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# Malonate decarboxylase of *Malonomonas rubra*, a novel type of biotin-containing acetyl enzyme

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Cell suspensions or crude extracts of *Malonomonas rubra* grown anaerobically on malonate catalyze the decarboxylation of this substrate at a rate of 1.7–2.5 μmol · min$^{-1}$ · mg protein$^{-1}$ which is consistent with the malonate degradation rate during growth. After fractionation of the cell extract by ultracentrifugation, neither the soluble nor the particulate fraction alone catalyzed the decarboxylation of malonate, but on recombination of the two fractions 87% of the activity of the unfractionated extract was restored. The decarboxylation pathway did not involve the intermediate formation of malonyl-CoA, but decarboxylation proceeded directly with free malonate. The catalytic activity of the enzyme was completely abolished on incubation with hydroxylamine or NaSCN. Approximately 50–65% of the original decarboxylase activity was restored by incubation of the extract with ATP in the presence of acetate, and the extent of reactivation increased after incubation with dithioerythritol. Reactivation of the enzyme was also obtained by chemical acetylation with acetic anhydride. These results indicate modification of the decarboxylase by deacetylation leading to inactivation and by acetylation of the inactivated enzyme specimens leading to reactivation. It is suggested that the catalytic mechanism involves exchange of the enzyme-bound acetyl residues by malonyl residues and subsequent decarboxylation releasing $CO_2$ and regenerating the acetyl-enzyme. The decarboxylase was inhibited by avidin but not by an avidin-biotin complex indicating that biotin is involved in catalysis. A single biotin-containing 120-kDa polypeptide was present in the extract and is a likely component of malonate decarboxylase.

In recent years, several anaerobic bacteria have been shown to conserve the free energy of decarboxylation reactions [1, 2]. Membrane-bound $Na^+$-translocating decarboxylases have been found for oxaloacetate in *Klebsiella pneumoniae* [3, 4] and *Salmonella typhimurium* [5], for methylmalonyl-CoA in *Veillonella alcalescens* [6] and *Propionigenium modestum* [7] and for glutaconyl-CoA in *Acidaminococcus fermentans* and other glutamate fermenting bacteria [8]. Upon decarboxylation of these substrates, an electrochemical gradient of $Na^+$ is established over the cytoplasmic membrane that drives various endergonic reactions, e.g. solute transport [9], ATP synthesis [10], or NADH synthesis by reversed electron transfer [11]. All $Na^+$-translocating decarboxylases are oligomeric enzymes with a similar subunit composition and all contain a prosthetic group, biotin [1, 2]. The reaction mechanism is also similar and involves carboxylation of enzyme-bound biotin by carboxyl transfer from the substrate and subsequent decarboxylation of carboxybiotin which is coupled to $Na^+$ translocation [1, 2].

The free energy of the decarboxylation of a carboxylic acid allows growth of *P. modestum* on succinate [7, 12], of *Oxalobacter formigenes* on oxalate [13] and of *Malonomonas rubra* on malonate [14]. *P. modestum* converts the decarboxylation energy by virtue of methylmalonyl-CoA decarboxylase into $\Delta\tilde{\mu}Na^+$, which serves a unique $Na^+$-translocating $F_1F_0$ ATPase as the driving force for ATP synthesis [7, 10]. An entirely different mode for conserving decarboxylation energy applies for *O. formigenes* [13]. Here, a $\Delta\tilde{\mu}H^+$ is generated by an oxalate/formate antiporter which receives its driving force from the low oxalate concentration and high formate concentration in the cytoplasm as a consequence of an efficient and irreversible decarboxylation of the substrate inside the bacteria.

To make the decarboxylation of these substrates chemically feasible, succinate is converted to methylmalonyl-CoA [7] and oxalate is converted to oxalyl-CoA [13]. By analogy, one might expect that in *M. rubra* malonate is converted to malonyl-CoA which is then decarboxylated to acetyl-CoA. Energy conservation in this organism could be analogous to that of *P. modestum* involving a membrane-bound decarboxylase or could apply to the *O. formigenes* type with an energy-generating antiporter. As an approach to elucidating the energy-conservation mechanism in *M. rubra*,

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*Enzymes.* Acetyl-CoA carboxylase (EC 6.4.1.2); adenosinetriphosphatase (EC 3.6.1.3); citramalate lyase (EC 4.1.3.22); citrate lyase (EC 4.1.3.6); deoxyribonuclease I (EC 3.1.21.1); glutaconyl-CoA decarboxylase (EC 4.1.1.70); malonate decarboxylase (EC 4.1.1.–); malonyl-CoA decarboxylase (EC 4.1.1.9); methylmalonyl-CoA decarboxylase (EC 4.1.1.41); oxaloacetate decarboxylase (EC 4.1.1.3).

we have determined the type of enzyme(s) catalyzing the decarboxylation of malonate.

