

Aspartate 203 of the oxaloacetate decarboxylase β -subunit catalyses both the chemical and vectorial reaction of the Na^+ pump

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We report here a new mode of coupling between the chemical and vectorial reaction explored for the oxaloacetate decarboxylase Na^+ pump from *Klebsiella pneumoniae*. The membrane-bound β -subunit is responsible for the decarboxylation of carboxybiotin and the coupled translocation of Na^+ ions across the membrane. The biotin prosthetic group which is attached to the α -subunit becomes carboxylated by carboxyltransfer from oxaloacetate. The two conserved aspartic acid residues within putative membrane-spanning domains of the β -subunit (Asp149 and Asp203) were exchanged by site-directed mutagenesis. Mutants D149Q and D149E retained oxaloacetate decarboxylase and Na^+ transport activities. Mutants D203N and D203E, however, had lost these two activities, but retained the ability to form the carboxybiotin enzyme. Direct participation of Asp203 in the catalysis of the decarboxylation reaction is therefore indicated. In addition, all previous and present data on the enzyme support a model in which the same aspartic acid residue provides a binding site for the metal ion catalysing its movement across the membrane. The model predicts that Asp203 in its dissociated form binds Na^+ and promotes its translocation, while the protonated residue transfers the proton to the acid-labile carboxybiotin which initiates its decarboxylation. Strong support for the model comes from the observation that Na^+ transport by oxaloacetate decarboxylation is accompanied by H^+ transport in the opposite direction. The inhibition of oxaloacetate decarboxylation by high Na^+ concentrations in a pH-dependent manner is also in agreement with the model. **Keywords:** bacterial Na^+ pump/coupling/mutagenesis/transport

Introduction

Sodium ion-translocating decarboxylases are among the simplest vectorial catalysts with respect to their structure and are, therefore, well suited to explore the relationship between the chemical and vectorial reaction. Oxaloacetate decarboxylase from *Klebsiella pneumoniae* is the best studied member of this family. Physiologically, this enzyme acts as generator of an electrochemical Na^+ potential during citrate fermentation (reviewed by Dimroth, 1987). The Na^+ gradient is required to drive citrate uptake (Dimroth and Thomer, 1986a; van der Rest *et al.*, 1992; Pos and Dimroth,

1996) and to gain NADH for biosynthesis by reversed electron transfer catalysed by a unique Na^+ -dependent NADH:ubiquinone oxidoreductase (Pfenninger-Li and Dimroth, 1992).

Oxaloacetate decarboxylase is composed of only three different subunits, the peripheral α -subunit and the integral membrane-bound subunits β and γ . The catalytic cycle is initiated by transfer of the carboxyl group from oxaloacetate to the prosthetic biotin group which is located within the C-terminal domain of the α -subunit. This step is catalysed by the N-terminal carboxyltransferase domain of the α -subunit and is independent of the presence of Na^+ ions (Dimroth and Thomer, 1983). The sequence between the two domains of the α -subunit consists of an extended stretch of mostly alanine and proline residues (Schwarz *et al.*, 1988). This is believed to form a flexible region required to bring the carboxybiotin residue to the catalytic centre of the decarboxylase located on the membrane-bound $\beta\gamma$ -subunits. The strict requirement for Na^+ ions for the decarboxylation of carboxybiotin suggests that this step is coupled to Na^+ translocation. The β -subunit is protected specifically from proteolysis by Na^+ ions, which is further evidence for an Na^+ binding site on this subunit (Dimroth and Thomer, 1983, 1992).

The *oadGAB* genes encoding the γ -, α - and β -subunits of the oxaloacetate decarboxylase from *K.pneumoniae* and *Salmonella typhimurium* were cloned and sequenced (Schwarz and Oesterhelt, 1985; Schwarz *et al.*, 1988; Laußermair *et al.*, 1989; Woehlke *et al.*, 1992a,b) and the sequences were compared with those of other members of the Na^+ -translocating decarboxylases, i.e. methylmalonyl-CoA decarboxylase from *Veillonella parvula* (Huder and Dimroth, 1993) and *Propionigenium modestum* (M.Bott, personal communication) and glutaconyl-CoA decarboxylase from *Acidaminococcus fermentans* (W.Buckel, personal communication). Remarkably, the β -subunits from all of these complexes contained extended stretches of sequence identity in the putative membrane-spanning segments. The only negatively charged residues in membrane-spanning domains are two conserved aspartate residues (Asp149 and Asp203, numbering according to the oxaloacetate decarboxylase β -subunit from *K.pneumoniae*), which therefore have been assumed to function in Na^+ binding and translocation across the membrane.

The role of Asp149 and Asp203 of the β -subunit has now been studied by mutagenesis with an *Escherichia coli* expression clone of the *oad* genes (Di Berardino and Dimroth, 1995). We show here that mutation of Asp149 had no effect on the function of the decarboxylase Na^+ pump, whereas Asp203 was absolutely essential. Based on these and additional biochemical data described here and elsewhere, we have built a model that accounts for the coupling between chemical and vectorial catalysis, i.e. the decarboxylation of the carboxybiotin and the transport

Table I. Enzymic properties of purified wild-type or mutant oxaloacetate decarboxylase

Purified oxaloacetate decarboxylase	Specific activity (U/mg)	Na ⁺ transport into membrane vesicles	Mol ¹⁴ C ₂ bound per mol enzyme
Wild-type enzyme	58.3	+	0.03
Asp149→Gln mutant	61.3	+	0.04
Asp149→Glu mutant	62.6	n.d.	n.d.
Asp203→Asn mutant	0.0	–	0.98
Asp203→Glu mutant	0.0	n.d.	n.d.

