Interactions of Anaerobic Propionate Formation and Acid-Base Status in *Arenicola marina*: An Analysis of Propionyl-CoA Carboxylase

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Abstract

The contribution of propionyl-CoA carboxylase (PCC) to the control of anaerobic metabolism by acid-base parameters (pH, P_{CO_2}, and $[HCO_3^-]$) was investigated with a purified enzyme preparation and isolated mitochondria from the body wall musculature of Arenicola marina. *The enzyme catalyzes the rate-limiting step in anaerobic propionate formation, namely, the carboxylation of methylmalonyl-CoA with concomitant formation of ATP and base equivalents (= HCO_3^-). Propionyl-CoA carboxylase is likely not saturated with its substrates methylmalonyl-CoA, ADP, and P_i under in vivo conditions, and propionate formation is therefore activated by a decreasing energy charge of the cell (i.e., increasing ADP and P_i concentrations). The effects of the individual acid-base parameters pH, P_{CO_2}, and $[HCO_3^-]$ on PCC activity have been determined. Stimulation of PCC by both high proton and low bicarbonate concentrations reflects an amplified control of propionate formation by the intracellular acid-base status. Nonrespiratory acidosis enhances the rate of decarboxylation of methylmalonyl-CoA, leading to a release of base equivalents. This mechanism has a strong stabilizing effect on the intracellular pH during long-term anaerobiosis. Without bicarbonate production by PCC, an additional pH drop of about 0.03 pH units per hour of anaerobiosis would be observed in* A. marina. *Our data support the hypothesis that, besides ionic transport mechanisms, metabolism itself contributes to cellular pH regulation.*

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Introduction

Propionyl-CoA carboxylase (PCC; E.C. 6.4.1.3.) catalyzes the reversible carboxylation of propionyl-CoA to methylmalonyl-CoA. Under aerobic conditions, this anaplerotic reaction serves to introduce C_3 skeletons, originating from fatty or amino acid metabolism, into the citric acid cycle (Davis, Spydevold, and Bremer 1980).

In the anaerobic metabolism of some invertebrates, however, PCC catalyzes the reverse reaction, the carboxylation of D-methylmalonyl-CoA to propionyl-CoA, which is an important step of propionate fermentation (Schöttler 1987):

$$\text{D-methylmalonyl-CoA} + \text{ADP} + P_i \rightarrow \text{propionyl-CoA} + \text{ATP} + HCO_3^-.$$

Propionate is the prominent anaerobic end product during long-term anaerobiosis in many facultative anaerobes, for example, the lugworm *Arenicola marina* (Kamp 1993). The advantages of propionate production for the anaerobic organism are associated with the decarboxylation reaction, catalyzed by PCC: First, the high Gibbs free energy (ΔG) of the decarboxylation reaction is used for additional ATP synthesis, so that the energy yield of propionate-producing fermentation (6.4 mol ATP $\cdot$ mol^{-1} glycogen) is relatively high compared to succinate production (4.7 mol ATP $\cdot$ mol^{-1} glycogen; Gnaiger 1983) and anaerobic glycolysis (3 mol ATP $\cdot$ mol^{-1} glycogen; Gnaiger 1983). Second, the degraded carboxyl group is released as bicarbonate, which takes up protons under physiological conditions. Thus, the net proton release during propionate production is reduced to 0.28 $H^+ \cdot$ mol^{-1} ATP compared to 0.8 $H^+ \cdot$ mol^{-1} ATP during succinate fermentation and 0.67 $H^+ \cdot$ mol^{-1} ATP in anaerobic glycolysis (Pörtner 1987).

Because of the enhanced proton load of the tissue caused by anaerobic metabolism, a decrease of the intracellular pH by about 0.2–0.5 pH units has been observed in all facultative anaerobes during hypoxia (Ellington 1983; Pörtner, Grieshaber, and Heisler 1984; Kamp and Juretschke 1989; Hardewig et al. 1991*b*). Since the pH drop plays an important role in the regulation and integration of anaerobic metabolism (Hand and Gnaiger 1988; Juretschke and Kamp 1990), the lower pH is not passively equilibrated, but must be actively regulated at a new steady state level (Pörtner 1993). According to the classical concept, pH homeostasis is exclusively achieved by ion exchange mechanisms. In recent years, however, the idea has evolved that metabolism itself may contribute to the regulation of acid-base balance (Atkinson and Bourke 1987; Pörtner 1989; Walsh and Mommsen 1992). This was first proposed by Atkinson and Camien in 1982 (Atkinson and Camien

1982). They postulated that bicarbonate consumption during urea synthesis is an important mechanism to maintain acid-base balance in terrestrial invertebrates. In subsequent years more evidence for metabolic pH regulation has been found. For example, in exercising rainbow trout, generation of NH_4^+ by protonation of NH_3 originating from AMP degradation removes a considerable amount of protons from the tissue (Mommsen and Hochachka 1988).