## MATERIALS AND METHODS

### Materials

DNase I was from Boehringer (Mannheim, FRG), avidin-peroxidase-labeled, 3.3′-dimethoxybenzidine and diisopropyl fluorophosphate were from Sigma (Buchs, Switzerland); prestained SDS/PAGE standards (low range) were from Bio-Rad. All other chemicals, as well as avidin, were from Fluka (Buchs, Switzerland).

### Growth of the organism

*M. rubra* was grown anaerobically at 30 °C with malonate as sole carbon and energy source essentially as described in [14]. The growth medium contained 40 mM malonate, 50 mM $NaHCO_3$, 340 mM NaCl, 5 mM $NH_4Cl$, 1.15 mM $KH_2PO_4$, 15 mM $MgCl_2$, 1 mM $CaCl_2$, 6.7 mM KCl, 1 mM cysteine and 0.5 ml/l each of filter-sterilized seven-vitamin solution [15], trace element solution SL 10 [16] and selenite/tungstate solution [17]. Stock solutions of neutralized malonate, bicarbonate and cysteine were autoclaved separately and the pH was adjusted to pH 7.1–7.3. The bacteria were transferred from a 50-ml stock culture (transferred into fresh medium once a month) into 0.5 l medium, and after 2 days this culture was used to inoculate 12 l medium in bottles sealed with gassing-tube-equipped rubber stoppers. After approximately 40 h, the culture was supplemented with another 50 mM malonate and allowed to grow for another 32 h. Cells were harvested by continuous centrifugation at $19000 \times g$ (Contifuge 17 RS, rotor 8575; Heraeus, Zürich). The yield was about 1 g wet packed cells/l medium. As the cells lysed during washing, even in the presence of up to 500 mM NaCl, they were frozen in liquid nitrogen without previous washing. The malonate decarboxylase was stable on storage under these conditions. Cultures were checked for purity with the agar shake culture method [18]. The appearence of single colonies was biconvex with a brownish-red color developing grey fuzzy edges with age.

### Preparation of cell extract and subcellular fractions

5 g frozen wet cells were suspended in 25 ml buffer A (50 mM potassium phosphate, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$), containing 5 mM dithioerythritol, 0.2 mM diisopropyl fluorophosphate and 1 mg DNase I.

The following treatments were performed at 4 °C. Cells were disrupted by two passages through a French press at 55 MPa. Whole cells and large debris were removed by centrifugation for 20 min at $15000 \times g$. The supernatant, referred to as extract, could be stored in liquid nitrogen without loss of malonate-decarboxylase activity. Fractionation of the extract by ultracentrifugation was carried out with a Beckman L8–70 ultracentrifuge at $200000 \times g$ for 30 min (rotor 70.1 Ti). The resulting membrane pellet was resuspended either in buffer A or, as control, in the cytoplasmic fraction using a motor-driven Teflon plunger (type RW 20 DZM, Janke & Kunkel).

### Determination of malonate-decarboxylase activity

Malonate-decarboxylase activity was determined by measuring $CO_2$ formation from malonate. The reactions were carried out in 13-ml serum bottles sealed with rubber stoppers at 30 °C. Decarboxylation was initiated by adding 3 µl 1 M sodium malonate, pH 7.5, to 0.1 ml extract (1.3 mg protein) in buffer A. In some experiments, the extract containing endogenous acetate from bacterial metabolism, was incubated for 2 min with 5 µl 100 mM ATP to reacetylate deacetylated enzyme specimens and thus restore catalytic activity. If not indicated otherwise, the reaction was terminated after 30 s by adding 10 µl 2 M HCl with a syringe. The amount of $CO_2$ formed and released into the gas phase was determined by injecting 0.3 ml gas phase onto a Poropak-N column maintained at 140 °C. The amount of $CO_2$ analysed was not dependent on the incubation time after acidification. The signal was recorded with a Hewlett Packard 5890 series II gas chromatograph equipped with a thermal-conductance detector. Peak areas were automatically calculated and converted into an amount of $CO_2$ by comparison with the peak areas of $CO_2$ standards. The stock mixture of $CO_2$ standard was a 1:200 dilution of $CO_2$ in $N_2$. In our measuring range of 10–120 nmol $CO_2$, the areas corresponded linearly to the amount of $CO_2$ analysed.

