A biotin-dependent sodium pump: glutaconyl-CoA decarboxylase from *Acidaminococcus fermentans*

Wolfgang Buckel and Roswitha Semmler

Biochemie I, Universität Regensburg, D-8400 Regensburg, FRG

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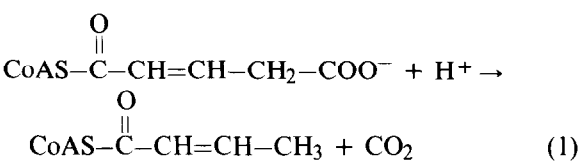
The decarboxylation of glutaconyl-CoA to crotonyl-CoA in the anaerobic bacterium *Acidaminococcus fermentans* is catalysed by a membrane-bound, biotin-dependent enzyme which requires Na^+ for activity. Inverted vesicles from *A. fermentans* accumulated Na^+ only if glutaconyl-CoA was decarboxylated. The Na^+ uptake was inhibited by avidin but not by the avidin biotin complex. Detergents and ionophores such as monensin also prevented the Na^+ transport. The results indicate that the enzyme is able to convert the free energy of decarboxylation ($\Delta G^{o\prime} \simeq -30$ kJ/mol) into a Na^+ gradient.

Glutaconyl-CoA decarboxylase *Biotin* *Sodium ion transport* *Avidin* *Triton X-100* *Monensin*

1. INTRODUCTION

During fermentation of glutamate to acetate and butyrate by the anaerobic bacterium *Acidaminococcus fermentans* glutaconyl-CoA is decarboxylated to crotonyl-CoA [1–3]:



$$\mathrm{CoAS{-}\overset{\overset{O}{\|}}{C}{-}CH{=}CH{-}CH_2{-}COO^- + H^+ \rightarrow \; CoAS{-}\overset{\overset{O}{\|}}{C}{-}CH{=}CH{-}CH_3 + CO_2} \qquad (1)$$

The enzyme catalysing this reaction has two distinctive properties: it contains biotin and it is bound to membranes. This led to the assumption that the free energy of decarboxylation ($\Delta G^{o\prime} \simeq -30$ kJ/mol) may be used to transport ions through the membrane [1,2]. In the meantime Dimroth has established that this hypothesis is correct. He discovered that the closely related enzyme oxaloacetate decarboxylase from *Klebsiella aerogenes* is a biotin-dependent sodium pump [4,5]. Furthermore, the pump could be reconstituted from the purified biotin enzyme and phospholipid vesicles [6,7]. In [8] methylmalonyl-CoA decarboxylase from *Veillonella alcalescens* was shown to act as a similar sodium pump. Stimulated by these results we investigated the cation requirements of glutaconyl-CoA decarboxylase and found that this enzyme represented the third example of a sodium pump in anaerobic bacteria driven by the free energy of decarboxylation.

2. MATERIALS AND METHODS

Acidaminococcus fermentans ATCC 25085 was grown on glutamate–yeast extract–biotin medium [2]. Inverted vesicles [9] were prepared from 30 g wet cells suspended in 120 ml 50 mM K-phosphate (pH 7) containing 5 mM $MgCl_2$ and desoxyribonuclease I (10 μg/ml). After two passages through a French-press cell at 20 000 lb/in.^{2}, cell debris was removed by centrifugation at 30 000 × *g* for 15 min. The supernatant was again centrifuged at 200 000 × *g* for 90 min. The pellet was homogenized in 5 ml 50 mM K-phosphate (pH 7) and washed once. The resulting vesicles (8 ml, 27 mg protein/ml and 1 U decarboxylase/mg protein) were stored in portions at −80°C. The auxiliary enzymes for the decarboxylase assay were prepared from the 200 000 × *g* supernatant by treatment with streptomycin sulfate (1.5%) followed by chromatography on the DEAE–Sephacell and

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Sephacryl columns as described for glutaconate CoA-transferase [3]. Finally, the concentrated eluate was dialysed against 50 mM K-phosphate (pH 7.0) containing 1 mM DTE and 1 mM K-EDTA. The enzyme mixture was stored at 4°C and was stable for several months. Sodium was determined by flame photometry. Traces of sodium were removed from NAD by a passage through AG 50 × 8, K^+, which was prepared with KOH containing <0.002% Na^+ (Merck, Darmstadt).

The assay of glutaconyl-CoA decarboxylase contained in a cuvette 50 mM K-phosphate (pH 7.0), 0.1% Triton X-100, 20 mM NaCl, 2 mM NAD, 2 mM DTE, 2 mM K-EDTA, 0.1 mM CoASH, 0.1 mM acetyl-CoA, auxiliary enzymes 0.2 mg/ml and decarboxylase up to 0.1 U/ml. The reaction was initiated by addition of 1 mM K_2-glutaconate and the formation of NADH was measured at 366 nm. The activities of the auxiliary enzymes were determined as in [10]. Sodium transport was estimated from appropriate samples which were passed over columns (0.5 × 2 cm) of AG 50 × 8, K^+, 200–400 mesh, to separate free $^{22}Na^+$ from $^{22}Na^+$ incorporated into vesicles [5,11]. Protein was determined by the biuret method.