The enzyme complex was purified by monomeric avidin–Sepharose affinity chromatography and the decarboxylation activity determined with the coupled spectrophotometric assay as described by Dimroth (1986). *Escherichia coli* DH5 α membrane vesicles (4.6 mg of protein) containing the expressed wild-type or mutant oxaloacetate decarboxylase were prepared according to Di Bernardino and Dimroth (1995) and Na⁺ transport was monitored using the ²²Na⁺ isotope (Dimroth, 1982b). Carboxylation of the enzyme complex with ¹⁴C₂ from [4-¹⁴C]oxaloacetate was performed as described (Di Bernardino and Dimroth, 1995) and the bound radioactivity determined after incubation of the purified enzyme samples (45–100 μ g of protein) with 0.05 μ mol [4-¹⁴C]oxaloacetate (5×10^5 c.p.m.) at 25°C for 1 min. n.d., not determined.

of Na⁺ ions across the membrane. In this model Asp203 in its dissociated form plays a central role in Na⁺ binding and translocation across the membrane, while the protonated Asp203 is essential for the catalysis of the decarboxylation of the acid-labile carboxybiotin. Strong support for this mechanism comes from the discovery that the oxaloacetate decarboxylase Na⁺ pump translocates protons in the opposite direction.

Results

Site-directed mutagenesis of Asp149 and Asp203 of the β -subunit

The sequences of the β -subunits from several members of the Na⁺ transport decarboxylase family are remarkably similar. The only conserved negatively charged residues within putative membrane-spanning domains are Asp149 and Asp203 of the β -subunit. These residues are located within highly conserved regions of the protein. They were therefore candidates to function as Na⁺ binding sites during transport. To investigate this hypothesis, each of these aspartic acid residues was exchanged by a number of other amino acids by site-directed mutagenesis with the mixed oligonucleotides oligo D149X and oligo D203X, respectively, as described in Materials and methods. Mutant clones were sequenced and the amino acid substitutions were identified. As there were no Asp→Glu mutations at both positions among those analysed, we constructed these with the specific oligonucleotides oligo D149E and oligo D203E, respectively. Mutations D149Q, D149E, D203N and D203E were investigated further because these amino acid substitutions are assumed to cause the least structural changes compared with the wild-type enzyme.

Enzymic properties of oxaloacetate decarboxylase with mutations in Asp149 or Asp203 of the β -subunit

The effect of the β -subunit mutations on oxaloacetate decarboxylase and Na⁺ transport activities was investigated after synthesis of the mutant proteins in *E. coli* and purification by avidin–Sepharose affinity chromatography. The oxaloacetate decarboxylase activities of the D149Q and D149E mutants were indistinguishable from that of the wild-type enzyme and so was the Na⁺ transport activity (determined with the D149Q mutation only; Table I). Therefore, Asp149 is not an essential residue either for

Na⁺ transport or for oxaloacetate decarboxylase activity. However, the D203N and D203E mutant proteins were completely inactive in oxaloacetate decarboxylation and Na⁺ transport activities. No trace of oxaloacetate decarboxylase activity of the D203E mutant protein was found between pH 5.5 and 9.0 with Na⁺ or Li⁺ concentrations up to 100 mM: This position of the enzyme is therefore apparently very sensitive to even minute structural changes such as the replacement of an aspartate by a glutamate residue. We assumed from these results that the first partial reaction, i.e. the carboxyltransfer from oxaloacetate to the prosthetic biotin group, was not affected by mutating Asp203 of the β -subunit. The enzyme with the D203Q mutation became rapidly labelled upon incubation with [4-¹⁴C]oxaloacetate (Table I), which indicates transfer of the labelled carboxylate to the biotin. As the subsequent decarboxylation of the carboxybiotin enzyme was impaired, Na⁺ ions were without effect on the labelling of the enzyme. In contrast, the carboxybiotin-containing protein of the wild-type or of the Asp149 mutant oxaloacetate decarboxylase was only obtained if the presence of Na⁺ ions was carefully excluded (Dimroth, 1982a; data not shown). These results show that Asp203 is an essential residue for the Na⁺-dependent decarboxylation of carboxybiotin.

Kinetic studies with wild-type oxaloacetate decarboxylase

The activity of wild-type oxaloacetate decarboxylase was inhibited in the presence of Na⁺ concentrations above 30 mM. The inhibitory effect was investigated over the pH range from 5.3 to 9.0 by comparing decarboxylase activities at 25 mM NaCl (V_{\max}) with those at 150 mM NaCl. The results shown in Figure 1 indicate 40–45% inhibition at the high Na⁺ concentration between pH 5.3 and 7.0 and a significant increase in this inhibition at higher pH values, to reach 85% at pH 9.0. In this region, the profile was that of a titration curve with a pK of ~8.0. No inhibition was observed with 130 mM KCl, indicating that it was specific for Na⁺ ions. These results might indicate a competition of Na⁺ and H⁺ for binding to the same amino acid residue. Protonation of this group, which is essential for the decarboxylation of the carboxybiotin, would be impaired by high Na⁺ concentrations and a high pH.

