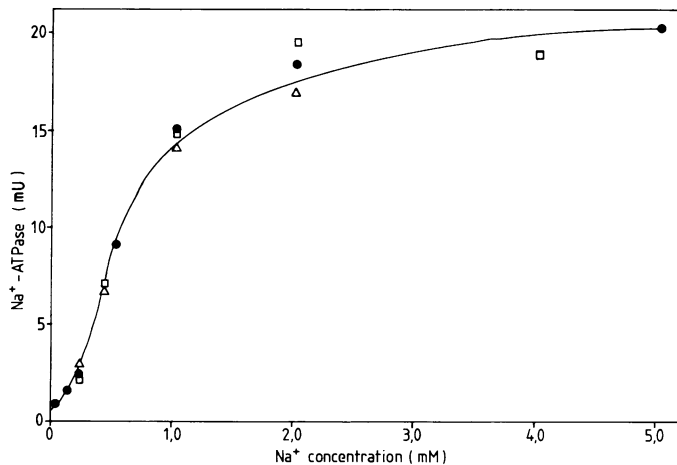


Fig. 4. Activation of the membrane-bound ATPase by Na^+ ions. The Na^+ salts were added as NaCl (●), Na_2SO_4 (□), and Na_2CO_3 (△) and the activities were determined as described under Materials and methods. The ATPase used was a soluble membrane extract, prepared with 2% octylglucoside.

ions to yield internal Na^+ concentrations which were about the same as when the transport was catalyzed by methylmalonyl-CoA decarboxylase (cf. Figure 3). The ATP-driven Na^+ transport was abolished by monensin but was not significantly affected by carbonylcyanide-*p*-trifluoromethoxy phenylhydrazone. These results indicate that the hydrolysis of ATP by the Na^+ -activated ATPase is coupled to the transport of Na^+ ions.

Table III. ATP synthesis by membrane vesicles of *P. modestum*

Additions and omissions	ATP formed nmol/mg protein/5 min
Complete	0.85 (5)
Monensin (50 μM) added	0.04 (5)
Malonyl-CoA omitted	0.07 (1)
ADP omitted	0.10 (1)

The measurements were performed as described under Materials and methods with the additions and omissions indicated. The number of independent experiments is given in the brackets and the mean values are recorded.

The ATP-dependent transport of Na^+ ions is the expected reversion of physiological ATP synthesis in this organism. When intact vesicles were incubated with malonyl-CoA, ADP and inorganic phosphate, ATP was in fact synthesized (Table III). This ATP synthesis was dissipated by monensin which indicates that the Na^+ gradient established upon decarboxylation of malonyl-CoA is required to make ATP. This is the most meaningful control to indicate membrane-linked ATP synthesis, since soluble enzymes (phosphotransacetylase and acetate kinase) can form ATP from ADP, phosphate and acetyl-CoA, which arises from the decarboxylation or malonyl-CoA. We have therefore included citrate synthase and oxaloacetate as an acetyl-CoA trapping system to reduce the soluble ATP synthesis as far as possible.

Recent studies in our laboratory have shown that malonyl-CoA decarboxylation by methylmalonyl-CoA decarboxylase can be reversed if a Na^+ gradient of proper direction and

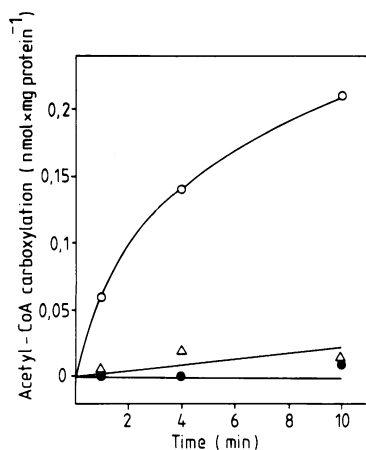
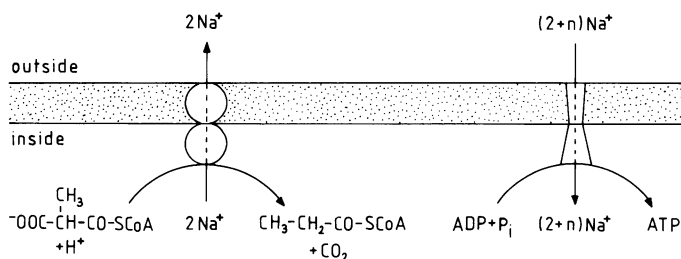


Fig. 6. Carboxylation of acetyl-CoA by membrane vesicles of *P. modestum*. Complete reaction mixture (○), complete reaction mixture plus 50 μM monensin (△), complete reaction mixture plus 2% Triton X-100 (●). For details see Materials and methods.