A prerequisite for metabolic pH regulation is the sensitivity of acid-base relevant reactions to changes in the acid-base status of the cell. This can be achieved by either (1) a strong pH sensitivity of the enzymes involved, (2) K_m values for the acid-base parameters CO_2 or HCO_3^- falling in the range of physiological concentrations for enzymes catalyzing carboxylation reactions, or (3) regulation of decarboxylation reactions via end product inhibition by CO_2 or HCO_3^-. Generally, the acid-base–sensitive enzymes must be rate limiting in their respective metabolic pathways, in order for reaction velocities to be responsive to acid-base disturbances in vivo.

Pyruvate carboxylase, the key enzyme for anaerobic ethanol production in crucian carp and goldfish, conforms to these requirements: low intracellular pH activates this enzyme and thus stimulates the proton consuming decarboxylation of pyruvate to ethanol, counteracting a further acidification of the cell (van Waarde, van den Thillart, and Verhagen 1993). A similar mechanism has been postulated by Pörtner for PCC (Pörtner 1987, 1989). On the basis of the participation of bicarbonate as a product of the PCC reaction, Pörtner suggested that this enzyme might be activated by nonrespiratory acidosis.

Since the decarboxylation reaction catalyzed by PCC influences the energy and the acid-base status of the cell, PCC is a prime candidate that may have a regulatory role in both energy and acid-base homeostasis. This hypothesis was tested in the following study. To this end, kinetic characteristics of PCC from the body wall of the lugworm *A. marina* were determined, with emphasis on the effects of the individual acid-base parameters pH, P_{CO_2}, and $[HCO_3^-]$ on the enzyme activity. The role of PCC in the anaerobic metabolism of *A. marina* is discussed.

Material and Methods

Chemicals

Biochemicals were obtained from Sigma (St. Louis) and Boehringer Mannheim (Mannheim, Germany).

Animals

Specimens of *Arenicola marina* were collected at the harbor of St. Pol (France). The animals were kept in seawater aquaria (36‰ salinity; 15°C) for several weeks.

Purification of PCC

Because PCC is located in the mitochondrial matrix, mitochondria were isolated from 10–20 g of cleaned muscle tissue of *A. marina*, as described by Völkel and Grieshaber (1994). The tissue was minced with dissection scissors in 20 mL of isolation buffer consisting of 20 mM Tris(hydroxymethyl)-aminomethane (Tris)-HCl (pH 7.5), 0.55 M glycine, 0.25 M sucrose, 4 mM ethylenediaminetetra-acetic acid (EDTA), and 0.2% bovine serum albumin (BSA) homogenized with a loose-fitting glass Teflon homogenizer. After an additional 480 mL of isolation medium was added, the homogenate was filtered through gauze, followed by centrifugation at 4,000 *g* for 15 min at 4°C. The resulting supernatant was centrifuged again at 16,000 *g* for 60 min at 4°C. The resulting pellet containing the mitochondria was dissolved in 5–10 mL of buffer A consisting of 20 mM Tris (pH 7.0), 1 mM dithiothreitol, 0.1 mM EDTA, and Triton was added to a concentration of 1%. The suspension was vigorously mixed and incubated on ice for 30 min to achieve disruption of the mitochondrial membranes. The suspension was then centrifuged at 13,000 *g* for 30 min and the supernatant (crude extract) was applied to a tetramethylaminoethyl anion exchanger (TMAE; Merck, Darmstadt, Germany) preequilibrated with buffer A. Propionyl-CoA carboxylase was eluted with a linear gradient of 0–200 mM $(NH_4)_2SO_4$ using a flow rate of 1 mL · min^{-1}. Fractions showing PCC activity were pooled and applied to a Superose 12 gel filtration column (Pharmacia, Freiburg, Germany) with a flow rate of 0.5 mL · min^{-1}. The eluted PCC was concentrated by Centricon 30 Ultrafiltration Units (Amicon, Danvers, Mass.). After addition of 50% (vol/vol) glycerol, the purified enzyme was stored for several weeks at −80°C without substantial loss of activity.

Assay of Enzyme Activity

The activity of PCC was determined on the basis of the method of Schulz, van Duin, and Zandee (1983) with minor modifications. The reaction mixture contained 100 mM N-[2-hydroxyethyl]piperazine-N-[2-ethane sulfonic acid] (HEPES; pH 7.0), 50 mM $MgSO_4$, 35 mM KH_2PO_4, 20 mM glucose, 1 mM ADP, 0.5 mM $NADP^+$, 4 U hexokinase, and 1 U glucose-6-phosphate-dehy-

drogenase (lyophilized, free from $(NH_4)_2SO_4$; Boehringer Mannheim, Mannheim, Germany). After stabilization of the absorbance at 339 nm, the reaction was initiated by the addition of 0.1 mM methylmalonyl-CoA. A slight contamination of the ADP solution by ATP caused a small increase in the extinction prior to the start of the PCC reaction. For measurements of enzyme activity during the purification procedure, 0.15 mM Di-adenosine-5-pentaphosphate (Ap5A) was added to the assay mixture after the absorbance had stabilized, which successfully eliminated the endogenous adenylate kinase activity.