The participation of protons (not H₂O) as a substrate of the decarboxylation of carboxybiotin with the formation

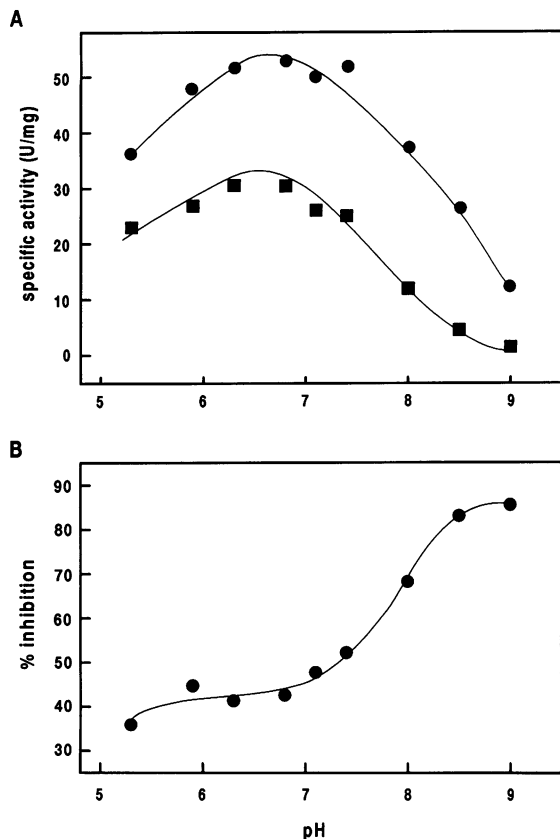


Fig. 1. Inhibition of the oxaloacetate decarboxylase activity by 150 mM NaCl. (A) The decarboxylase activity was determined with the simple spectrophotometric assay as described (Dimroth, 1986) in 40 mM Mes/Tris buffer in the presence of 25 mM NaCl (●) or 150 mM NaCl (■) at the pH values indicated. The values are means of two measurements. (B) The activities in the presence of 25 mM NaCl (0% inhibition) and 150 mM NaCl (x% inhibition) were compared.

of CO_2 (not HCO_3^-) has been demonstrated previously (Dimroth and Thomer, 1983). Protonation of carboxybiotin is known to increase its rate of decarboxylation dramatically via a process involving concerted proton transfer to yield CO_2 and the isourea form of biotin (Figure 4B). Conceivably, the enzyme performs a similar protonation of the carboxybiotin from a protonated amino acid residue at the active site.

After transfer of the proton from Asp203 to the carboxybiotin, the aspartate could be re protonated from either of the two sides of the membrane. If the proton derived from the side to which Na^+ ions were pumped, the interior of proteoliposomes accumulating Na^+ ions should become alkaline. One way of testing this is by comparing the effect of an uncoupler with that of valinomycin on Na^+ transport. Both ionophores dissipate the rate-limiting membrane potential and should be equipotent in stimulating Na^+ transport if no pH gradient is involved. This has in fact been observed with the reconstituted Na^+ -pumping F_1F_0 ATPase from *P.modestum* (Laubinger and Dimroth, 1988). The effect of these ionophores on Na^+ transport by reconstituted oxaloacetate decarboxylase is shown in Figure 2. While the rate of Na^+ transport was about double in the presence of valinomycin, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) did not stimulate the transport except in the early stage of the development

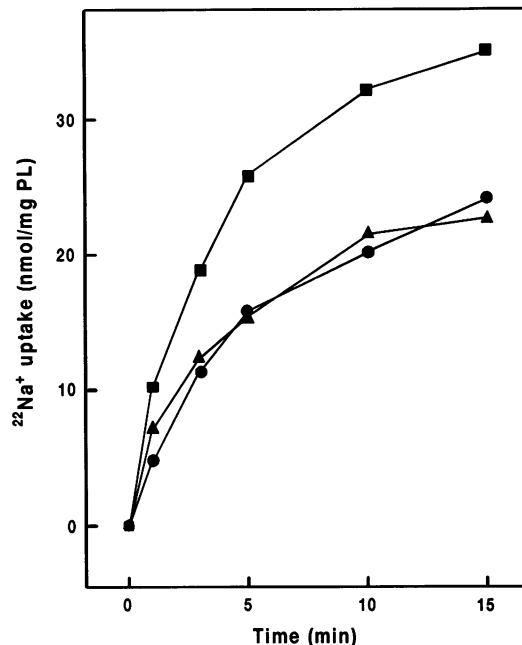


Fig. 2. Effect of the ionophores valinomycin and CCCP on Na^+ uptake into proteoliposomes. Oxaloacetate decarboxylase was reconstituted into proteoliposomes by the detergent dilution method as described in Materials and methods. To the proteoliposomes (2.65 mg of phospholipids) which were diluted into the assay buffer containing 0.5 mM $^{22}\text{Na}_2\text{SO}_4$ (270 d.p.m./nmol), 0.2 μM valinomycin (■) or 0.3 μM CCCP (▲) was added and the decarboxylation reaction started by the addition of 1 mM oxaloacetate (at time = 0 min). (●) Proteoliposomes without the addition of ionophores.

of the Na^+ gradient. These results are expected if the decarboxylation of oxaloacetate forms a H^+ gradient opposite to the Na^+ gradient. Movement of H^+ against ΔpH is unfavourable and may not lead to a (complete) dissipation of the rate-limiting membrane potential. In contrast, no such restraints exist for the valinomycin-induced K^+ movement.