Scheme 1. Hypothetical mechanism of energy conservation in *P. modestum*.

magnitude is applied to reconstituted proteoliposomes (unpublished results). Acetyl-CoA should therefore be carboxylated by *P. modestum* membrane vesicles after a Na^+ ion gradient was established by ATP hydrolysis. The results shown in Figure 6 are in agreement with this expectation. Intact vesicles capable of generating a Na^+ ion gradient upon ATP hydrolysis catalyzed the carboxylation of acetyl-CoA, but no acetyl-CoA carboxylation occurred after disruption of the membrane integrity with Triton X-100 or in presence of monensin. In summary, these results indicate that the decarboxylation of methylmalonyl-CoA is linked in a reversible manner to the phosphorylation of ADP through a circuit of Na^+ ions over the membrane.

Discussion

We conclude from the results presented here that energy metabolism in *P. modestum* proceeds by the four enzyme reactions given in eqn. 1–4. The central energy converting steps are catalyzed by methylmalonyl-CoA decarboxylase, which builds up a Na^+ ion gradient over the cytoplasmic membrane, and the Na^+ -dependent ATPase which uses this Na^+ gradient to drive ATP synthesis (Scheme 1). These conclusions are supported by several lines of independent evidence. (i) The bacteria grow by the fermentation of succinate to propionate and CO_2 , and cell-free extracts catalyze the degradation of succinate via succinyl-CoA, methylmalonyl-CoA and propionyl-CoA which allows neither substrate nor electron transport phosphorylations. (ii) The extracts contain a methylmalonyl-CoA decarboxylase and an ATPase which are both membrane-bound and specifically activated by Na^+

ions. (iii) Membrane vesicles catalyze the transport of Na^+ ions upon decarboxylation of methylmalonyl-CoA or upon ATP hydrolysis. The transport by either of these substrates is sensitive to the Na^+ carrier monensin but not to the uncoupler carbonyl cyanide-*p*-trifluoromethoxy phenylhydrazine. (iv) The vesicles catalyze both monensin-sensitive ATP synthesis by malonyl-CoA decarboxylation and acetyl-CoA carboxylation by ATP hydrolysis.

The rates of these transport-coupled syntheses (0.06–0.17 nmol/min/mg protein) appear to be far too low if compared with malonyl-CoA decarboxylation (780 nmol/min/mg protein) and ATP hydrolysis (100 nmol/min/mg protein) as determined with the same vesicles. One should keep in mind, however, that the transport of Na^+ ions is highly uncoupled from the chemical reactions (Dimroth, 1982b; Hilpert and Dimroth, 1983). A rough estimate of the transport rate from the results shown in Figure 4 yields a value of ~ 1.5 nmol/min/mg protein which is not unreasonably high in comparison with the rates of ATP or malonyl-CoA syntheses.

The Na^+ pump methylmalonyl-CoA decarboxylase was detected in *V. alcalescens* (Hilpert and Dimroth, 1982) which performs succinate degradation to propionate (eqn. 1–4) as part of the lactate fermentation pathway, but is unable to grow on succinate (Schink and Pfennig, 1982). Probably, the Na^+ -activated ATPase is lacking in this bacterium. Other sodium transport decarboxylases are oxaloacetate decarboxylase of *Klebsiella aerogenes* (Dimroth, 1980, 1982a, 1982b) and glutaconyl-CoA decarboxylase of *Acidaminococcus fermentans* (Buckel and Semmler, 1982). The decarboxylation-dependent energy transductions are distinct from all other membrane-linked energy conservation mechanisms since no electron transport chains are involved and since Na^+ ions and not protons are functioning as coupling ions.