Absorbance at 339 nm was recorded with a dual wavelength Sigma ZWS II spectrophotometer (Sigma Instruments, Berlin). The quantity 1 U PCC is defined as the amount of enzyme that catalyzes the decarboxylation of 1 µmol methylmalonyl-CoA per minute at 25°C.

Determination of K_m and Inhibition Constant (K_i) Values

The K_m values of PCC for methylmalonyl-CoA, ADP, and P_i were estimated by extrapolation of Lineweaver-Burk linear reciprocal plots.

The influence of HCO_3^- and CO_2 on PCC activity at constant pH was determined by equilibrating the reaction mixture with different gas mixtures (0%–10% CO_2 in N_2) delivered by a gas mixing pump (Wösthoff, Bochum, Germany). The Henderson-Hasselbalch equation was used to calculate the amount of HCO_3^- needed to keep the pH of the reaction mixture constant at pH 7.0. Results were linearized by the Hill equation to determine K_i values.

Incubation of Isolated Mitochondria

To check the integrity of the isolated mitochondria, the respiratory control index (RCI = $\dot{V}O_2$ state 3/$\dot{V}O_2$ state 4) of each preparation was determined as described by Völkel and Grieshaber (1994).

For incubations at different pH values, the mitochondrial pellet was resuspended in 0.17 mL incubation medium per gram of muscle tissue. The incubation medium consisted of 10 mM Tris-HCl (pH 7.0), 0.55 M glycine, 0.25 M sucrose, 5 mM $MgCl_2$, and 5 mM K_2HPO_4. The suspension was diluted 1:3 with different isolation media of pH values ranging from 6.0 to 8.0 (10 mM Tris was replaced by 40 mM imidazole buffer). In 50-mL siliconized Erlenmeyer flasks, 0.5 mL of the different mitochondrial suspensions were mixed with 10 mM succinate, 2 mM malate, and 2 mM ADP. During the incubation (3 h at 15°C), the flasks were equilibrated with pure nitrogen.

The P_{O_2} of the incubation mixture was found to be below 0.4 kPa, while the P_{CO_2} was nominally zero.

For incubation of the mitochondria at constant pH but at different CO_2 concentrations, the incubation medium (see above) was buffered with 40 mM Tris-HCl (pH 7.0). The Erlenmeyer flasks were equilibrated with various gas mixtures of N_2 and 0–2 kPa CO_2. To keep the pH of the incubation mixture constant at pH 7.0, the appropriate amounts of bicarbonate were added (see above).

At the end of each experiment, the pH of the incubation mixture was measured with a microelectrode BMS 3Mk2 (Radiometer, Copenhagen). The incubation was terminated by the addition of perchloric acid (PCA) to a final concentration of 0.6 M. After centrifugation of the PCA extract at 13,000 *g* for 15 min, the protein pellet was resuspended in 1 M NaOH. The supernatant was neutralized with KOH, centrifuged again, and stored at −80°C.

Protein concentrations were determined according to Bradford (1976), using solutions of γ-globulin in 0.1 M NaOH as standards. Propionate concentration was measured as described by Hardewig et al. (1991*a*).

Calculation of Intracellular and Mitochondrial Metabolite Concentrations

Metabolite concentrations in the intracellular water were calculated as

$$C_i = \frac{TC}{F \cdot (1 - Q)},$$

where C_i (mM) is the intracellular metabolite concentration, TC ($\mu mol \cdot g^{-1}$) is the tissue content of the metabolite, F is fraction of water in tissue (= 0.77; Reitze 1987), and Q is the fraction of extracellular water in tissue water (= 0.22; Reitze 1987).

Estimates of metabolite concentrations in the mitochondrial matrix were calculated as

$$C_m = \frac{TC}{F \cdot (1 - Q) \cdot 0.02},$$

where C_m is the metabolite concentration in the mitochondrial matrix. The insertion of the factor 0.02 in the denominator is based on the assumption that the mitochondria represent 2% of the cell volume. Mitochondrial volume ranges between 1% and 4% in the body wall muscle of different

annelids (Mattisson and Birch-Andersen 1962; Staubesand and Kersting 1964; Wissocq 1967).

Results

Purification of PCC

The PCC activity in the body wall musculature of *Arenicola marina* was 28.7 ± 3.6 mU · g^{-1} ($\bar{X}$ ± SD; n = 6).

Table 1 shows the results of a typical purification procedure. The specific activity of PCC in the crude extract amounted to 37 mU · mg^{-1} protein and was enhanced 17-fold by anion exchange chromatography. The subsequent gel filtration led to a further sixfold purification with a recovery rate of 69%. The purified enzyme had a specific activity of 3,720 mU · mg^{-1} protein. The final enzyme preparation showed no myokinase contamination (data not shown).