### Determination of acetate concentration

Acetate concentration was determined by GC using a Perkin Elmer 8700 gas chromatograph equipped with a flame-ionization detector. Samples were acidified with 10% $H_3PO_4$ and centrifuged for 5 min at $15000 \times g$. 1 µl supernatant was injected and separated on a Chromosorb-WAW column (10% SP 1200, 1% $H_3PO_4$; 120 °C). Standards were treated similarly.

### SDS/PAGE and Western blotting

The extract was separated by SDS/PAGE using a Midget apparatus (Pharmacia/LKB). The sample gel was prepared with 4% acrylamide and 0.125% bisacrylamide whereas the separating gel was prepared with 10% acrylamide, 0.3% bisacrylamide and either 6 M urea or 13% glycerol according to [19]. The polypeptide bands were either visualized by staining with Coomassie brillant blue [20] or the gel was blotted onto a nitrocellulose membrane as described [21]. After washing the blot twice for 5 min with buffer B (10 mM Tris/Cl, pH 7.5 and 150 mM NaCl), it was blocked 1 h with 2% bovine serum albumin, following incubation with approximately 0.2 mg of an avidin-peroxidase conjugate in 20 ml buffer B for another hour. Subsequently, the blot was washed three times for 10 min with buffer B, and biotin-containing polypeptides were visualized by incubation with 20 ml buffer B, containing 5 mg 3,3′-dimethoxybenzidine (solubilized in 1 ml methanol) and 20 µl 35% $H_2O_2$.

### Protein determination

Protein was determined according to Bradford [22], using the Bio-Rad protein-assay reagent mixture. Bovine serum albumin served as a standard.

## RESULTS

### Demonstration of a malonate decarboxylase in cell-free extracts of *M. rubra*

Exponentially growing cells of *M. rubra* consume malonate at a considerable rate. The malonate-decar-

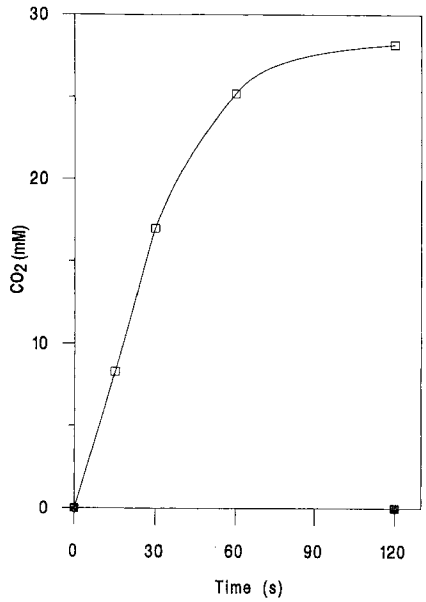


**Fig. 1. Kinetics of $CO_2$ formation from malonate, catalyzed by malonate decarboxylase.** The decarboxylation of malonate by an extract of *M. rubra* was determined from the amount of $CO_2$ liberated as described in Materials and Methods. Assays were performed with 30 mM malonate (□) or without substrate (■).

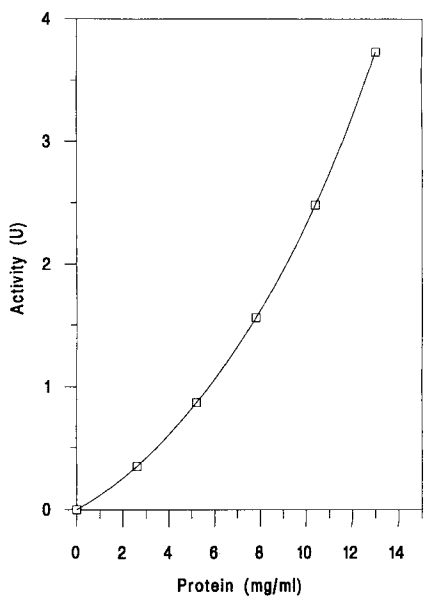


**Fig. 2. Dependence of malonate-decarboxylase activity on protein concentration.** The extract of *M. rubra* with an initial protein concentration of 13 mg/ml was diluted with buffer A to the concentrations indicated. Malonate decarboxylation was determined with 0.1 ml of each diluted extract as described under Materials and Methods. The reactions were terminated after 30 s, in the linear part of the kinetics of $CO_2$ formation (Fig. 1). The activities are therefore based on initial rates. Values are means of duplicate assays.

