On the Mechanism of Sodium Ion Translocation by Oxaloacetate Decarboxylase of *Klebsiella pneumoniae*

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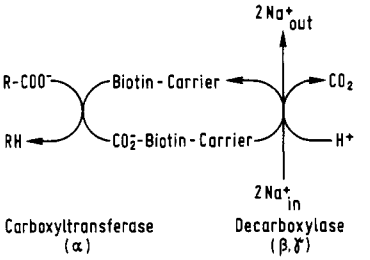
ABSTRACT: Proteoliposomes reconstituted with purified oxaloacetate decarboxylase of *Klebsiella pneumoniae* catalyzed the uptake of Na^+ ions upon oxaloacetate decarboxylation. The degree of coupling between the chemical and the vectorial reaction is dependent on the reconstitution conditions, and with the best preparations approaches a stoichiometry of two Na^+ ions per decarboxylation of one oxaloacetate. This coupling ratio is observed only in the absence of a $\Delta\tilde{\mu}_{Na^+}$, immediately after oxaloacetate addition. The ratio gradually declines during development of the electrochemical Na^+ ion gradient and becomes zero in the steady state. The Na^+ pump, however, continued to decarboxylate oxaloacetate and to catalyze Na^+ influx at the apparent stoichiometry of two Na^+ ions per decarboxylation event. During the steady state, this influx must be compensated by Na^+ efflux of the same size. The efflux is catalyzed by the Na^+ pump upon oxaloacetate decarboxylation, because in the absence of the substrate the efflux rate dropped to less than 10%. Proteoliposomes loaded with Na_2SO_4 catalyzed a bicarbonate-dependent uptake of $^{22}Na^+$ that was completely abolished after incubation with avidin. These results suggest coupling of Na^+ translocation to the carboxylation/decarboxylation of the biotin prosthetic group without the requirement for the oxaloacetate/pyruvate interconversion. The oxaloacetate-dependent transport of Na^+ into proteoliposomes was inhibited by the additional presence of the $\beta+\gamma$ subunits of oxaloacetate decarboxylase. A model of Na^+ translocation by oxaloacetate decarboxylase based on these experimental results is proposed.

Oxaloacetate decarboxylase of *Klebsiella pneumoniae* is a membrane-bound biotin-containing enzyme that pumps Na^+ ions across the membrane at the expense of the decarboxylation energy (Dimroth, 1982a,b). The enzyme consists of three different subunits, α, β, γ with molecular weights of 63 600, 44 900 and 8900, respectively (Laussermair et al., 1989; Woehlke et al., 1992). The enzyme complex has been dissociated by freezing and thawing in the presence of $LiClO_4$, and the biotin-containing α subunit has been separated by avidin–Sepharose affinity chromatography from subunits β and γ. The catalytically active decarboxylase was subsequently reconstituted from a mixture of the isolated α and $\beta+\gamma$ subunits (Dimroth & Thomer, 1988).

An analysis of the reactions catalyzed by the isolated subunits has led to a dissection of the overall catalysis into the events depicted in Scheme I [for a review, see Dimroth (1987, 1990)]. The first reaction is catalyzed by the α subunit and involves carboxyl transfer from oxaloacetate to the biotin prosthetic group (Dimroth & Thomer, 1983). This is located on the C-terminal domain of the α subunit, while the carboxyltransferase activity is believed to reside on its N-terminal domain (Schwarz et al., 1988). In the next step, the carboxybiotin moves from the carboxyltransferase site on the α subunit to the decarboxylase site on the β or $\beta+\gamma$ subunits, where it is decarboxylated in a Na^+-dependent fashion. Under physiological conditions, the decarboxylation is accompanied by the transport Na^+ ions across the membrane.

The related sodium ion translocating decarboxylases methylmalony-CoA decarboxylase (Hilpert & Dimroth, 1983; Hoffmann et al., 1989) and glutaconyl-CoA decarboxylase (Buckel & Semmler, 1983) have a similar subunit organization as oxaloacetate decarboxylase and perform a similar catalysis

Scheme I: Partial Reactions Catalyzed by Individual Subunits of Oxaloacetate Decarboxylase



(cf. Scheme I). More recently, insight into the mechanism of sodium ion translocation was obtained with methylmalonyl-CoA decarboxylase reconstituted into proteoliposomes (Hilpert & Dimroth, 1991). The decarboxylation is strictly coupled to Na^+ influx and not to net uptake. The decarboxylation does not stop, therefore, when the $\Delta\mu_{Na^+}$ has been fully developed, but catalyzes a turnover between external and internal Na^+ ions. It was the aim of the present work to compare these data with the mode of Na^+ translocation by oxaloacetate decarboxylase-containing proteoliposomes and to obtain further insight into the Na^+ translocation mechanism.