The alkalization of the inner compartment of the proteoliposomes upon oxaloacetate decarboxylation was monitored directly by measuring $[1-^{14}\text{C}]$ acetate uptake. The undissociated acetic acid but not the acetate anion diffuses over the membrane into the inner compartment of the proteoliposomes, where it dissociates and accumulates if this compartment is more alkaline than the environment. Simple dilution of the proteoliposomes into 5 mM potassium phosphate buffer, pH 7.0, did not lead to acetate accumulation on the inside, as expected (Figure 3, data points before oxaloacetate addition). The acetate concentration in the inner compartment increased rapidly, however, after oxaloacetate addition, indicating an alkalization of the inner compartment. This alkalization was due to oxaloacetate decarboxylation because no acetate was accumulated if the decarboxylase was inhibited by 1.5 mM oxalate. Dissipation of the membrane potential generated by the Na^+ pump by adding valinomycin did not significantly affect the acetate accumulation within the proteoliposomes. The pH gradient (inside alkaline) is therefore not generated by the membrane potential-directed passive proton movement across the membrane. Rather, the oxaloacetate decarboxylase catalyses proton pumping in the opposite direction to Na^+ pumping. After ~20 min,

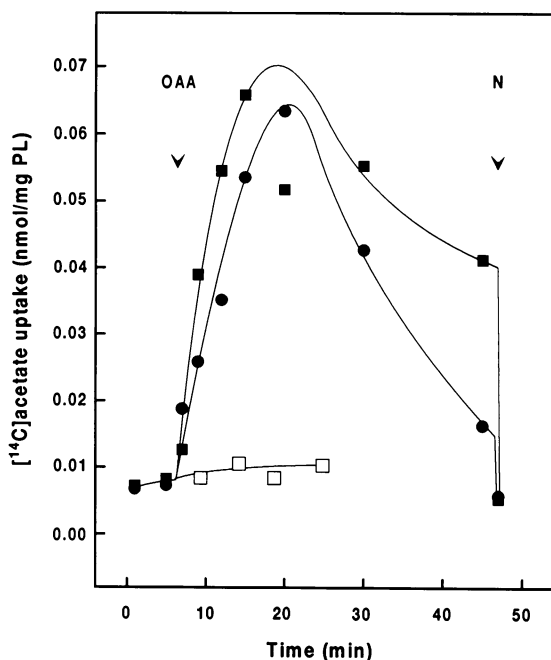


Fig. 3. Decarboxylation-dependent [^{14}C]acetate uptake into proteoliposomes. Oxaloacetate decarboxylase was reconstituted into proteoliposomes as described in Materials and methods. The proteoliposomes (2.2 mg of phospholipids, 215 mU oxaloacetate decarboxylase) were diluted into 5 mM potassium phosphate buffer, pH 7.0, containing 42.8 μM [^{14}C]acetate (120 000 d.p.m./nmol) in the presence (●) or absence (■) of 0.12 μM valinomycin. 1 mM oxaloacetate (OAA) was added to start the decarboxylation reaction. In a parallel assay, 1.5 mM oxalate was added prior to the addition of OAA (□). By the addition of 0.75 μM nigericin (N), which promotes the exchange of H^+ for K^+ across the membrane, the previously formed ΔpH is dissipated, leading to the efflux of the labelled acetate.

when the oxaloacetate was completely decarboxylated, the pH gradient decreased continuously, indicating that the protons could leak slowly through the membrane of the proteoliposomes. This is not the case for Na^+ ions, because the Na^+ gradient remains stable for ~ 1 h after completion of oxaloacetate decarboxylation. The addition of nigericin completely collapsed the pH gradient, as shown by the complete reversal of [^{14}C]acetate accumulation. In control experiments with proteoliposomes containing the Na^+ -pumping F_1F_0 ATPase from *P.modestum*, Na^+ transport into the interior volume was not accompanied by its alkalization, and no [^{14}C]acetate was accumulated (data not shown).

Discussion

In designing a mechanism for Na^+ transport by oxaloacetate decarboxylase, the following observations are important. (i) The membrane-bound subunits β and γ alone are unable to catalyse Na^+ translocation (counterflow) across the membrane (Di Berardino and Dimroth, 1995). (ii) Na^+ transport (counterflow) is catalysed by the $\alpha\beta$ complex in the absence of oxaloacetate, but only under conditions where the biotin can be carboxylated. Na^+ counterflow, therefore, required the presence of bicarbonate and was abolished in the presence of avidin (Dimroth and Thomer, 1993). These results suggest that the transport of Na^+ ions is coupled obligatorily to the carboxylation

of biotin or the decarboxylation of carboxybiotin and that the latter species directly participates in the translocation events. (iii) High Na^+ concentrations (>25 mM) are required to protect the β -subunit from proteolysis (Dimroth and Thomer, 1983, 1992), which indicates a low affinity binding site for Na^+ on this subunit. In contrast, the K_m for Na^+ during oxaloacetate decarboxylation is <1 mM, which suggests a second Na^+ binding site in the carboxylated enzyme specimen. The most likely interpretation of these results is that the carboxybiotin, when bound to the β -subunit, directly participates in the high affinity binding of Na^+ ions.

On comparing the primary structures and the predicted topological models of the β -subunits from oxaloacetate decarboxylase from *K.pneumoniae* and *S.typhimurium* (Woehlke *et al.*, 1992a,b) and the related Na^+ pump methylmalonyl-CoA decarboxylase from *V.parvula* (Huder and Dimroth, 1993), only two conserved negatively charged amino acid residues are located in putative membrane-spanning helices. These residues (Asp149 and Asp203) of the β -subunit were therefore considered as key candidates to participate in Na^+ binding and translocation across the membrane. Consequently, we replaced both aspartic acid residues individually with glutamine, asparagine or glutamic acid residues by site-directed mutagenesis and studied the properties of the resultant mutant decarboxylases. The two Asp149 mutations were without detectable effect on oxaloacetate decarboxylase or Na^+ transport activities, clearly indicating that Asp149 is not a Na^+ binding site. However, the two Asp203 mutations led to a completely inactive oxaloacetate decarboxylase. The reason for this inactivation was the inability to catalyse the Na^+ -dependent decarboxylation of the carboxybiotin enzyme, while the carboxyltransfer from oxaloacetate to the prosthetic biotin group was not affected. As the Asp \rightarrow Asn or Asp \rightarrow Glu mutations are unlikely to induce major structural changes that could abolish the decarboxylase activity, the best interpretation of these results is a direct participation of Asp203 in the catalysis of the Na^+ -dependent decarboxylation of the carboxybiotin and in the transport of Na^+ that is linked to it.