boxylating activity of these cells was calculated from the growth parameters $Y_s$ = 1.9 g/mol and $\mu$ = 0.154 $h^{-1}$ [14] to be 2.5 $\mu$mol · $min^{-1}$ · mg $protein^{-1}$. A suspension of cells harvested in the stationary growth phase catalyzed malonate decarboxylation at a rate of 1.9 $\mu$mol · $min^{-1}$ · mg $protein^{-1}$.

Cell-free extracts prepared with a French pressure cell retained the malonate-decarboxylase activity. The kinetics of $CO_2$ formation from malonate under optimized conditions are shown in Fig. 1. Within the first 30 s, the amount of $CO_2$ formed increased linearly with time yielding a specific activity of the enzyme of 2.7 U/mg protein. This activity is therefore commensurate with the malonate-decarboxylation rate of whole cells. After 30 s the decarboxylation of malonate slowed down and after 120 s the substrate was completely decarboxylated.

These results indicate that *M. rubra* contains an enzyme system that catalyzes the cleavage of the unmodified substrate according to Eqn 1:

$$\text{malonate} + H^+ \rightarrow \text{acetate} + CO_2. \quad (1)$$

Acetyl-CoA was without effect on malonate decarboxylation, and ATP did not stimulate the activity of a freshly prepared cell extract. A complex enzyme system catalyzing malonate decarboxylation via malonyl-CoA to acetyl-CoA is thus not indicated by our experiments.

The dependence of malonate-decarboxylase activity on protein concentration was not linear but showed an increase of specific activity with increasing protein concentration (Fig. 2). The concentration-dependent increase of decarboxylase activity could indicate the presence of an endogenous cofactor or a multicomponent enzyme system being subject to a concentration-dependent dissociation/association equilibrium [23]. The requirement for an exchangeable low-molecular-mass cofactor was excluded by demonstrating malonate-decarboxylase activity in dialyzed extracts (see below).

**Table 1. Disappearance of malonate-decarboxylase activity after separation of the extract into cytoplasmic and membrane fractions by ultracentrifugation and reconstitution of enzyme activity by combining these fractions.** Centrifugation of the crude extract was performed at 200000 × *g* for 30 min, as described in Materials and Methods.

| Fraction | Malonate decarboxylase activity | |
|---|---|---|
| | U/mg | (%) |
| Extract | 2.83 | (100) |
| Membrane | 0.09 | ( 3) |
| Cytoplasm | 0.02 | ( 1) |
| Membrane/cytoplasm | 2.45 | ( 87) |

## The malonate-decarboxylase activity depends on the soluble and particulate fractions of the cell extract

Evidence for a multicomponent enzyme system for the decarboxylation of malonate was obtained after separating soluble and particulate fractions of the extract by ultracentrifugation. The results in Table 1 indicate that neither of these separated fractions retained any significant amount of malonate-decarboxylase activity. On recombination of both fractions, however, 87% of the activity of the non-fractionated extract was recovered. Thus, the enzyme, after disruption of the cells, consists of a soluble and a membrane-bound component. While it is unclear whether the soluble part of the enzyme has been sheared off during cell rupture, the results clearly indicate that a membrane-bound component is involved. Location of the enzyme in the membrane is expected if energy conservation proceeds by a direct malonate-decarboxylase-dependent ion-pumping mechanism.