EXPERIMENTAL PROCEDURES

Materials. Oxaloacetate decarboxylase of *Klebsiella penumoniae* was prepared by affinity chromatography of a solubilized membrane extract on monomeric avidin–Sepharose (Dimroth, 1986).

The purified enzyme was dissociated by freezing and thawing in the presence of $LiClO_4$, and the biotin-containing α subunit was separated from $\beta+\gamma$ by chromatography on monomeric avidin–Sepharose as described (Dimroth & Thomer, 1988).

The lipids used for reconstitution experiments were either soybean phosphatidylcholine (Sigma, type II-S), purified by

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Table I: Oxaloacetate Decarboxylation and Na^+ Transport by Reconstituted Proteoliposomes[a]

reconstitution method	treatment of proteoliposomes	oxaloacetate decarboxylation [μmol min^{-1} (mg of protein)$^{-1}$] without Triton	oxaloacetate decarboxylation [μmol min^{-1} (mg of protein)$^{-1}$] with Triton	Na^+ transport [μmol min^{-1} (mg of protein)$^{-1}$] without valinomycin	Na^+ transport [μmol min^{-1} (mg of protein)$^{-1}$] with valinomycin
A		3.85	27.3	2	4
	avidin	0.63	0	0	0
B		8.9	29.8	0.7	0.9

[a] The reconstitutions were performed by method A or B as described under Experimental Procedures at phospholipid to protein ratios of 130:1. The oxaloacetate decarboxylase activity was determined in the absence or presence of 0.05% Triton X-100. Pretreatment of proteoliposomes with avidin was performed with 0.15 mL of proteoliposomes (4 mg of phospholipid) and 0.1 mg of avidin for 90 min at 0 °C. The amount of valinomycin added to the Na^+ uptake experiments was 20 nmol in a 1.0-mL volume containing proteoliposomes with 3.1 mg of phospholipid.

extraction with acetone (Kagawa & Racker, 1971), or *Klebsiella pneumoniae* lipids, prepared as described (Dimroth & Hilpert, 1984).

Reconstitution of Oxaloacetate Decarboxylase into Proteoliposomes. Method A. The previously described reconstitution by the detergent dilution method with *n*-octyl glucoside as detergent (Dimroth, 1981a) was modified to yield optimum Na^+ transport activities. The purified soybean phospholipids or *K. pneumoniae* lipids (40 mg) were dissolved in 0.5 mL of *n*-hexane. The solvent was removed with a stream of N_2 and subsequently by applying a vacuum for 1 h. The lipids were dissolved in 1.2 mL of 30 mM potassium phosphate buffer, pH 7.5, containing 1 mM Na_2SO_4 and 32 mg of *n*-octyl glucoside. The reconstitution mixture was completed by the addition of 50 μL of oxaloacetate decarboxylase (0.3 mg of protein, 20–40 units/mg of protein). After 2 min at 0 °C, the mixture was dialyzed for 16 h at 4 °C against 1 L of 30 mM potassium phosphate buffer, pH 7.5, containing 1 mM Na_2SO_4. The proteoliposomes thus formed were subsequently used for Na^+ transport experiments. In the range of protein to phospholipid ratios between 1:1000 and 1:90, the rate of Na^+ transport increased linearly with the amount of oxaloacetate decarboxylase applied during reconstitution. If the soybean phosphatidylcholine was not purified prior to the reconstitution experiments, or if the lipids were sonicated for 5 min at 80 W prior to the addition of octyl glucoside and the decarboxylase, the Na^+ transport activity of the proteoliposomes could be about 2–3 times lower.