In the following we want to describe a model for the decarboxylation of enzyme-bound carboxybiotin and the coupled transport of Na^+ , in which Asp203 of the β -subunit plays a key role in both the chemical and the vectorial reaction. In this model (Figure 4A) two different Na^+ binding sites are envisaged, one with high affinity at the β -subunit-bound carboxybiotin and one with low affinity at Asp203 of this subunit. The high affinity site is accessible from the cytoplasm (under physiological conditions) and must be occupied by Na^+ if decarboxylation is to occur. The low affinity site at Asp203 is accessible from the periplasm and may be occupied by Na^+ , H^+ , or remain empty. It is assumed further in this model that only one of the two Na^+ binding sites can be occupied by this alkali ion at any time, but that protonation of Asp203 does not prevent occupation of the high affinity site by Na^+ . The catalytic cycle may occur through steps A-E (Figure 4A) as follows. As soon as the biotin prosthetic group on the α -subunit becomes carboxylated by the carboxyltransfer from oxaloacetate (E \rightarrow A), the negatively charged carboxybiotin could move into a binding pocket on the β -subunit where it becomes part of the

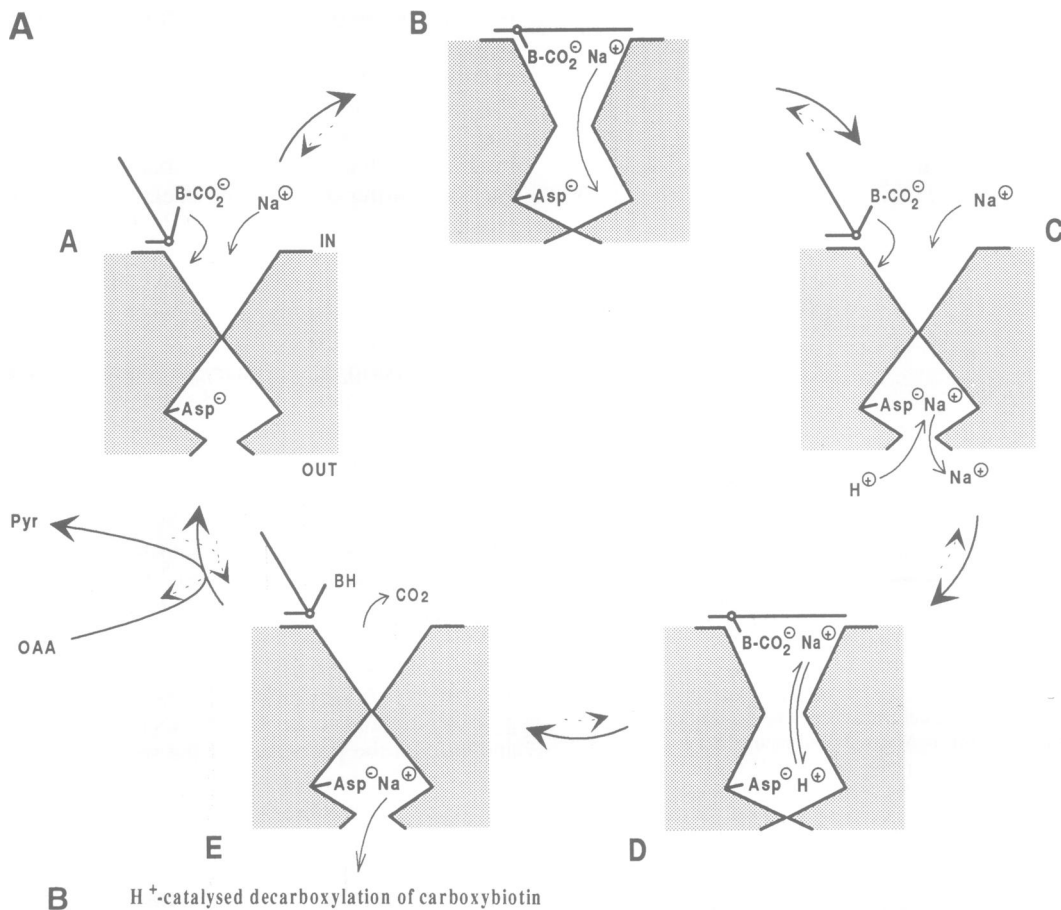


Fig. 4. (A) Model for the coupling between oxaloacetate decarboxylation and Na⁺ translocation: (for details see text). (B) Hypothetical reaction mechanism for the decarboxylation of carboxybiotin.

Na⁺ binding site. This movement of the (carboxy) biotin group between the catalytic centres on the α- and β-subunits, respectively, is facilitated by the extended alanine/proline-rich sequence between the carboxyltransferase and the biotin binding domain of the α-subunit (Schwarz *et al.*, 1988). The binding of Na⁺ at the high affinity site of the β-subunit requires the simultaneous presence of carboxybiotin at this site and is fundamental for the subsequent transfer of the alkali ion to Asp203 (state B). In this state, the protein assumes a closed conformation, where an exchange of Na⁺ is only possible between the two sites but not with the surrounding fluid. The Na⁺ transfer may be facilitated by releasing the carboxybiotin residue from the binding pocket in the hydrophobic environment of the β subunit (B→C). The associated conformational change of the protein exposes the Na⁺ binding site at Asp203 to the outside of the membrane, where it can be displaced by another Na⁺ ion or by a

proton. Only the latter substitution initiates the decarboxylation of carboxybiotin through steps D–E and leads to a net Na⁺ translocation. To reach step D, it is further necessary that the high affinity site on the carboxybiotin is occupied by a new Na⁺ ion. The protein thereby resumes the closed conformation (state D) in which an exchange of Na⁺ and H⁺ between the two sites becomes possible. Upon acceptance of the proton from Asp203, the carboxybiotin undergoes immediate decarboxylation which opens the conformation so that the Na⁺ ion can be released from its low affinity site at Asp203 to the periplasmic side of the membrane. Thus, when the cycle reaches state E, a net extrusion of two Na⁺ ions and the transport of one H⁺ in the opposite direction per decarboxylation of one oxaloacetate to pyruvate and CO₂ is achieved.