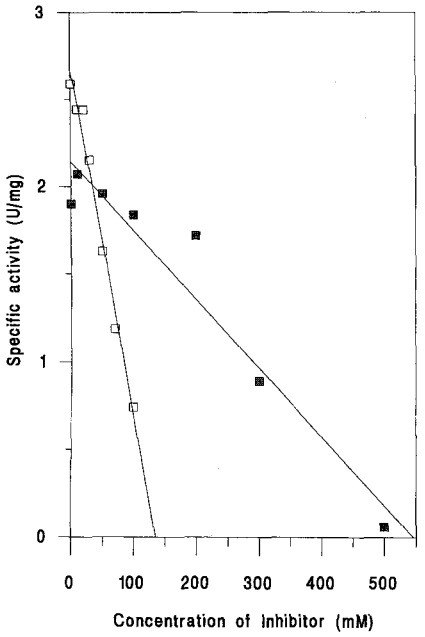


**Fig. 3. Inhibition of malonate decarboxylase by hydroxylamine and thiocyanate.** The bacterial extract (75 µl, 1 mg protein) was mixed with 25 µl hydroxylamine in buffer A to yield the inhibitor concentration indicated. After 5 min at 25°C the decarboxylase activity was determined (■). Alternately, 90 µl bacterial extract was mixed with 10 µl NaSCN in buffer A to yield the final inhibitor concentration indicated. The malonate-decarboxylase activity was subsequently determined as described above (□). Values represent means of duplicate assays.

**Table 2. Reactivation of inactivated malonate decarboxylase by acetylation.** The freshly prepared enzyme of specific activity 2.67 U/mg protein (not increased by incubation with ATP) was completely inactivated by incubation with 100 mM hydroxylamine for 45 min followed by dialysis for 4 h (A), by incubation with 100 mM NaSCN for 15 min followed by dialysis for 3 h (B), or by dialysis for 18 h (C). The dialysis buffer was buffer A in all cases. As a control for the last experiment (C), the extract was kept without dialysis for 18 h at 4°C. For the chemical acetylation of the enzyme with acetic anhydride, 1 µl 100 mM acetic anhydride in $H_2O$ (diluted immediately before) was added to 100 µl inactivated enzyme and the mixture was incubated for 1 min at 30°C. The residual malonate decarboxylase activity was 0.5 U/mg protein and could not be increased by incubation with ATP. n.d., not determined. For (A) and (B), activities are given as percentage initial activity, while for (C) activity is given as percentage control.

| Additions | Malonate decarboxylase activities | | | | | |
|---|---|---|---|---|---|---|
| | A | | B | | C | |
| | U/mg | (%) | U/mg | (%) | U/mg | (%) |
| Inactivated enzyme | 0 | ( 0) | 0 | ( 0) | 0 | ( 0) |
| 5 mM ATP/5 mM sodium acetate | 1.20 | (45) | 1.32 | (49) | 0.30 | (60) |
| 5 mM ATP/20 mM dithioerythritol | 1.73 | (65) | n.d. | (n.d.) | n.d. | (n.d.) |
| 1 mM acetic anhydride | 0.44 | (16) | 1.04 | (39) | 0.23 | (46) |

## Evidence for an acetyl enzyme

The above results, indicating decarboxylation of free malonate were surprizing from a chemical point of view, and a malonate-decarboxylase enzyme has never been found before. Malonyl-CoA, however, is decarboxylated by a variety of enzymes [24] including the $Na^+$-translocating methylmalonyl-CoA decarboxylase of *V. alcalescens* [6]. The chemical problem of decarboxylating free malonate might be overcome by forming the malonyl thioester group transiently on the enzyme by an exchange of malonate for an enzyme-bound acetyl thioester residue. Decarboxylation of the malonyl thioester on the enzyme would regenerate the acetyl enzyme. This mechanism would imply that malonate decarboxylase is active only in an acetylated form.

To test this hypothesis the enzyme was incubated with hydroxylamine to remove putative acetyl thioester residues by formation of acetyl hydroxamates. The results in Fig. 3 show that the decarboxylase was inactivated on incubation with hydroxylamine. The activity decreased with inreasing hydroxylamine concentrations, complete inactivation being observed after 5 min incubation with 500 mM hydroxylamine. Inactivation of the decarboxylase was also observed on incubation with thiocyanate at approximately three times lower concentrations than with hydroxylamine.

Enzyme specimens thus inactivated were dialyzed for 4 h to remove the inhibitor, and malonate decarboxylase activity was subsequently determined in the presence of the compounds listed in Table 2. Enzyme completely inactivated by hydroxylamine regained 45% of the original activity after 2 min incubation with 5 mM ATP. The activity increased further to 65% if the extract was incubated with 20 mM dithioerythritol, indicating the importance of thiol groups for this reactivation. With extracts containing 0.18 mM endogenous acetate, the additional presence of 1 – 5 mM sodium acetate was without effect on the reactivation of decarboxylase activity. With extensively dialyzed extract, however, the activation with acetate plus ATP was 2.2 times higher than with ATP alone. After these treatments, 11% of the original enzyme activity was recovered. A control extract, that was not treated with hydroxylamine but kept overnight at 4°C, lost 80% of its activity which could not be restored to any significant extent by incubation with ATP. The enzyme thus lost activity on storage which was not due to a deacetylation event. If this irreversible loss of activity on storage is taken into account, the proportion of decarboxylase that was reactivated by ATP plus acetate was 60%.