Method B. Purified soybean phosphatidylcholine (28 mg) was suspended in 2.32 mL of 30 mM potassium phosphate buffer, pH 7.5, containing 0.5 mM Na_2SO_4 and sonicated at 80 W for about 3 min in intervals of 20 s using a tip-type sonicator with cooling in an ice/water bath. The suspension was mixed with 280 μL of 10% (w/v) Triton X-100 and incubated for 10 min at 20 °C. To each 650 μL was added 10 μL of oxaloacetate decarboxylase (50 μg of protein, 20–40 units/mg of protein) or 10 μL of oxaloacetate decarboxylase plus 20–80 μL (10–40 μg of protein) of the isolated β and γ subunits. The mixtures were recycled 15 times over columns containing 0.6 g (wet weight) of Bio-beads in Pasteur pipetts. The proteoliposomes formed during this treatment were frozen in liquid N_2 and thawed at room temperature. The vesicles were subsequently sonicated for 10 s at 80 W with a microtip. The Na^+ transport activity of the proteoliposomes eluted from the Bio-bead columns decreased during freezing and thawing and increased again to about 2 times the original activity after the sonication step. The activity decreased again if the sonication was continued for another 10 s. Method B is of advantage for reconsitutions including the isolated β and γ subunits which contain about 2% Triton X-100 from the isolation procedure.

Na^+ Transport Assay. If not indicated otherwise, the incubation mixtures contained the following in 2.4 mL at 25 °C: 25 mM potassium phosphate buffer, pH 7.5; 0.42 mM $^{22}Na_2SO_4$ (660 cpm/nmol of Na^+); the reconstituted proteoliposomes (7 mg of phospholipid); and 3.7 mM potassium oxaloacetate which was used to initiate the reactions. The uptake of $^{22}Na^+$ was determined with samples (55 μL) taken at different incubation times and passed over Dowex 50, K^+, as described (Dimroth, 1986).

Na^+ Counterflow Experiments. Oxaloacetate decarboxylase was reconstituted into proteoliposomes by method A at a phospholipid to protein ratio of 160:1 in 30 mM potassium phosphate buffer, pH 7.5, containing 20 mM Na_2SO_4. The proteoliposomes were collected by centrifugation (1 h, 220000*g*), resuspended in the same volume of 30 mM potassium phosphate buffer, pH 7.5, containing 20 mM K_2SO_4, and used immediately for $^{22}Na^+$ uptake. The incubation mixtures for these assays contained the following in 0.5 mL at 25 °C: 30 mM potassium phosphate buffer, pH 7.5; 20 mM K_2SO_4; the proteoliposomes (7 mg of phospholipid); 1.3 mM $^{22}Na_2SO_4$ (230 cpm/nmol of Na^+); and 50 mM $KHCO_3$. $^{22}Na^+$ uptake into the proteoliposomes was determined with samples (100 μL) passed over Dowex 50, K^+, as described (Dimroth, 1986). All the solutions used in these experiments were degassed in vacuo to keep the endogenous CO_2 concentrations as low as possible.

Other Analytical Methods. Sodium ion concentrations were determined by atomic absorption spectroscopy (Hilpert & Dimroth, 1991). Protein was determined as described by Lowry et al. (1951).

RESULTS

Rate of Na^+ Transport with Reconstituted Proteoliposomes. Most of the studies reported in this paper have been performed with proteoliposomes prepared from purified oxaloacetate decarboxylase and phospholipids by removal of *n*-octyl glucoside by dialysis (method A). The conditions that have been found to yield optimum Na^+ transport activities are described under Experimental Procedures. An alternate method (B) was used for reconstitutions including solutions of the $\beta+\gamma$ subunits of oxaloacetate decarboxylase that contained about 2% Triton X-100 from the isolation procedure. A mixture was prepared from phospholipid, Triton X-100, and oxaloacetate decarboxylase with or without additional $\beta+\gamma$ subunits and recycled over a small column of Bio-beads to remove the Triton X-100 and allow the proteoliposomes to be formed. The rate of Na^+ transport upon oxaloacetate decarboxylation of these proteoliposomes increased after freezing and thawing and a short sonication as described under Experimental Procedures.

The initial rates of oxaloacetate decarboxylation and Na^+ transport obtained with proteoliposomes prepared by method A or B are summarized in Table I. With proteoliposomes prepared by method A, the decarboxylation of oxaloacetate