Characteristics of PCC

The PCC from the body wall musculature of *A. marina* exhibited Michaelis-Menten kinetics with respect to all substrates (fig. 1). The apparent K_m for D-methylmalonyl-CoA was estimated to be 4.1 ± 1.0 μM (n = 4), taking into account that the commercially available methylmalonyl-CoA is a racemic mixture of D- and L-enantiomers. The K_m values for ADP and P_i were 25.7 ± 5.3 μM and 27.2 ± 7.2 mM, respectively (n = 4).

The pH dependence of PCC was determined at subsaturating concentrations of substrates (5 μM D-methylmalonyl-CoA, 30 μM ADP, 50 mM P_i)in

TABLE 1
Purification procedure of propionyl-CoA carboxylase from mitochondria of the total body wall musculature of Arenicola marina

	Specific Activity (mU · mg^{-1} protein)	Purification Factor	Recovery (%)
Mitochondrial suspension	37	...	100.0
Anion exchanger	645	17.3	38.4
Gel filtration	3,720	99.7	26.5

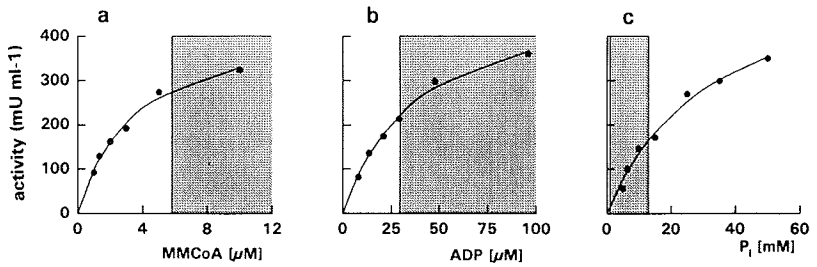

Fig. 1. Substrate saturation curves of PCC from the body wall of Arenicola marina *with respect to (a) methylmalonyl-CoA, (b) ADP, and (c) inorganic phosphate (P_i). Hatched areas represent reasonable estimates of the expected range of physiological concentrations of the respective substrate in the mitochondria of* A. marina.

order to mimic near-physiological conditions. The P_{CO_2} of the reaction mixture was about 0.03 kPa (= air saturation) and the bicarbonate concentrations ranged between 0.01 mM and 0.28 mM depending on the pH. Propionyl-CoA carboxylase exhibited a pronounced pH sensitivity (see fig. 2). The activity of the enzyme was maximal between pH 6.5 and pH 6.8. At pH 6.2, it dropped to 55% and at pH 7.5 to 25% of the maximal value.

Elevation of both CO_2 and HCO_3^- concentrations at constant pH 7.0 caused

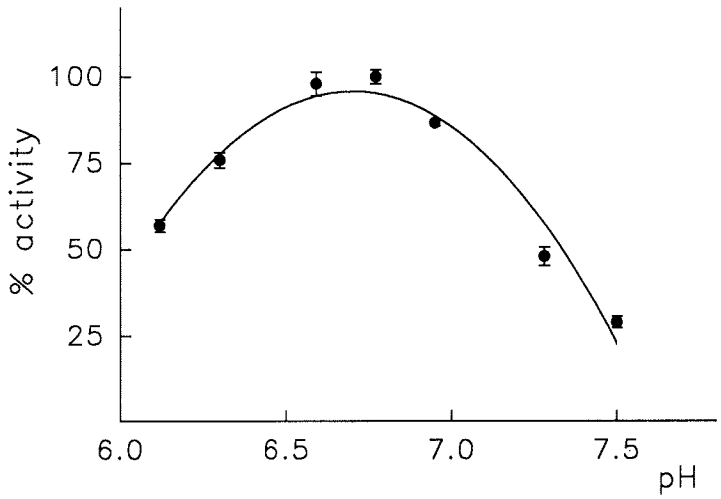

Fig. 2. The pH sensitivity of PCC from Arenicola marina *at subsaturating substrate concentrations (5 μM D-methylmalonyl-CoA, 30 μM ADP, 50 mM P_i); P_{CO_2} = 0.03 kPa (= air saturation); [HCO_3^-] ranges between 0.01 and 0.28 mM, depending on the pH ($\bar{X} \pm SD$, n = 3).*

a decrease of the enzyme activity down to 40% of the control value (see fig. 3). The K_i for bicarbonate was 5.8 ± 0.9 mM (n = 4).

Figure 4 illustrates the influence of monovalent ions on PCC activity. The addition of either NaCl or KCl resulted in a significant inhibition of the enzyme. Substitution of Cl^- by isethionate did not influence this effect. However, replacement of Na^+ and K^+ ions by choline released the inhibition, resulting in a higher PCC activity.

Propionate Production by Isolated Mitochondria

The integrity of isolated mitochondria was checked by measuring state 3 and state 4 respiration rates using malate and succinate as substrates. Respiratory control indices were 2.7 ± 0.8 ($\bar{X}$ ± SD; n = 6).