These results suggested that an enzyme-catalyzed acetylation of specific SH groups of the decarboxylase with ATP and acetate is responsible for the observed reactivation. Convincing evidence for catalytically competent acetyl residues bound to the decarboxylase was obtained by reactivation of the inactivated enzyme with acetic anhydride. This chemical acetylation restored 36 – 77% of the activity that could be obtained by enzymic acetylation with ATP and acetate. Similar results on reactivation by acetylation were obtained with enzyme samples that were inactivated with *thiocyanate*, indicating that this treatment likewise leads to a loss of catalytically important acetyl residues.

Results on substrate specificity of the acetylating enzyme (Table 3) indicate that the decarboxylase is reactivated with ATP, ADP, or GTP with decreasing efficiency in that order, but not with AMP or with acetyl-CoA. As ATP may be formed from ADP by adenylate kinase but not from AMP, the results suggest that ATP is the physiological substrate of the enzyme acetylating deacetylmalonate decarboxylase.

## Evidence for a biotin enzyme

A possible involvement of biotin in the reaction catalyzed by malonate decarboxylase was determined by measuring the

**Table 3. Specificity of the malonate-decarboxylase-acetylating enzyme for ATP.** The enzyme of specific activity 2.67 U/mg protein was completely inactivated by incubation with 100 mM hydroxylamine (45 min) followed by dialysis (4 h). Activities are given as U/mg and percentage of initial activity.

| Additions | Malonate-decarboxylase activities | |
|---|---|---|
| | U/mg | (%) |
| Inactivated enzyme | 0 | ( 0) |
| 5 mM ATP | 1.20 | (45) |
| 5 mM ADP | 0.84 | (31) |
| 5 mM AMP | 0 | ( 0) |
| 5 mM GTP | 0.72 | (27) |
| 5 mM acetyl-CoA | 0 | ( 0) |

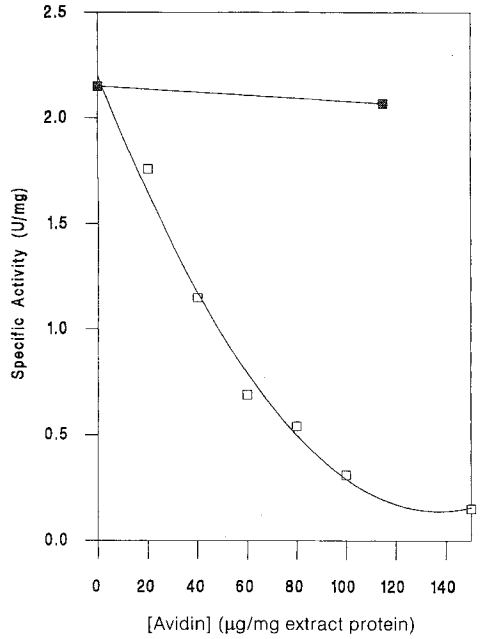


**Fig. 4. Inhibition of malonate decarboxylase by avidin.** The bacterial extract (100 µl, 1.3 mg protein) was mixed with 20 µl avidin of the appropriate concentration in buffer A to yield the amount shown. After 15 min at 25 °C, residual biotin-binding sites of the avidin were blocked by adding 10 µl biotin (7.5 mg/ml, final concentration 2.4 mM). After another 15 min the malonate-decarboxylase activity was determined (□). In the control (■) avidin was incubated with biotin for 30 min prior to the addition to the cell extract. Values are means of duplicate assays.

effect of avidin on catalytic activity. The results in Fig. 4 show decreasing malonate decarboxylase activities after incubation of the cell extract with increasing amounts of avidin. With 45 µg avidin/mg extract protein, the inhibition was 50%, and 150 µg avidin/mg extract protein destroyed the decarboxylase activity almost completely. Incubation with an avidin-biotin complex performed as a control, however, was without effect on the enzyme activity. These results strongly indicate a biotin prosthetic group on malonate decarboxylase. Accordingly, we found a single biotin-containing protein band after SDS/PAGE. After blotting the separated polypeptides onto nitrocellulose membranes, labelling with an avidin-peroxidase conjugate and locating the peroxidase with $H_2O_2$/3,3′-dimethoxybenzidine, only a single stained band became visible. Comparison of the mobility of this band with those of