The model described above applies to situations of modest $\Delta\mu_{\text{Na}^+}$ values. At high $\Delta\mu_{\text{Na}^+}$ in reconstituted

proteoliposomes decarboxylation was observed that involved an exchange of external and internal Na^+ ions but no net accumulation (Dimroth and Thomer, 1993). This uncoupled reaction sequence must therefore include steps that lead to Na^+ movement in the opposite direction to Na^+ pumping. This Na^+ movement can be explained by the preferential occupation of Asp203 in state C by Na^+ rather than by H^+ , leading to the reverse reaction sequence $\text{C} \rightarrow \text{B} \rightarrow \text{A} \rightarrow \text{C}$ rather than continuing from state C to state E (see below).

Observations in accord with the model

The experimental data that were collected over the years on the catalytic mechanism of the oxaloacetate decarboxylase Na^+ pump are in agreement with our model. Some of the observations will be listed below and will be discussed with respect to the model.

(i) In the absence of a $\Delta\bar{\mu}\text{Na}^+$, the oxaloacetate decarboxylase Na^+ pump operates at an Na^+ to oxaloacetate stoichiometry of ~ 2 (Dimroth and Thomer, 1993). This observation can be explained by the operation of the Na^+ pump through a complete reaction cycle (steps A–E). This mechanism is also in accord with H^+ movement in the opposite direction and with the electrogenicity of Na^+ transport. The latter observation clearly implies that the number of Na^+ ions moving from the inside to the outside exceeds the number of protons moving in the opposite direction.

(ii) At high $\Delta\bar{\mu}\text{Na}^+$, the Na^+ to oxaloacetate stoichiometry drops and the pump catalyses an exchange of internal and external Na^+ ions (Dimroth and Thomer, 1993). This observation can be explained if reaction steps are included by which Na^+ ions can move in the opposite direction to Na^+ pumping. Forced by the high Na^+ concentration and the positive electrical potential on the outside, Asp203 will be occupied preferentially by Na^+ in states where this residue is accessible (C and/or E). According to our model, occupation of Asp203 by Na^+ prevents binding of this alkali ion to carboxybiotin from the inside of the cells and favours, therefore, the back reaction ($\text{C} \rightarrow \text{A}$) which leads to Na^+ movement from the outside to the inside. The Na^+ movement in this direction continues until a proton is bound to Asp203 in state C and initiates continuation of the pump cycle through steps D–E. A competition between Na^+ and H^+ for the binding to Asp203 in state C is also in accord with the inhibition of oxaloacetate decarboxylation by high Na^+ concentrations, especially at high pH. These conditions favour occupation of Asp203 by Na^+ rather than by H^+ , which prevents the enzyme from proceeding in the direction of decarboxylation through steps D and E.

(iii) In the presence of a large $\Delta\bar{\mu}\text{Na}^+$, pyruvate is carboxylated to oxaloacetate (Dimroth and Hilpert, 1984). This observation can be explained by the complete reversal of the pump cycle ($\text{E} \rightarrow \text{D} \rightarrow \text{C} \rightarrow \text{B} \rightarrow \text{A} \rightarrow \text{E}$).

(iv) The pump catalyses Na^+ counterflow in the absence of oxaloacetate, dependent on CO_2 and sensitive to avidin (Dimroth and Thomer, 1993; Di Berardino, unpublished data). This observation is in accord with the reversible operation of the pump through steps $\text{E} \rightarrow \text{C}$ or $\text{E} \rightarrow \text{A}$, but not involving an $\text{A} \rightarrow \text{E}$ interconversion. After Asp203 has been loaded with the Na^+ from the side with the high Na^+ concentration, and the free biotin and CO_2 have

been bound to the binding pocket on the β -subunit, the carboxylation of biotin occurs. The reaction is favoured by moving the proton liberated in the carboxylation reaction to Asp203 and moving the Na^+ to the binding site on the newly formed carboxybiotin. After opening the conformation (state C), this Na^+ can be equilibrated with the external Na^+ pool. The reaction sequence could then follow steps $\text{C} \rightarrow \text{A}$ or $\text{C} \rightarrow \text{E}$ again in the opposite direction, depending on the occupation of Asp203 by Na^+ or H^+ , respectively. In the back reaction from A to E or C to E being coupled to the decarboxylation of carboxybiotin, the Na^+ ions are transported from the inside back to the outside and a proton is transported from the outside back to the inside.

(v) The model is also in agreement with the strict dependence on Na^+ for the decarboxylation of the carboxybiotin intermediate (Dimroth and Thomer, 1986b), the requirement for a proton (not H_2O) as the second substrate (Dimroth and Thomer, 1983) and for H^+ movement in the opposite direction to Na^+ movement (this paper). Only after Na^+ binding to the carboxybiotin can the conformation change and Na^+ ions move to Asp203, from where they can gain access to the other side of the membrane. The obligatory requirement for carboxybiotin (which is bound to the α -subunit) for Na^+ translocation is in accord with the notion that reconstituted ($\beta\gamma$) subunits alone were unable to catalyse Na^+ transport into liposomes (Di Berardino and Dimroth, 1995). Steps D and E explain how the decarboxylation of the carboxybiotin is coupled to proton movement from the outside to the inside.