Under anaerobic conditions, malate and succinate were metabolized not only to propionate, but also to acetate. Figure 5*a* shows the propionate production of isolated mitochondria at various pH values. The CO_2 and HCO_3^- concentrations were nominally zero, because of N_2 bubbling of the mitochondrial suspension. At pH 6.8–7.0, propionate synthesis was maximal at 264 ± 162 nmol · h^{-1} · mL^{-1} mitochondrial suspension or 49 ± 3

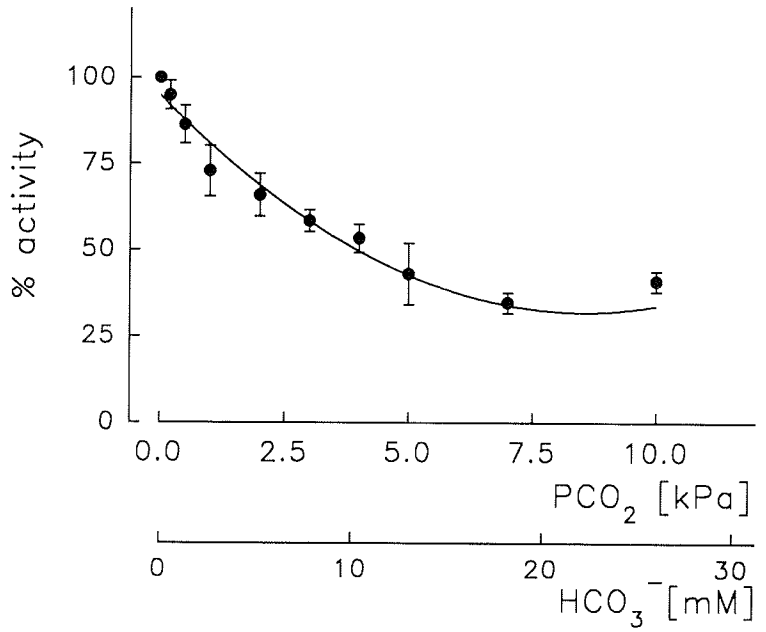

Fig. 3. Inhibition of PCC by CO_2/HCO_3^-. The pH of the reaction mixture was kept constant at pH 7.0. Since HCO_3^- partakes in the actual reaction, it is very likely that bicarbonate inhibits PCC activity (see text).

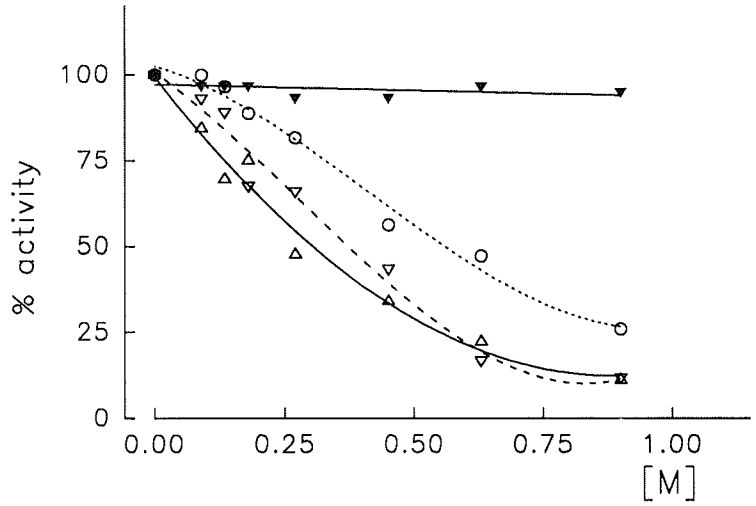

Fig. 4. Influence of NaCl (△), KCl (▽), Na-isethionate (○), and choline-chloride (▼) on the activity of PCC from Arenicola marina.

nmol · h^{-1} · mg^{-1} protein ($\bar{X} \pm$ SD; $n = 6$). Below and above these pH values, propionate synthesis showed a steep decline. This result corresponds to the pH profile of purified PCC, as can be seen from the dashed line in figure 5*a*.

An increase of the concentrations of CO_2 and HCO_3^- in the mitochondrial suspension caused a decline of propionate formation. Changes of CO_2 and HCO_3^- levels had a stronger effect on mitochondrial propionate production than on the activity of isolated PCC (see fig. 5*b*).

The formation of acetate showed a similar pH dependence as propionate synthesis. It was maximal between pH 6.8 and 7.0 (131 ± 39 nmol · h^{-1} · mL^{-1} or 21 ± 6.4 nmol · h^{-1} · mg^{-1} protein). Elevation of the P_{CO_2} had no effect (data not shown).