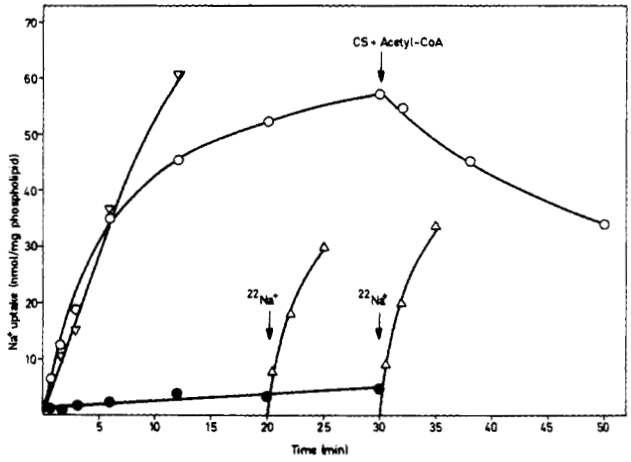


FIGURE 1: Correlation between oxaloacetate decarboxylation, Na^+ uptake, Na^+ influx, and Na^+ efflux at different incubation times. The proteoliposomes were prepared by method A with lipids from *K. pneumoniae* at a phospholipid to protein ratio of 173:1. The incubation mixtures contained in 1.0 mL at 15 °C the following: 30 mM potassium phosphate buffer, pH 7.0; 1.85 mM $^{22}Na_2SO_4$ (310 cpm/nmol of Na^+); the reconstituted proteoliposomes (13.2 mg of phospholipid), 0.5 mM dithioerythritol; and 1 mM oxaloacetate which was used to initiate the reactions. Oxaloacetate decarboxylation (▽) and net Na^+ uptake (○) were determined from samples (0.09 mL) taken at the times indicated. At the arrow, 0.9 μmol of acetyl-CoA and 5 units of citrate synthase (CS) were added to convert residual oxaloacetate to citrate in order to determine the efflux of $^{22}Na^+$ after the Na^+ pump was switched off. In parallel experiments not containing the radioactive tracer, the influx of Na^+ into the proteliposomes was measured by adding $^{22}Na^+$ at 20- or 30-min incubation times (arrows) and determining the uptake of radioactivity into the proteoliposomes (△). Na^+ uptake in the absence of oxaloacetate (●). For details, see Experimental Procedures.

increased 7-fold by destruction of the proteoliposomal membranes with Triton X-100. Incubation with avidin completely inhibited the oxaloacetate decarboxylase activity in the presence of Triton, but caused only partial inhibition in the absence of the detergent, while the Na^+ transport was completely abolished. These data indicate that part of the oxaloacetate diffuses across the membrane where it is decarboxylated from enzyme facing the substrate binding site to the interior. The data suggest a highly asymmetric reconstitution of the enzyme with only about 14% facing the exterior compartment. The ratio of oxaloacetate decarboxylation to Na^+ transport with 1.9 with these proteoliposomes. Under optimized conditions the coupling ratio approaches 0.5 (Figure 1). Valinomycin, which destructs the membrane potential generated by the Na^+ pump, increased the Na^+ transport rate by a factor of 2.

The proteoliposomes reconstituted by method B have a higher percentage of externally oriented oxaloacetate decarboxylase (about 30%) than those prepared by method A. However, the ratio of Na^+ transport to oxaloacetate decarboxylation is only 0.08, and the stimulation of Na^+ transport by valinomycin is only 1.3-fold. Only part of the decarboxylase molecules appear to be properly reconstituted by this method, therefore, to participate in the vectorial catalysis.

Coupling between Oxaloacetate Decarboxylation and Na^+ Transport. The fact that oxaloacetate passes through the proteoliposomal membrane so that internally oriented oxaloacetate decarboxylase will pump Na^+ outward complicates the determination of coupling between the chemical and transport reactions. In order to minimize these complications, the proteoliposomes were prepared at a low protein to phospholipid ratio (1:173), so that per proteoliposome on average only 1–2 decarboxylase molecules were present and the number of proteoliposomes carrying decarboxylase molecules in both orientations was low. We also conducted the transport experiments at 15 °C rather than the usual 25 °C to slow down the passive diffusion of oxaloacetate through the membrane. The results of $^{22}Na^+$ uptake and oxaloacetate decarboxylation measured in parallel are shown in Figure 1. In the initial phase of the transport, the ratio of Na^+ uptake to oxaloacetate decarboxylation was about 1.7. This ratio gradually declined as the internal Na^+ concentration increased. Fast decarboxylation was observed even when the internal Na^+ concentration was approaching the steady state [see Figure 9 of Dimroth (1987)]. The rate of Na^+ influx was determined from the uptake of $^{22}Na^+$, added after different incubation periods to parallel transport assays not containing the radioactive tracer. The results (Figure 1) show that the rate of Na^+ influx at the beginning was not significantly different from that close to the steady state after 20- or 30-min incubation. In the steady state, the influx of Na^+ must be compensated by an equal Na^+ efflux. The rate of Na^+ efflux in the absence of oxaloacetate was determined with proteoliposomes loaded with $^{22}Na^+$ by converting the residual substrate enzymically to citrate. This efflux rate was less than 10% of the influx of Na^+ ions, suggesting that the pump catalyzes a turnover between external and internal Na^+ ions at the expense of oxaloacetate decarboxylation.