3. RESULTS

3.1. *Assay of the decarboxylase activity*

Glutaconyl-CoA decarboxylase was assayed spectrophotometrically through the formation of NADH by the aid of 5 enzymes which were partially purified together from *Acidaminococcus fermentans*. The mixture contained glutaconate CoA-transferase (16 U/mg protein [3]), enoyl-CoA hydratase (EC 4.2.1.17, 12 U/mg), 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35, 23 U/mg), thiolase (acetyl-CoA acetyltransferase, EC 2.3.1.9, 12 U/mg) and phosphate acetyltransferase (EC 2.3.1.8, 6 U/mg). The first enzyme, CoA-transferase was necessary to produce the substrate glutaconyl-CoA from acetyl-CoA and glutaconate. The next two enzymes were essential for measuring the decarboxylase reaction itself whereby the product crotonyl-CoA was hydrated and oxidized by NAD to acetoacetyl-CoA.

The latter compound was removed from the equilibrium by cleavage to 2 molecules of acetyl-CoA. Finally, CoASH was regenerated by the phosphate acetyltransferase reaction. In summary, glutaconate is oxidized to CO_2, acetate and acetylphosphate in the presence of catalytic amounts of acetyl-CoA:

$$\text{Glutaconate}^{2-} + \text{NAD}^+ + \text{H}_2\text{O} + \text{HPO}_4^{2-} \rightarrow \text{Acetate}^- + \text{CO}_2 + \text{NADH} + \text{acetylphosphate}^{2-} \quad (2)$$

With [2,4-^{14}C]glutaconate [2] as substrate, the product [2-^{14}C]acetate was identified by steam distillation and chromatography on amberlite IRC 50 [12]. It was degraded to unlabelled CO_2 and [^{14}C]-methylamine [13].

The other product acetylphosphate was estimated by the hydroxamic acid method [14]. The rate of NADH formation was proportional to the amount of added decarboxylase within a wide range. The assay was also used to determine glutaconate concentrations.

3.2. *Activation by cations and detergents*

The activity of glutaconyl-CoA decarboxylase was reduced significantly if Na^+ was carefully omitted from the assay system. Readdition of 20 mM NaCl or 10 mM Na_2SO_4 resulted in a 6–8-fold increase in activity. Since the 'sodium-free'

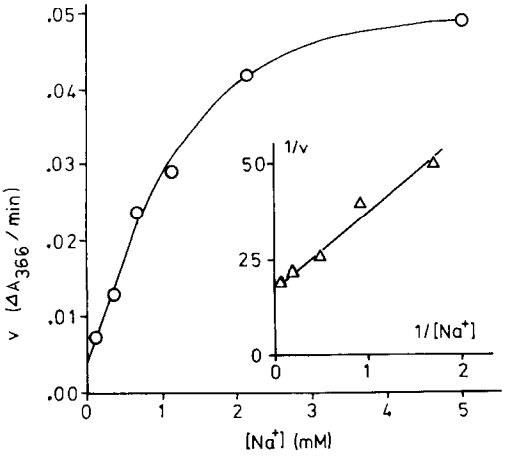

Fig.1. Dependence of glutaconyl-CoA decarboxylase activity on [Na^+]. Inverted vesicles (17 μg protein) were assayed as in section 2 but in the presence of varying [NaCl] as indicated. The $1/v$ values of the inserted graph were calculated from the differences of the observed velocities and the velocity at [Na^+] = 0 (0.004 ΔA_{366}/min).

assay system still contained 0.1 mM Na^+ as determined by flame photometry, the residual activity at $[Na^+] = 0$ was obtained by extrapolation. Thus the overall activation was 8–10-fold with an app. K_M of ~1.0 mM (fig.1). Lithium ions also activated the enzyme 5-fold, however, the app. K_M was 100 mM. The activation by Na^+ was inhibited by Li^+, e.g., in the presence of 20 mM Li^+ the app. K_M for Na^+ was raised from 1 to 5 mM. The effect of other cations was not investigated, but all these results were obtained in the presence of ~100 mM K^+.

The activity of the decarboxylase in inverted vesicles was increased 2-fold in the presence of 0.1% Triton X-100. Therefore 0.1% Triton was added to routine assays. This activation appeared to be specific, since 0.1% Brij 35 activated only 1.3-fold whereas *n*-octylglucoside and desoxycholate 0.1% each were inhibitory (30% and 100% inhibition, respectively).