According to the proposed mechanism, it is clear that the Asp203 mutation is unable to decarboxylate the carboxybiotin. As asparagine cannot deliver a proton, the essential exchange between Na^+ and H^+ (step D) is not possible. With respect to the mutation of Asp203 to Glu, one could envisage that the larger glutamate residue leads to a smaller metal ion binding pocket, not allowing Na^+ to bind, which again makes the decarboxylation impossible.

In summary, the oxaloacetate decarboxylase Na^+ pump seems to have adopted an ingeniously simple solution to the problem of vectorial catalysis, i.e. to achieve coupling between the chemical reaction and the vectorial ion movement across the membrane. The central feature of this mechanism is to use a membrane buried acidic amino acid residue (Asp203 of the β -subunit) for the vectorial as well as for the chemical reaction. Asp203 of the β -subunit in its protonated state is envisaged to take Na^+ ions from the carboxybiotin binding site and deliver them to the opposite side of the membrane. Simultaneously, the protons move to the carboxybiotin, where they catalyse the immediate decarboxylation of this acid-labile compound. This most elegant mechanism of vectorial catalysis is without precedent.

Materials and methods

Materials

The lipid used for reconstitution experiments was soybean phosphatidylcholine (Sigma, type II-S). CCCP and valinomycin were purchased from Sigma. $[1,5\text{-}^{14}\text{C}]$ citrate and $[1\text{-}^{14}\text{C}]$ acetate were purchased from NEN.

Site-directed mutagenesis of residues βD149 and βD203

All *E. coli* strains and plasmids required for site-directed mutagenesis are listed in Table II. Mutagenesis of the Asp149 and Asp203 residues

Table II. Bacterial strains and plasmids used in this work

Strain/plasmid	Genotype/description	Source/reference
<i>E. coli</i> strains		
DH5 α	<i>supE44 lacU169(80lacZM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Bethesda Research Laboratories
BL21(DE3)pLysS	<i>Cm^R; hsdS gal (λclts857 ind1 Sam7 nin5 lac UV5-T7 gene 1) T7 lysozyme gene in pACYC184</i>	Dunn and Studier (1983) Studier and Moffat (1986)
TG1	<i>supE hsdΔ5 thi Δ(lac-proAB) Δ[traD36 proAB lacI^q lacΔM15]</i>	Sambrook <i>et al.</i> (1989)
Plasmids		
M13mp18	sequencing vector	Norlander <i>et al.</i> (1983)
pKS-BXY	Ap ^R ; <i>oadB</i>	Di Berardino and Dimroth (1995)
pT7-BXY	Ap ^R ; <i>oadB</i>	Di Berardino and Dimroth (1995)
M13mp18B	Ap ^R ; <i>SacI-EcoRI</i> fragment from pKS-BXY containing <i>oadB</i>	this work
pSK-GAB	Ap ^R ; <i>oadGAB</i>	Di Berardino and Dimroth (1995)
pSK-GABd149q	Ap ^R ; <i>oadGAB</i> , D149Q mutation of the β -subunit	this work
pSK-GABd149e	Ap ^R ; <i>oadGAB</i> , D149E mutation of the β -subunit	this work
pSK-GABd203n	Ap ^R ; <i>oadGAB</i> , D203N mutation of the β -subunit	this work
pSK-GABd203e	Ap ^R ; <i>oadGAB</i> , D203E mutation of the β -subunit	this work

Ap^R, ampicillin resistance; Cm^R, chloramphenicol resistance.

of the oxaloacetate decarboxylase β -subunit was performed using the oligonucleotide-directed *in vitro* mutagenesis system (Amersham, version 2) as described by the manufacturer. The following oligonucleotides were designed to generate completely random amino acid changes at these two positions. Oligo D149X (5'-CGG GTT AGC TAG CAG CGG GCC GAA NNN GGT CAT CGC TCC G-3') was used to mutate β D149. A silent mutation GAC \rightarrow GAT in the non-coding strand (underlined in the oligonucleotide sequence) was introduced to create a new *NheI* site (bold letters). Oligo D203X (5'-AT CGC CGT CGG GCC NNN GGC GCC GCC GAT GAT GCC GAT CGC CGC CGC CT-3') was used to mutate β D203. The silent mutation CGG \rightarrow CGC in the non-coding strand introduced a new *PvuI* site (bold letters). Oligo D149E (5'-AG CGG GCC GAA TTC GGT CAT CGC-3') was synthesized for the Asp \rightarrow Glu exchange at position 149 of the β -subunit, generating with the mutation CTA \rightarrow CTT in the non-coding strand simultaneously the Asp \rightarrow Glu exchange and a new *EcoRI* site (bold letters). Similarly, oligo D203E (5'-CGT CGG GCC CTC GGC GCC GCC GAT-3') generated with the mutation CTG \rightarrow CTC in the non-coding strand an Asp \rightarrow Glu exchange at position 203 and a new *ApaI* site (bold letters).