Na^+ Uptake into Proteoliposomes in the Absence of Oxaloacetate. The efflux of Na^+ from Na^+-loaded proteoliposomes after removal of oxaloacetate suggested that the decarboxylase could catalyze an oxaloacetate-independent Na^+ translocation, because liposomes not containing the decarboxylase are very tight for Na^+ ions. A substantial uptake of $^{22}Na^+$ into the decarboxylase-containing proteoliposomes was in fact observed, if low concentrations of the labeled cation were added to proteoliposomes that had been loaded with 20 mM Na_2SO_4 (Figure 2). Interestingly, the $^{22}Na^+$ uptake was strictly dependent on the presence of $KHCO_3$. After equilibration of the internal and external Na^+ ion concentration, the $KHCO_3$-dependent $^{22}Na^+$ uptake was considerably reduced. The control in the absence of $KHCO_3$ yielded the same results as with the Na^+-loaded proteoliposomes.

The effect of various oxaloacetate decarboxylase inhibitors on the rate of $^{22}Na^+$ uptake into Na^+-loaded proteoliposomes in the presence of $KHCO_3$ is shown in Table II. The transport activity disappeared after incubation of the enzyme with avidin or $Hg(NO_3)_2$. The transport activity was also destructed by limited proteolytic degradation with trypsin, which cleaves the α subunit between the biotin binding domain and the carboxyltransferase domain (Schwarz et al., 1988). Under all these conditions, the oxaloacetate decarboxylase activity was abolished. The presence of as much as 5 mM oxalate, which reduces the oxaloacetate decarboxylase activity by more than 95% (Dimroth, 1981b), was without effect on the $KHCO_3$-dependent $^{22}Na^+$ uptake. Thiocyanate, which inhibits the oxaloacetate decarboxylase by about 95% at 50 mM concentration, reduced the rate of the $KHCO_3$-dependent $^{22}Na^+$ uptake to 25% of the control values.

Effect of the β+γ Subunits on Na^+ Transport into Oxaloacetate Decarboxylase-Containing Proteoliposomes. The translocation of Na^+ ions must proceed through the membrane-bound subunits β and γ. An interesting question therefore is whether these subunits can alone catalyze Na^+ translocation across the membrane, analogous to proton conduction through the isolated F_o moiety of the F_1F_o ATPase [for a review, see Schneider and Altendorf (1987)]. An

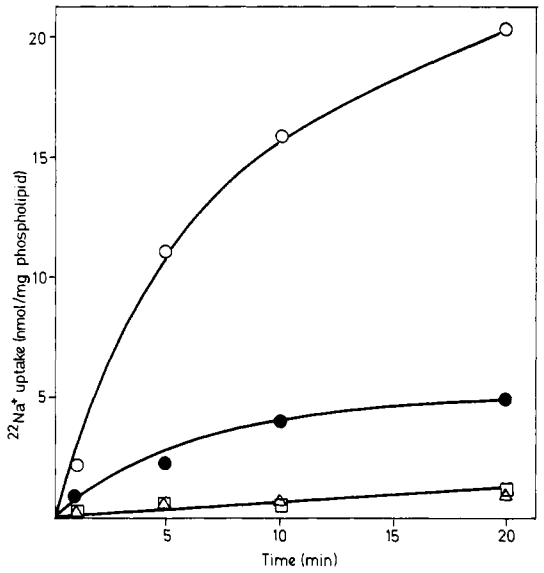


FIGURE 2: Effect of $KHCO_3$ on $^{22}Na^+$ uptake by oxaloacetate decarboxylase-containing proteoliposomes. The proteoliposomes were prepared by method A in 30 mM potassium phosphate buffer, pH 7.5, containing 20 mM Na_2SO_4, centrifuged, and resuspended in 30 mM potassium phosphate buffer, pH 7.5, containing 20 mM K_2SO_4, immediately before the $^{22}Na^+$ uptake experiments. The external Na^+ concentration in the incubation mixtures for $^{22}Na^+$ uptake was 2.3 mM, as determined by atomic absorption spectroscopy. $^{22}Na^+$ uptake into the proteoliposomes was determined in the presence (○) or absence (△) of 50 mM $KHCO_3$. Part of the proteoliposomal suspension was kept overnight at 4 °C to equilibrate the external and internal Na^+ concentrations prior to the transport assays that were performed in the presence (●) or absence (□) of 50 mM $KHCO_3$. For details, see Experimental Procedures.