3.3. *Sodium transport*

On incubation of inverted vesicles with glutaconyl-CoA generated from glutaconate and acetyl-CoA, Na^+ were rapidly incorporated (fig.2). Maximum uptake was reached within 1–2 min during which time the decarboxylation reaction (2) went to completion. Thereafter Na^+ was released again from the vesicles, probably due to leaky membranes. The other two experiments in fig.2 demonstrated that Na^+ was only transported if glutaconyl-CoA was present. In one incubation acetyl-CoA was omitted. Nevertheless, transport was observed but with a lower rate and a lag phase of ~2 min. Presumably, tiny amounts of an acyl-CoA, necessary to start the reaction, were present as contaminants in the auxiliary enzymes. However, if both acetyl-CoA and NAD were omitted, the formation of glutaconyl-CoA was not possible and no transport was observed until acetyl-CoA was added.

Glutaconyl-CoA decarboxylase was inhibited by avidin but remained active in the presence of an avidin–biotin complex [2]. Na^+ transport was influenced by these agents in the same manner (table 1).

This result confirmed the decarboxylation dependence pumping activity. However, if the membrane was destroyed by 0.1% Triton the Na^+ transport was completely abolished whereas the

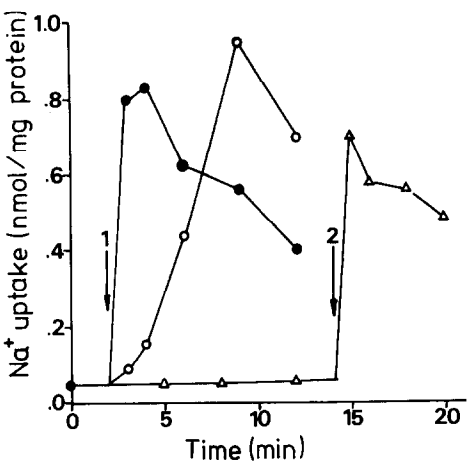

Fig.2. The complete incubation contained at 25°C: 75 mM K-phosphate (pH 7.0), 1.5 mM DTE, 1.5 mM K-EDTA, 0.12 mM acetyl-CoA, 0.1 mM CoASH, 1.2 mM NAD, auxiliary enzymes 0.15 mg/ml and 1.6×10^6 cpm ^{22}Na/ml, spec. act. 5×10^3 cpm/nmol. Inverted vesicles were added at time zero. Samples of 50 µl were withdrawn at times indicated and analysed for $^{22}Na^+$ incorporation into vesicles: transport was initiated with 1.2 mM K_2-glutaconate (1→); at (2→) 0.12 mM acetyl-CoA was added; complete incubation (•); acetyl-CoA omitted (○); acetyl-CoA and NAD omitted (△).

Table 1

Inhibitors of sodium transport

Exp. no.	Additions	Maximum sodium uptake	
		cpm	nmol/mg protein
1	None	2650	1.09
	Avidin (0.5 mg/ml)	325	0.14
	Avidin and biotin (3 mM)	2767	1.13
	Glutaconate omitted	154	0.06
2	None	2164	0.89
	Monensin 0.5 nM	2279	0.94
	Monensin 5.1 nM	1804	0.74
	Monensin 51 nM	286	0.12
	Monensin 510 nM	85	0.03

The incubations were performed as in fig.2. Maximum sodium uptake was obtained within 1–2 min after the addition of glutaconate

decarboxylase activity was increased (see section 3.2.). Ionophores such as gramicidin A (60 μM) and monensin (table 1) prevented the accumulation of Na^+ in the vesicles, as expected. The latter compound [15] was extremely active. Only 10 nM was required for 50% inhibition. Valinomycin (30–3000 nM) in the presence of 100 mM K^+ and carbonylcyanide – *p*-trifluoromethylphenylhydrazone (FCCP 80 μM) had no effect on the Na^+ transport into inverted vesicles.

4. DISCUSSION

These results show clearly that the biotin-dependent glutaconyl-CoA decarboxylase is a sodium pump. It closely resembles oxaloacetate and methylmalonyl-CoA decarboxylases. Thus the apparent K_M-values for Na^+ for all 3 enzymes (~ 1 mM) and even the amount of Na^+ transported into the vesicles (~ 1 nmol/mg membrane protein) are identical. Differences such as the failure to stimulate the Na^+ uptake by valinomycin with glutaconyl-CoA decarboxylase presumably show differences in the membrane components rather than in the enzymes themselves. Furthermore, preliminary experiments demonstrate that an active sodium pump can be reconstituted from the purified decarboxylase and phospholipid vesicles. In this system valinomycin (25–250 nM in the presence of 100 mM K^+ stimulates the initial velocity of Na^+ uptake by a factor of 2. This experiment indicates that the Na^+ transport rate is limited in reconstituted vesicles by the potential induced by the influx of Na^+, whereas valinomycin compensates this potential by catalysing an efflux of K^+.

This paper demonstrates the conversion of the free energy of decarboxylation into a Na^+ gradient. It was believed that such gradients are only established by the hydrolysis of ATP [16]. However, there are also examples of anabolic processes which are driven by decarboxylations: gluconeogenesis and the biosynthesis of fatty acids, sphingosin and porphyrins.

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