Na^+ ion gradients across membranes are of utmost importance for almost every living cell and the importance of Na^+ for a great variety of transport processes is also well known (Lanyi, 1979). However, the direct participation of Na^+ in a membrane-linked phosphorylation mechanism, as described here, is without precedent. Protons are therefore not the only coupling ions in biological energy conservations and it may be adequate to consider still other cations and anions as mediators of membrane-linked energy conservation mechanisms. The preference of Na^+ ions over protons in decarboxylation-dependent energy conservations is not at all clear. If energy-rich ion gradients have to be maintained in an alkaline environment, Na^+ ions are certainly of advantage. Dissipation of ΔpH by the large amounts of CO_2 formed in decarboxylation reactions may be another reason for the specific involvement of Na^+ ions in these reactions (Hilpert and Dimroth, 1983). Generally, primary active Na^+ transport in bacteria appears to be rare. Other examples are an ATP-driven Na^+ transport in *Streptococcus faecalis* (Heefner and Harold, 1982) and the respiratory Na^+ pump of *Vibrio alginolyticus* (Tokuda and Unemoto, 1982) which may be responsible under certain conditions for cation transport (Tokuda and Unemoto, 1983) or motility (Chernyak *et al.*, 1983).

The energy liberated upon decarboxylation of 1 mol methylmalonyl-CoA (-27.6 kJ/mol) is not sufficient to support the synthesis of a total mol of ATP. A substrate-linked phosphorylation mechanism is therefore not applicable for energy conservation in *P. modestum*. However, with the Na^+ coupling mechanism, ATP can be synthesized by consuming the

decarboxylation energy of >1 mol methylmalonyl-CoA if the two membrane-linked processes are coupled to different stoichiometries of Na⁺ ions. Methylmalonyl-CoA decarboxylase of *V. alcalescens* pumps 2 mol Na⁺ ions through the membrane per mol methylmalonyl-CoA (Hilpert, 1983) generating a sodium motive force ($\Delta\mu_{\text{Na}^+}$) of ~110 mV (Hilpert, 1983). According to $\Delta G^{\circ} = -nF\Delta E$ this is equivalent to -21.2 kJ. This amount of energy is not sufficient to drive ATP synthesis. However, if 3 or 4 mol Na⁺ ions were traversing the membrane at the given $\Delta\mu_{\text{Na}^+}$, the available energy would increase to -31.8 kJ and -42.4 kJ, respectively, which could energetically balance the synthesis of 1 mol ATP from ADP and inorganic phosphate. These results indicate that the smallest quantum of biologically useful energy is not the free energy of ATP hydrolysis but that of an ion which forms a gradient over a membrane.

The mechanism of ATP synthesis in *P. modestum* has significance for our understanding of the life of a number of other microbes which grow from fermentations with a free energy change insufficient to allow the synthesis of a total mol of ATP per fermentation cycle. Examples for such fermentations are the anaerobic decarboxylation of oxalate to formate (Dawson *et al.*, 1980) and the formation of methane from acetate (Mah *et al.*, 1978; Zehnder *et al.*, 1980). A number of organisms besides *P. modestum* conserve decarboxylation energy by converting it into Na⁺ ion gradients (Dimroth, 1980; Hilpert and Dimroth, 1982; Buckel and Semmler, 1982), but none of these depends exclusively on this kind of energy conservation. It is not even known whether these bacteria which perform classical substrate-linked or electron transport phosphorylations are actually able to utilize the decarboxylation-derived Na⁺ gradients for ATP synthesis. *P. modestum* is therefore the first known organism which gains its total energy for life from a decarboxylation reaction.

Materials and methods

The sources of chemicals were the following: enzymes and nucleotides (Boehringer Mannheim), radiochemicals (NEN), Dowex (Bio-Rad). All other chemicals were of analytical grade.

Enzymes

Acetate kinase (EC 2.7.2.1); ATPase (EC 3.6.1.3); citrate synthase (EC 4.1.3.7); fatty acid synthetase (EC 2.3.1.38, EC 2.3.1.39, EC 2.3.1.41, EC 1.1.1.100, EC 4.2.1.58, EC 1.3.1.10); glutaconyl-CoA decarboxylase (EC 4.1.1.-); hexokinase (EC 2.7.1.1.); lactate dehydrogenase (EC 1.1.1.27); methylmalonyl-CoA decarboxylase (EC 4.1.1.41); methylmalonyl-CoA mutase (EC 5.4.99.2); methylmalonyl-CoA racemase (EC 5.1.99.1); oxaloacetate decarboxylase (EC 4.1.1.3); pyruvate kinase (EC 2.7.1.40); succinyl-CoA propionate: CoA transferase (EC 2.8.3.-).