Table II: Effect of Various Inhibitors on the Uptake of $^{22}Na^+$ by Oxaloacetate Decarboxylase-Containing Proteoliposomes in the Presence of 50 mM $KHCO_3$[a]

expt	rel rate of $^{22}Na^+$ uptake (%)	expt	rel rate of $^{22}Na^+$ uptake (%)
A	100	E	113
B	0	F	93
C	0	G	25
D	7		

[a] Proteoliposomes loaded with Na_2SO_4 were prepared, and $^{22}Na^+$ uptake in the presence of 50 mM $KHCO_3$ was determined as described in the legend of Figure 2 and under Experimental Procedures. The following conditions apply to experiments A–F: A, control; B, the proteoliposomes (5 mg of phospholipid) were preincubated for 1 h at 25 °C with 25 μg of avidin; C, oxaloacetate decarboxylase was incubated with 1 mM $Hg(NO_3)_2$ for 15 min at 25 °C prior to the reconstitution; D, the decarboxylase (0.25 mg, 14 units) was incubated for 10 min at 25 °C with 2 μg of trypsin, and the digestion was terminated with 10 mM diisopropyl fluorophosphate prior to the reconstitution; the trypsin completely inactivated the decarboxylase activity; E, addition of 1 mM oxalate to the transport assay; F, addition of 5 mM oxalate; G, addition of 50 mM KSCN. 100% activity corresponds to an initial rate of Na^+ uptake of 2 nmol min^{-1} (mg of phospholipid)$^{-1}$.

alternative mechanism would be an obligatory coupling of Na^+ translocation to carboxylation or decarboxylation of the prosthetic biotin group on the α subunit. In this case, the isolated β+γ subunits should not conduct Na^+. An initial attempt to resolve these ambiguities was made by measuring oxaloacetate-dependent Na^+ uptake into proteoliposomes that had been coreconstituted with oxaloacetate decarboxylase and the free β+γ subunits. The results shown in Figure 3indicate a marked decrease in the apparent rate and extent of Na^+ uptake with increasing amounts of β+γ subunits present. The kinetics of Na^+ uptake into proteoliposomes containing β+γ subunits that were denatured by heating at 100 °C for 10 min

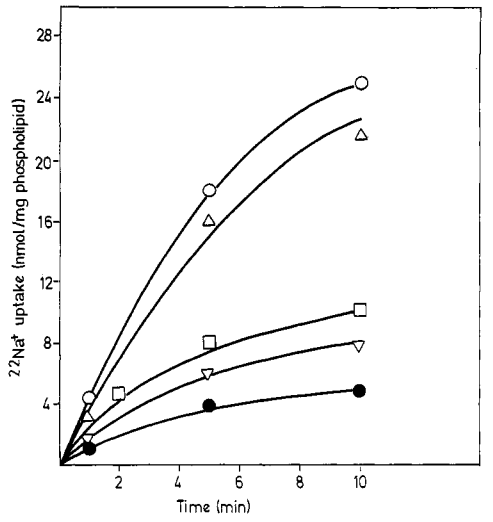


FIGURE 3: Effect of the isolated β+γ subunits on the uptake of Na^+ into reconstituted proteoliposomes. The proteoliposomes were reconstituted by method B in 30 mM potassium phosphate buffer, pH 7.5, containing 0.5 mM Na_2SO_4 with 7 mg of phospholipid and 48 μg of oxaloacetate decarboxylase (25 units/mg of protein) (○), or in the additional presence of the isolated β+γ subunits in the following amounts: 10 μg (△); 20 μg (□); 30 μg (▽); 40 μg (●). The transport assays were performed in a total volume of 2.4 mL at 25 °C containing 25 mM potassium phosphate, pH 7.5, 0.42 mM $^{22}Na_2SO_4$ (660 cpm/nmol of Na^+), the reconstituted proteoliposomes (7 mg of phospholipid), and 3.7 mM potassium oxaloacetate which was used to initiate the reactions. The uptake of $^{22}Na^+$ was determined with samples (55 μL) taken at the times indicated.