For mutagenesis, the *EcoRI-SacI* fragment from pKS-BXY (Di Berardino and Dimroth, 1995) containing the *oadB* gene was moved into M13mp18, single-stranded DNA was isolated and the mutagenic oligonucleotides annealed. Mutated double-stranded DNA was transformed into strain TG1, and replicative form (RF) phage DNA was prepared from selected plaques. The isolated clones were screened by digestion with the enzymes, whose sites should have been generated using the mutant oligonucleotides. Single-stranded DNA was prepared from plaques which gave RF phage with the newly introduced restriction sites and was analysed by DNA sequencing. RF phage containing the desired mutations were digested with *MscI* and *DsaI*, and the resulting 0.36 kb fragments containing the mutations were used to replace the corresponding wild-type fragments in pT7-BXY. The plasmids obtained this way were digested with *BspEI* and *BclI* and the 0.77 kb fragments cloned into pSK-GAB (Di Berardino and Dimroth, 1995), replacing the corresponding wild-type fragments. The resulting plasmids, pSK-GABd149x and pSK-GABd203x (d = Asp, x = any amino acid), were transformed into the *E. coli* strains DH5 α or BL21(DE3)pLysS, and the mutated oxaloacetate decarboxylase synthesized by these clones was purified as described (Dimroth, 1986). The region containing the cloned 0.77 kb mutant fragment was sequenced again in the expression vector to exclude other mutations that might have occurred during the mutagenesis and cloning procedures.

Screening of oxaloacetate decarboxylase activity from mutant clones and purification of mutant enzymes

Before a large-scale purification of the mutant proteins was performed, small-scale cultures of the respective clones were used to measure oxaloacetate decarboxylase activity. For this purpose, 5 ml LB cultures of *E. coli* BL21(DE3)pLysS harbouring the mutated expression plasmids were grown overnight from single colonies. After centrifugation, the sedimented cells were resuspended in 50 mM Tris-HCl buffer, pH 7.5, containing 2 mM Mg-EDTA, 5 mM MgCl₂ and 0.2% Triton X-100

(100 μ l of buffer for cells from 1 ml of culture with an A₆₀₀ of 0.85). The cell suspension was frozen subsequently in liquid N₂ and thawed at room temperature. After the addition of traces of DNase and a further incubation for 10 min on ice, the decarboxylation activity was determined with the simple spectrophotometric assay at 265 nm as described (Dimroth, 1986).

For a large-scale purification of the mutant enzymes, 2 l LB cultures containing 100 μ g/ml ampicillin were inoculated with a single colony of the desired clones (*E. coli* DH5 α carrying the mutant expression plasmids) and grown overnight. Mutant oxaloacetate decarboxylases were purified on monomeric avidin-Sepharose following essentially the procedure described by Dimroth (1986).

Labelling of the α -subunit of mutant enzyme with ¹⁴C₂ from [4-¹⁴C]oxaloacetate

[4-¹⁴C]oxaloacetate was prepared from [1,5-¹⁴C]citrate with citrate lyase and the transfer of the radioactive carboxyl residue from [4-¹⁴C]oxaloacetate to the prosthetic biotin group was performed as described (Di Berardino and Dimroth, 1995).

Determination of oxaloacetate decarboxylase activity at various Na⁺ concentrations and pH values

The decarboxylation activity of wild-type oxaloacetate decarboxylase was measured at different pH values in the range between pH 9.0 and pH 5.3 in 40 mM Mes/Tris buffer containing variable Na⁺ concentrations with the simple spectrophotometric assay according to Dimroth (1986).

Reconstitution of oxaloacetate decarboxylase into proteoliposomes

A suspension of 40 mg of phospholipids in 1 ml of the appropriate reconstitution buffer (see below) was prepared by vortexing and sonication for 3 \times 1 min with a bath-type sonicator. To 250 μ l of this suspension (10 mg of phospholipids) 40 μ l of 20% *n*-octylglucoside (final concentration = 2.67%) was added and the mixture incubated for 10 min on ice. Subsequently, 50–75 μ g of purified oxaloacetate decarboxylase (50 U/mg) was added and the reconstitution mixture incubated for another 5 min on ice. The mixture was finally diluted into 12 ml of the same reconstitution buffer and centrifuged for 1 h at 20 000 *g*. The proteoliposomes were resuspended in 100 μ l of reconstitution buffer and used for the different transport assays.

Acetate diffusion into proteoliposomes

Proteoliposomes were prepared in 5 mM potassium phosphate buffer, pH 7.0 containing 5 mM MgCl₂ and 2 mM NaCl as described above. Acetate uptake was performed at 25°C and started by diluting the resuspended proteoliposomes into reconstitution buffer (final volume = 1 ml) containing 21.3 μ mol [1-¹⁴C]acetate (120 000 d.p.m./nmol) and valinomycin (50 pmol/mg phospholipids) where indicated. The decarboxylation reaction was started by the addition of 1 mM oxaloacetate. After various times, 100 μ l samples were removed and filtered through Millipore GSTF02500 filters (0.22 μ m). The proteoliposomes which were retained on the filters were washed three times with 600 μ l

of 0.1 M LiCl each and the filter-bound radioactivity was determined by liquid scintillation counting.

Effects of ionophores on $^{22}\text{Na}^+$ uptake into proteoliposomes

Proteoliposomes used for the Na^+ transport assay were prepared in 50 mM potassium phosphate buffer, pH 7.5 containing 5 mM MgCl_2 . The Na^+ transport assay contained in a final volume of 1 ml: 50 mM potassium phosphate buffer, pH 7.5, 5 mM MgCl_2 , 0.5 mM $^{22}\text{Na}_2\text{SO}_4$ (270 d.p.m./nmol Na^+) and the ionophores valinomycin (70 pmol/mg phospholipids) or CCCP (115 pmol/mg phospholipids). The $^{22}\text{Na}^+$ uptake was started by the addition of 1 mM oxaloacetate.

Analytical procedures

The protein content of samples was determined according to Bradford (1976). Oxaloacetate decarboxylase activity was determined as described (Dimroth, 1986). DNA sequence analysis was performed according to the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using a *Taq* Dye-Deoxy terminator cycle sequencing kit and the model 370A DNA sequencer from Applied Biosystems.

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