Growth conditions for *Propionigenium modestum*

The conditions for growing *P. modestum* were the same as described (Schink and Pfennig, 1982). For mass production, cells were grown in a fermentor with 20 l growth medium containing per liter: 2.75 g KH₂PO₄, 0.5 g NH₄Cl, 20 g NaCl, 3.0 g MgCl₂ × 6 H₂O, 0.15 g CaCl₂ × 2 H₂O, 0.23 g Na₂S × 9 H₂O, 16.2 g disodium succinate, 1 g yeast extract, 2 mg FeSO₄ × 7 H₂O, 70 µg ZnCl₂, 6 µg H₂BO₃, 100 µg MnCl₂ × 4 H₂O, 36 µg Na₂MoO₄, 3 µg CuSO₄ × 5 H₂O, 224 µg CoSO₄ × 7 H₂O and 26 µg NiSO₄ × 6 H₂O. The pH was adjusted to 7.0 with NaOH. After inoculation with 10% of a culture of *P. modestum*, the bacteria were grown under anaerobic conditions at 30°C with gentle stirring for 20 h. They were collected by centrifugation and washed with 20 mM potassium phosphate buffer, pH 7.0 containing 15 mM MgCl₂ and 300 mM NaCl.

Preparation of cell-free extract, membrane vesicles and membrane extract

The cell-free extract was prepared with buffers containing 1 mM dithioerythritol, 1 mM MgCl₂ and either 250 mM Tris-HCl, pH 7.0 (buffer A), or 50 mM Tris-HCl, pH 7.0 (buffer B), or 50 mM potassium phosphate, pH 7.0 (buffer C). *P. modestum* cells (1 g wet weight) and traces of deoxyribo-

nuclease were suspended in 2.5 ml of the extraction buffer and passed three times through a French press at 6000 p.s.i. (41 MPa). Unbroken cells and large debris were removed by centrifugation at 25 000 g for 15 min. The cell-free extract was fractionated into a particulate and a soluble fraction by high-speed centrifugation (150 000 g, 40 min). The particulate fraction containing the membrane vesicles was carefully homogenized with 0.5 ml extraction buffer and used for the transport experiments. Buffers A and B were usually used for the preparation of methylmalonyl-CoA decarboxylase and buffer C for ATPase preparations to obtain optimized activities of the respective enzyme. Methylmalonyl-CoA decarboxylase and the Na⁺-activated ATPase were extracted from the membranes by incubation with 2% octylglucoside and separated from insoluble material by centrifugation at 150 000 g for 40 min. These extracts were used for determining Na⁺ dependencies.

Determination of enzyme activities

Methylmalonyl-CoA decarboxylase was determined with malonyl-CoA as substrate as described (Hilpert and Dimroth, 1983). As shown with a partially purified enzyme, the activity determined with malonyl-CoA was ~4 times lower than that determined with methylmalonyl-CoA. Crude extracts, however, contain methylmalonyl-CoA mutase which competes for methylmalonyl-CoA but not for malonyl-CoA as substrate. In these extracts the activity had therefore to be determined with malonyl-CoA.

Succinyl-CoA propionate: CoA transferase was determined in cuvettes containing in 1.0 ml at 25°C: 10 mM sodium arsenate buffer, pH 7.0, 50 mM KCl, 20 U phosphotransacetylase, 0.09 mM succinyl-CoA and the enzyme (~70 mU). After determining the decrease of thioester absorbance at 232 nm ($\Delta E_1/\text{min}$), the CoA transfer was initiated by adding 1 mM propionate. The new rate ($\Delta E_2/\text{min}$) diminished by $\Delta E_1/\text{min}$ was used to calculate the CoA transferase activity. Since methylmalonyl-CoA decarboxylase in combination with methylmalonyl-CoA mutase and methylmalonyl-CoA racemase severely interferes with the determination of the CoA transferase, the decarboxylase was inhibited by avidin treatment (100 µl of the soluble cell fraction containing 1.5 mg protein were incubated with 50 µg avidin for 20 min at 0°C) prior to the activity determination.

Methylmalonyl-CoA mutase was determined as described above for the CoA transferase but by replacing the succinyl-CoA with methylmalonyl-CoA. The activity found by this coupled assay yields a minimal value, since the mutase may not be rate determining.