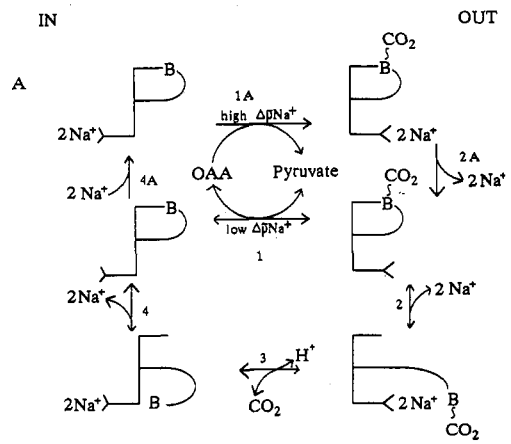
were the same as with proteoliposomes containing only oxaloacetate decarboxylase, and the rate of oxaloacetate decarboxylation was the same in proteoliposomes with or without the β+γ subunits (data not shown). The results could indicate that the native β+γ subunits create a leak in the membrane of the proteoliposomes by which the Na^+ ions pumped into the interior by oxaloacetate decarboxylase flow back out downhill the electrochemical gradient. Alternatively, Na^+ uptake by the decarboxylase could be specifically inhibited by an excess of the free β+γ subunits. Preliminary results with proteoliposomes containing only β+γ subunits indicated no significant increase of Na^+ conductivity as compared to liposomes without β+γ in response neither to ΔpNa^+ nor to a potassium diffusion potential (Di Berardino and Dimroth, unpublished results).

DISCUSSION

All experimental results on the translocation of Na^+ ions by oxaloacetate decarboxylase, methylmalonyl-CoA decarboxylase, or the isolated β+γ subunits reported here and elsewhere (Dimroth & Hilpert, 1984; Hilpert & Dimroth, 1991) can be described by the tentative mechanism depicted in Scheme II. Important differences are apparent under conditions of low and high $\Delta\tilde{\mu}_{Na^+}$, respectively. The two different subcases are therefore discussed separately.

(A) Na^+ Transport at Low $\Delta\tilde{\mu}_{Na^+}$. Under these conditions, decarboxylation of oxaloacetate is strictly coupled to Na^+ transport with a Na^+ to oxaloacetate stoichiometry of 2 (Dimroth, 1987). The reaction sequence can be described by steps 1–4 of Scheme II. In the first step, the prosthetic biotin group (B) is bound to the carboxyltransferase site of the α-subunit, where it is carboxylated with the simultaneous decarboxylation of oxaloacetate to pyruvate (step 1) (Dimroth & Thomer, 1983). This reaction is completely independent from the presence of Na^+, and no further reactions take place in the absence of this alkali ion (Dimroth & Thomer, 1983).

Scheme II: Hypothetical Mechanism of Na^+ Translocation by Oxaloacetate Decarboxylase[a]



[a] The enzyme is symbolized with a Na^+ binding site (on $\beta+\gamma$) that may be oriented to the internal or external surface of the membrane. The biotin prosthetic group (B) that is covalently bound to the biotin domain of the α subunit (indicated by the bar in the center of the enzyme molecule) moves between the carboxyltransferase domain of the α subunit (top of symbolized enzyme) and the lyase catalytic site on the $\beta+\gamma$ subunits (bottom of symbolized enzyme). In a proteoliposomal system, the interior is on the left, and the exterior with an accessible oxaloacetate/pyruvate binding site is on the right.

The next step (2), therefore, is supposed to be the binding of Na^+ ions from the exterior of the proteoliposomes to the Na^+ binding site on the $\beta+\gamma$ subunits (Dimroth & Thomer, 1983, 1992) and the movement of the carboxybiotin to the lyase catalytic site on these subunits. Decarboxylation of carboxybiotin may trigger a conformational change by which the Na^+ ions become exposed to the inner surface of the proteoliposomal membrane (step 3). In step 4, the Na^+ ions dissociate to the inside of the proteoliposomes, and the free biotin prosthetic group moves back into the carboxyltransferase site. In the following carboxylation of biotin with the carboxyl group of oxaloacetate (step 1), the Na^+ binding site is supposed to switch back, becoming exposed again to the outside.