Discussion

Kinetic Characteristics of PCC

Propionyl-CoA carboxylase from mitochondria of the body wall musculature of *Arenicola marina* was purified 100-fold to a final specific activity of 3.7 U · mg^{-1} protein. Schulz et al. (1983) isolated PCC from a crude extract of mantle tissue of *Mytilus edulis*. A 400-fold purification resulted in an enzyme preparation with a specific activity of only 0.9 U · mg^{-1} protein. This indicates that the purification of PCC from isolated mitochondria is more efficient than the extraction of the whole tissue as performed by Schulz and co-

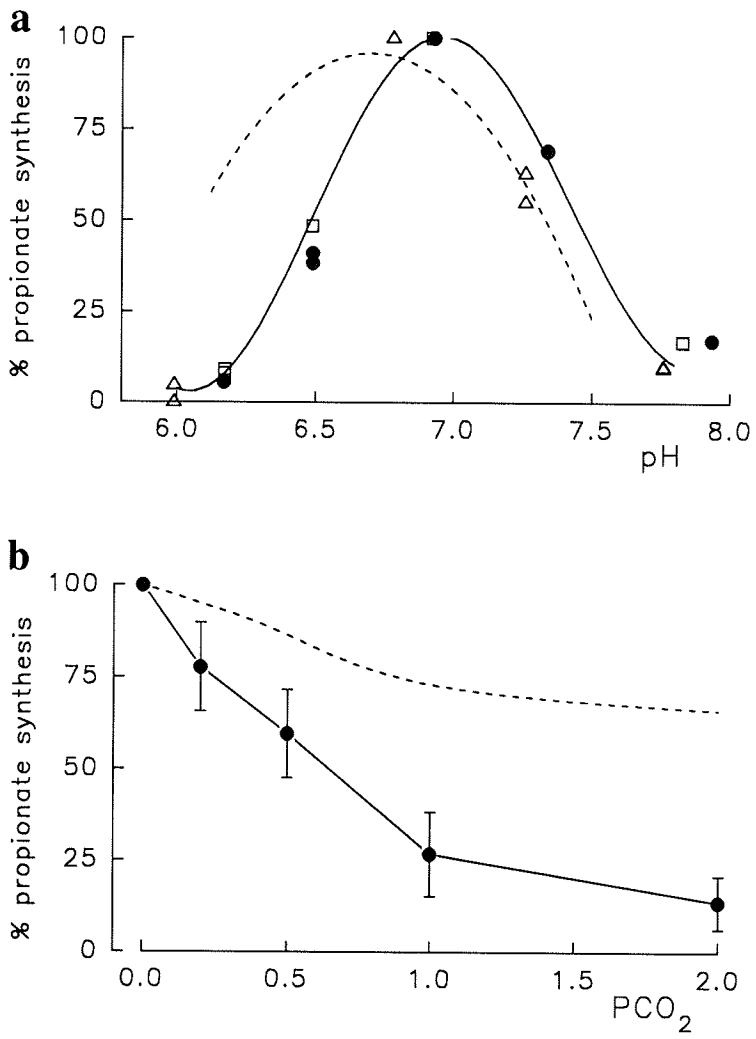

Fig. 5. Propionate synthesis of isolated mitochondria from the body wall of Arenicola marina *at different* (a) *pH* (P_{CO_2}; $[HCO_3^-]$ = *nominally zero; symbols correspond to replicates) and* (b) P_{CO_2} ($\bar{X} \pm SD$, n = 3). *Dashed lines represent the data from figs. 2 and 3 using isolated PCC.*

workers (1983). Hsia, Scully, and Rosenberg (1979) isolated PCC from human fibroblasts with a specific activity of maximally 1.62 $U \cdot mg^{-1}$ protein. The purest PCC has been obtained from pig heart by protein crystallization (as reported by Kaziro et al. [1961]). The homogeneous enzyme had a specific activity of 16.4 $U \cdot mg^{-1}$ protein.

The K_m values of PCC for methylmalonyl-CoA, ADP, and P_i are all in the range of the respective substrate concentrations in the body wall of *A. marina*

(see fig. 1): Levels of methylmalonyl-CoA between 0.15 and 0.5 nmol · g^{-1} were measured in the anaerobic muscle tissue of *A. marina* (Hardewig 1993), corresponding to an intracellular concentration of 0.25–0.8 μM. Taking into account that methylmalonyl-CoA is exclusively located in the mitochondrial compartment, an intramitochondrial concentration of 12.5–40 μM can be estimated (for calculations see Material and Methods). Since methylmalonyl-CoA constitutes a racemic 1:1 mixture of D- and L-isomers in vivo (Allen et al. 1963), the concentration of D-methylmalonyl-CoA (the substrate of PCC) ranges between 6.25 and 20 μM. The K_m value for this substrate was determined to be 4.1 μM.

With data for ATP and taurocyamine concentrations and H^+ activities from Pörtner, Surholt, and Grieshaber (1979) and Kamp and Juretschke (1989), the concentrations of free ADP in the tissue of *A. marina* were estimated from the equilibrium of taurocyamine kinase (K_{eq} = [taurocyamine][ATP]/[taurocyaminephosphate][ADP][H^+] = 6.48 · 10^8 at 4 mM total Mg^{2+}; Lawson and Veech 1979; Ellington 1989). In the anaerobic muscle of *A. marina* the free levels of ADP increase from about 30 μM to 160 μM (K_{mADP} = 25.7 μM). These values represent the concentration of ADP in the cytoplasm. In fact, ADP is not distributed homogeneously between the two compartments, but rather intramitochondrial concentrations exceed that of the cytosol. In isolated hepatocytes matrix ADP levels are 2.8–3.1-fold higher than cytosolic ones (Aw, Andersson, and Jones 1987). In rat liver in vivo a difference of a factor of 7.5–10 has been observed (Schwencke et al. 1981). In both studies, this ratio dropped under conditions of inhibited oxidative phosphorylation such as anoxia (in isolated cells) or anesthesia (in rats in vivo). With the scarcity of the available information we can only speculate that mitochondrial ADP concentrations are in the range of the K_{mADP} of PCC. They may, however, be beyond the levels where they play an important role in the fine control of this enzyme.