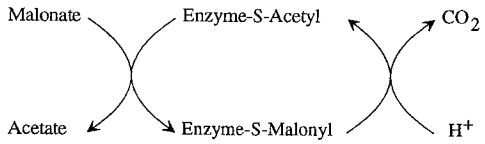


**Fig. 5. hypothetical reaction mechanism of malonate decarboxylase.**

marker proteins indicated a molecular mass for the biotin-containing polypeptide of approximately 120 kDa. The relative mobility of this band was not affected if the SDS gel contained additions of 6M urea or 13% glycerol.

## DISCUSSION

On decarboxylation of a carboxylic acid, the carboxyl group is replaced by a proton. An electron-withdrawing substituent on the carbon at which this replacement occurs therefore greatly facilitates these decarboxylation reactions. For catalysis of decarboxylations under physiological conditions, the electron-attracting substituent is provided, e.g. by a carbonyl group in 3-oxoacids, a thioester residue, the pyridinium cation of pyridoxal phosphate, or the thiazolium cation of thiamine pyrophosphate.

*M. rubra* grows using malonate as the sole carbon and energy source and thereby decarboxylates the dicarboxylic acid to acetate and $CO_2$ [14]. A chemically feasible mechanism for malonate decarboxylation in these cells (Eqn 1) would be the conversion of malonate to malonyl-CoA by a CoA transferase with acetyl-CoA as the second substrate (Eqn 2), and subsequent decarboxylation of malonyl-CoA to acetyl-CoA (Eqn 3). Malonyl-CoA could alternately be formed under ATP consumption by malonyl-CoA synthetase.

$$\text{malonate} + \text{acetyl-CoA} \rightleftharpoons \text{malonyl-CoA} + \text{acetate} \quad (2)$$

$$\text{malonyl-CoA} + H^+ \rightarrow \text{acetyl-CoA} + CO_2 \quad (3)$$

$$\text{malonate} + H^+ \rightarrow \text{acetate} + CO_2 \quad (1)$$

Malonyl-CoA decarboxylase is present in many organisms including plants, animals and bacteria [24]. The enzymes isolated from the uropygial gland of geese or from *Mycobacterium tuberculosis* are not biotin dependent, are located in the cytoplasm and do not participate in energy conservation [24]. Therefore, the decarboxylase of *M. rubra* could more likely be related to methylmalonyl-CoA decarboxylase of *V. alcalescens* which accepts malonyl-CoA as an alternate substrate [6]. This enzyme is firmly bound to the membrane, contains biotin as prosthetic group and conserves energy by generating an electrochemical gradient of $Na^+$ across the membrane [6].

An investigation of the enzyme(s) involved in malonate decarboxylation by *M. rubra*, however, led to the discovery that the decarboxylase reacts with free malonate and that malonyl-CoA is not involved in this catalysis. Based on these results, an alternative mechanism was considered in which malonate was activated by its covalent attachment to the enzyme instead of its linkage to CoA. A catalytic sequence analogous to that shown in Eqn (2) and Eqn (3) could result, with acetyl-enzyme and malonyl-enzyme derivatives as shown in Fig. 5. This mechanism consists of an exchange of malonate for enzyme-bound acetyl-residues and the subsequent decarboxylation of the malonyl-enzyme intermediate to yield $CO_2$, thereby regenerating the acetyl-enzyme. Evidence for

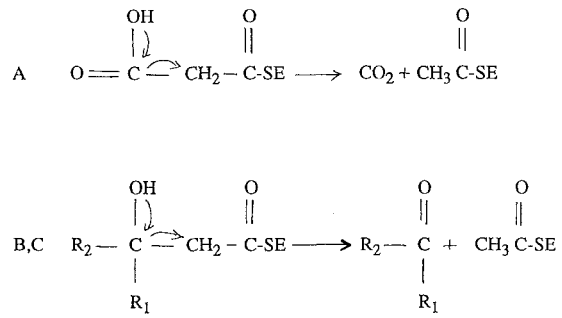


**Fig. 6. A comparison of the reactions of malonate decarboxylase, citrate lyase and citramalate lyase involving enzyme-bound thioester derivatives.** (A) Malonate decarboxylase. (B) Citrate lyase: $R_1$, COOH; $R_2$, $CH_2-COOH$. (C) Citramalate lyase: $R_1$, COOH; $R_2$, $CH_3$.