The Na⁺-activated ATPase was determined with an ATP generating system. The cuvette contained in 1.0 ml at 25°C: 50 mM Tris-HCl buffer, pH 8.0, 2.5 mM MgCl₂, 5 mM NaCl, 2.5 mM ATP, 3 mM phosphoenolpyruvate, 0.2 mM NADH, 50 mM KCl, 10 U pyruvate kinase, 40 U lactate dehydrogenase and enzyme. The rate of NADH oxidation was recorded at 340 nm and corrected for NADH oxidation in the absence of ATP. To determine activation of the ATPase by Na⁺ ions, all anions were added as potassium salts except for NADH which was the cyclohexylammonium salt. The enzymes were dialyzed against 50 mM potassium phosphate, pH 7.0 of low Na⁺ content and all solutions were kept in plastic vessels to reduce the Na⁺ content as far as possible. The endogenous Na⁺ content of the reaction mixture was determined by atomic absorption spectroscopy and the amount of Na⁺ added was corrected by this value.

Conversion of succinate to propionate by a cell-free extract

The incubation mixture contained in 5.0 ml at 25°C: 50 mM Tris-HCl buffer, pH 7.0, 1 mM dithioerythritol, 1 mM MgCl₂, the cell extract prepared with buffer B (26 mg protein), 50 mM succinate (disodium salt) and 0.1 mM propionyl-CoA. Samples (0.7 ml) were acidified after appropriate incubation periods with 70 µl 1 M H₂SO₄, the denatured protein was removed by centrifugation and the propionate content was determined by gas chromatography (Schink and Pfennig, 1982).

Sodium transport assay

The uptake of ²²Na⁺ into membrane vesicles was determined in incubation mixtures containing in a total volume of 0.6 ml at 25°C: 250 mM Tris-HCl buffer, pH 7.0, 1 mM dithioerythritol, 1 mM MgCl₂, 0.7 mM ²²NaCl (720 c.p.m./nmol), vesicles prepared in buffer A (10.2 mg protein) and 1.2 mM malonyl-CoA or 1.1 mM ATP to initiate the uptake reaction. The amount of ²²Na⁺ uptake was determined in samples (0.1 ml) from the amount of radioactivity passing through Dowex 50, K⁺ columns as described (Dimroth, 1982b).

ATP synthesis

The incubation mixtures contained in 0.2 ml at 25°C: 125 mM Tris-H₂SO₄ buffer, pH 7.0, 0.5 mM Na₂SO₄, 0.5 mM MgSO₄, 0.5 mM dithioerythritol, 5 mM ADP, 100 mM glucose, 5 U hexokinase, 5 U citrate synthase, 5 mM oxaloacetate, 2 mM [³²P]phosphate (1170 c.p.m./nmol) and vesicles prepared with buffer A containing 0.7 mg protein. Immediately after ADP addition, malonyl-CoA (1.0 µmol in 0.03 ml) was continuously added over a period of 4.5 min with a peristaltic pump. After 5 min, the reaction was terminated

with 1 ml ammonium molybdate in H₂SO₄ (Kagawa and Sone, 1979) and the amount of ATP synthesized was determined from the radioactivity incorporated into glucose-6-phosphate as described (Kagawa and Sone, 1979). Citrate synthase and oxaloacetate were present in the reaction mixture to prevent ATP synthesis from acetyl-CoA (generated from the decarboxylation of malonyl-CoA) as catalyzed by phosphotransacetylase and acetate kinase. The continuous addition of malonyl-CoA assured a continuous Na⁺ pumping during the entire incubation period.

Carboxylation of acetyl-CoA

The incubation mixtures contained in 1.05 ml at 25°C: 200 mM Tris-H₂SO₄ buffer, pH 7.0, 0.8 mM Na₂SO₄, 0.8 mM MgSO₄, 0.8 mM dithioerythritol, 1 mM ATP, 5 mM phosphoenolpyruvate, 2 U pyruvate kinase, 10 mM KHCO₃, 0.6 mM NADPH, 0.24 U fatty acid synthetase, 0.25 mM [1-¹⁴C]acetyl-CoA (6800 c.p.m./nmol) and vesicles prepared in buffer A (0.7 mg protein). The reactions were terminated after appropriate incubation periods by placing samples (0.3 ml) into a boiling water bath and the radioactivity incorporated into long-chain fatty acids was subsequently determined as described (Lynen, 1969).

Other determinations

Protein was determined by the method of Lowry *et al.* (1951) and radioactivity was determined by liquid scintillation counting.

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