The content of free inorganic phosphate is assumed to be about 1 mM in resting muscles (determined in the adductor of *Mytilus edulis* by ^{31}P NMR spectroscopy; Schanck et al. 1986). During 24 h of anaerobiosis free phosphate concentrations in the tissue of *A. marina* may increase up to 13 mM because of phosphagen hydrolysis (calculated with data from Pörtner et al. [1979]). The K_m value of PCC for inorganic phosphate amounts to 27.2 mM.

The comparison of the physiological concentrations of methylmalonyl-CoA, ADP, and P_i with the respective K_m values reveals that PCC is not saturated with either methylmalonyl-CoA or P_i, so that its activity in vivo is strongly dependent on the availability of these substrates. Physiological ADP concentrations are in the same order of magnitude as the K_m value and might have a regulatory effect on PCC activity.

Acid-base parameters pH, P_{CO_2}, and HCO_3^- strongly influence the activity of PCC from *A. marina*. The pH profile shows maximal enzyme activity at values between 6.5 and 6.8. On either side of the optimum the PCC activity drops markedly. The pH-induced changes in bicarbonate concentrations in the reaction mixture did not significantly influence PCC activity since HCO_3^- levels remained well below the $K_{i\ bicarbonate}$ at all pH values. The PCC of *M. edulis* is less sensitive to pH changes (Schulz et al. 1983). In the range between pH 6.7 and 7.5, the enzyme activity remains above 80% of its maximum activity. In the aerobic muscle tissue of *A. marina* the intracellular pH, which represents the cytosolic proton concentration, is about 7.3 and may drop as low as 6.7 during anaerobiosis (Juretschke and Kamp 1990). However, the pH of the mitochondrial matrix, where the PCC is located, is significantly higher than the cytosolic pH. Proton gradients of 0.4–0.8 pH units between cytosol and mitochondria have been determined in vertebrate tissues (Padan and Rottenberg 1973; Andersson, Aw, and Jones 1987). Hypoxia does not cause a disturbance of the transmembrane gradient in rat hepatocytes, even though the phosphorylation potential of the cells drops drastically (Andersson et al. 1987). The maintenance of the pH gradient, which is probably due to decreased ion permeability of the mitochondrial membranes, is discussed as a protective mechanism during anoxia. It is likely that facultative anaerobes like *A. marina* use the same mechanism, which allows fast recovery upon reoxygenation. Therefore, a decrease of intramitochondrial pH from 7.9 to 7.1 can be expected to occur in anaerobic muscle tissue of *A. marina*. This would lead to a strong stimulation of the PCC under these conditions.

At a constant pH of 7.0, elevation of CO_2 and HCO_3^- concentrations has an inhibitory effect on the PCC activity of *A. marina*. Since an equilibrium is established between both substances in aqueous solutions, their concentrations cannot be varied independently at constant pH. Thus, our data do not give clear information whether CO_2 or HCO_3^- exerts the inhibitory effect. However, since HCO_3^- is one of the products of the decarboxylation reaction catalyzed by PCC, it is likely that the HCO_3^- molecule causes the noted inhibition in enzyme activity (product inhibition). For different vertebrates, where PCC catalyzes the reverse reaction, K_m values for HCO_3^- between 4.5 and 7 mM have been determined (Hsia et al. 1979; Garrastazu et al. 1991). These results are in good agreement with the K_i value for HCO_3^- (5.8 ± 0.9 mM) determined for the PCC of *A. marina*. This observation supports the assumption that the inhibitory effect is caused by binding of HCO_3^- to the active site, that is, the biotin group of the enzyme. In the body wall of *A. marina*, intracellular bicarbonate concentrations between 2.3 and 4.5 mM have been determined within the same range as $K_{i\ bicarbonate}$. This indicates that PCC activity is modulated by HCO_3^- in vivo.

In addition to acid-base parameters, PCC is influenced by changing ion concentrations. The KCl as well as NaCl revealed an inhibitory effect on the activity of PCC. Substitution of either Na^+ and K^+ with isethionate, or Cl^- by choline, revealed that the inhibitory effect is caused by the cations Na^+ and K^+. This phenomenon has not been observed in other species. Monovalent cations were shown to have a stimulating effect on the PCC of the nematode *Turbatrix aceti* and the mussel *M. edulis* (Meyer and Meyer 1978; Schulz et al. 1983). In *A. marina,* however, the inhibition of PCC by K^+ ions might be responsible for the observed increase of propionate synthesis under hypoosmotic stress (Schöttler, Daniels, and Zapf 1990). Since *A. marina* is an osmoconformer, incubation in hypoosmotic seawater causes a decrease of the intracellular potassium concentration (Reitze and Schöttler 1989) and, therefore, an activation of PCC.