this type of catalysis is presented in this paper by the demonstration of an acetyl-enzyme as the active catalyst. The decarboxylase was completely inactivated by hydroxylamine and no activity was restored by subsequent removal of the inhibitor. Under the same conditions, an acetyl thioester would be converted into acetyl hydroxamate and the free mercaptane [25]. The enzyme was also inactivated by a variety of other conditions, e.g. incubation with NaSCN, prolonged dialysis, or a high pressure during rupture of the cells. In all these cases, the inactivation was mainly due to deacetylation, as these inactive enzyme specimens recovered activity upon chemical or enzymic acetylation with acetic anhydride or ATP plus acetate, respectively. Acetylation of a mercaptane residue of the decarboxylase is indicated by the observed increase of restored catalytic activity if the enzyme was incubated with dithioerythritol. As the crude extract dialyzed for 3–4 h contained about 0.2 mM acetate and as the enzyme which acetylates the inactive decarboxylase apparently has a low $K_m$ for acetate, the extract had to be extensively dialyzed to demonstrate the acetate requirement of this enzymic reactivation.

These findings on activation and inactivation of malonate decarboxylase by acetylation or deacetylation are reminiscent of similar observations with citrate lyase [25–27] and citramalate lyase [28]. These lyases are confronted with an analogous chemical problem to that of malonate decarboxylase: a C–C bond next to the methylene group of a non-activated acetyl-residue must be cleaved through replacement by a proton. This heterologous C–C bond cleavage creates an electron gap at the replaced carbon atom which is filled by the conversion of an hydroxyl into a carbonyl bond. The problem of low chemical reactivity for these lyase reactions is solved in all three cases by the formation of thioester bonds between the acetate-yielding carboxyl group of the substrate and a thiol group of the enzyme. A comparison of these reactions is given (Fig. 6) for the enzyme-bound thioester derivatives.

The thioester linkage creates an electron sink at the adjacent methylene carbon and thereby makes the replacement of its carbon substituent by a proton chemically feasible. Very similar conditions to those reported here for malonate decarboxylase apply for the deacetylation associated with inactivation of citrate lyase and citramalate lyase with hydroxylamine, and for the acetylation associated with reactivation of these enzymes with acetic anhydride or with acetate and ATP in the presence of a specific ligase [25–29]. It will be interesting to determine whether this analogy extends to the structure of the catalytically competent thiol-carrying residue which in the lyases is a phosphoribosyl-dephospho CoA prosthetic group bound as a phosphodiester to a specific serine residue of the proteins [30–32].

*M. rubra* grows by decarboxylation of malonate to acetate and $CO_2$ and must therefore conserve energy from this reaction [14]. Inhibition of the decarboxylase by avidin and demonstration of a single biotin-containing 120-kDa polypeptide by SDS/PAGE of the crude extract indicate that this polypeptide belongs to the decarboxylase. From these results, one may speculate that malonate decarboxylase performs the same type of energy conservation as the biotin-containing $Na^+$-transport decarboxylases, oxaloacetate decarboxylase, methylmalonyl-CoA decarboxylase and glutaconyl-CoA decarboxylase [1, 2], although with the additional requirement for substrate activation on the enzyme (see above). An energy conservation through a malonate-decarboxylase-catalyzed ion translocation is also indicated by the location of a component of this enzyme within the membrane.

The presence of only a single biotin-containing polypeptide in *M. rubra* growing on malonate is notable. Most bacteria require the biotin enzyme acetyl-CoA carboxylase to synthesize malonyl-CoA for fatty-acid biosynthesis. In *M. rubra*, malonyl-CoA could be readily provided by forming the thioester from malonate, and acetyl-CoA carboxylase may therefore be repressed by malonate. The enzyme should be present, however, if the bacteria are grown on a different substrate, e.g. fumarate [14]. It appears plausible that the malonate-decarboxylase mechanism with enzyme-bound thioesters is advantageous over the alternate route via malonyl-CoA and acetyl-CoA because competition with fatty-acid synthetase for the substrate malonyl-CoA is avoided.

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