The Regulatory Role of PCC in Metabolism

In order to investigate whether the regulation of the PCC by pH, P_{CO_2}, and HCO_3^- influences propionate production of *A. marina* in vivo, we examined the effect of these parameters on the mitochondrial synthesis of propionate. Anaerobic mitochondria metabolized added succinate and malate to the volatile fatty acids acetate and propionate. Schulz, Kluytmans, and Zandee (1982) demonstrated that the addition of small amounts of malate enhances propionate production from succinate by mitochondria from *M. edulis.* They showed that malate is oxidized via 2-oxo-glutarate to succinyl-CoA, which initiates the synthesis of propionate. Once the production process is started, succinyl-CoA is built by transfer of CoA from propionyl-CoA to succinate. Besides its primer function for propionate synthesis, malate serves as a substrate for mitochondrial acetate production by malic enzyme and pyruvate dehydrogenase (Schöttler 1980).

Changes in CO_2 concentrations and in pH showed a similar effect on propionate production by isolated mitochondria as on the activity of purified PCC. However, the sensitivity of the mitochondria to CO_2 fluctuations is more pronounced than that of the isolated enzyme. This is likely due to pH effects for the following reason. Even though enzyme kinetics and mitochondria incubations were both carried out at pH 7.0, the intramitochondrial pH under these conditions is probably slightly higher than the pH of the incubation medium, that is, if the transmembrane proton gradient is maintained under anoxia (see above). Therefore, at identical P_{CO_2} the concentration of HCO_3^- (as the actual inhibitor of PCC) was likely higher in the mitochondrial matrix than in the reaction mixture of the PCC assay. This may explain the stronger CO_2 effect on isolated mitochondria. In any case,

since the inhibition by either protons or CO_2/bicarbonate obviously decreased propionate production by purified or mitochondrial PCC, this enzyme is likely to contribute to the regulation of propionate synthesis in *A. marina*. It can not be ruled out, however, that the observed influence of pH on mitochondrial propionate synthesis is due to indirect pH effects on transport mechanisms. But at least the ADP/ATP translocase does not seem to be substantially affected by pH changes since in fish mitochondria respiration rates remain constant between 6.5 and 7.3 even at suboptimal ADP levels (Moyes, Schulte, and Hochachka 1992). Most mitochondrial transport systems like ADP/ATP translocase and P_i transporter are influenced by the transmembrane pH gradient rather than the pH itself.

A regulatory role of a particular enzyme in metabolism is also suggested by its low specific activity, which is usually close to the maximum flux through the respective metabolic pathway (Newsholme, Zammit, and Crabtree 1978). In this case the enzyme is rate limiting and has, therefore, a regulatory function. The maximum specific activity of PCC in the body wall of *A. marina* amounts to 28.7 ± 3.6 $mU \cdot g^{-1}$ body wall musculature, which is equivalent to a turnover rate of 1.7 $\mu mol \cdot h^{-1} \cdot g^{-1}$ body wall musculature. This is close to the in vivo rate of propionate production in *A. marina*. (0.43 $\mu mol \cdot h^{-1} \cdot g^{-1}$ body wall musculature; Schöttler, Wienhausen, and Zebe 1983).

The flux rates of ATP-generating pathways, such as propionate synthesis, have to be adapted to the changing energy demand of the organism. Propionyl-CoA carboxylase is sensitive to the cellular energy status, since its in vivo activity is enhanced by P_i and possibly ADP, both of which increase when the energy charge of the cell is low. In addition to its contribution to the regulation of the energy status, PCC also plays an important role in the maintenance of acid-base balance. The opposite effects of physiological proton and bicarbonate concentrations on the activity of PCC amplify the influence of acid-base disturbances on this enzyme. During nonrespiratory (e.g., metabolic) acidosis, high proton *and* low bicarbonate levels both activate PCC and enhance the decarboxylation of methylmalonyl-CoA, leading to a release of base equivalents. Alkalosis, on the other hand, inhibits PCC via low proton concentration *and* via high bicarbonate levels, thus preventing an additional alkalinization of the tissue. This mechanism provides a strong stabilizing effect on intracellular pH. In anaerobic *A. marina* an additional drop in pH_i of about 0.03 pH units per hour of anaerobiosis would be observed without bicarbonate production by PCC (based on a propionate production rate of 0.43 $\mu mol \cdot h^{-1} \cdot g^{-1}$ body wall musculature; Schöttler et al. 1983; and a buffer capacity of $\beta = 16.0$ $mmol \cdot kg^{-1} \cdot pH\ unit^{-1}$, determined for *Sipunculus nudus* by H. O. Pörtner, unpublished results).

From our results we conclude that PCC contributes to the regulation of both the energy and acid-base status of *A. marina* during long-term anaerobiosis.

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