(B) Na^+ Transport at High $\Delta\tilde{\mu}_{Na^+}$. The situation becomes more complicated at high $\Delta\tilde{\mu}_{Na^+}$, because under these conditions decarboxylation proceeds at least in part apparently decoupled from transport. It has been observed that while net Na^+ accumulation slows down to reach zero in the steady state, the decarboxylation of oxaloacetate or methylmalonyl-CoA by the appropriate decarboxylase (Hilpert & Dimroth, 1991) continues at a high rate. Importantly, even in this situation, the systems operate strictly coupled with respect to Na^+ influx. However, as this influx does not contribute to Na^+ accumulation during the steady state, it must be compensated by an equal Na^+ efflux. It has been shown here and elsewhere (Hilpert & Dimroth, 1991) that this efflux of Na^+ cannot be accounted for by a leak, but appears to be catalyzed by the pump itself. We suggest therefore that at high $\Delta\mu_{Na^+}$ the switch of the internally to the externally exposed Na^+ binding site upon the carboxylation of biotin by oxaloacetate may proceed with Na^+ ions bound to this site. The alternate sequences, 4A, 1A, and 2A, may thus be operating at high $\Delta\tilde{\mu}_{Na^+}$. Together with the reaction steps 2, 3, and 4, it would account for the observed cycling of Na^+ between exterior and interior vesicular spaces in the steady state with the stoichiometry of 2 Na^+ ions cycling per decarboxylation event. The model presented here is consistent with all our experimental results: (i) carboxyl transfer from oxaloacetate to carboxybiotin is independent of $[Na^+]$, and decarboxylation of carboxybiotin is $[Na^+]$-dependent; (ii) oxaloacetate-dependent Na^+ influx into the proteoliposomes always occurs at a Na^+ to oxaloacetate stoichiometry of 2; (iii) at low $\Delta\tilde{\mu}_{Na^+}$, this influx leads to a net accumulation, while at high $\Delta\tilde{\mu}_{Na^+}$ in the steady state the influx is compensated by an equal Na^+ efflux; (iv) as this efflux is not observed after cessation of oxaloacetate-dependent Na^+ pumping, it must be catalyzed by a step of the pump cycle; (v) the translocation of Na^+ by oxaloacetate decarboxylase depends on the carboxylation of biotin by oxaloacetate or CO_2 or on the decarboxylation of the carboxybiotin enzyme derivative. Other models which have been considered were not found to be consistent with all of these data.

Na^+ Translocation in the Absence of Oxaloacetate. It is clear that the sequence 4A, 1A, 2A is irreversible because no driving force exists to compensate the uphill transport of Na^+ ions in the reverse reaction (2A → 4A). All steps of the reaction sequence 1–4 are readily reversible, however, as shown by the carboxylation of pyruvate or acetyl-CoA with proteoliposomes to which a $\Delta\tilde{\mu}_{Na^+}$ of proper direction and magnitude was applied (Dimroth & Hilpert, 1984). We have shown here that oxaloacetate decarboxylase catalyzes a bicarbonate-dependent translocation of Na^+ ions in the absence of oxaloacetate. Bicarbonate is anticipated to serve as a source of CO_2 that is the substrate (product) of the lyase reaction (Dimroth & Thomer, 1983). Importantly, this Na^+ translocation is completely inhibited by blocking the prosthetic biotin group with avidin. These results suggest that the translocation of Na^+ from the inside to the outside of the proteoliposomes occurs through steps 4, 3, and 2 and includes the carboxylation of biotin with CO_2. In the reverse, external Na^+ is transported to the inside of the proteoliposomes through steps 2, 3, and 4, including the decarboxylation of carboxybiotin. The rate of $^{22}Na^+$ uptake into the proteoliposomes was significantly higher under "counterflow conditions" ($[Na^+]_{in} > [Na^+]_{out}$). This observation is also in accord with the mechanism shown in Scheme II. One would expect that a gradient $[Na^+]_{in} > [Na^+]_{out}$ shifts the equilibrium to the side of the otherwise unfavorable formation of carboxybiotin (steps 4 → 2). Once this has formed, $^{22}Na^+$ is readily translocated to the inside through steps 2 → 4 and is trapped there by dilution with the large pool of unlabeled Na^+ ions. This counterflow activity was not influenced by oxalate, which is a transition-state analogue of the oxaloacetate/pyruvate interconversion and binds tightly to the carboxyltransferase site on the α subunit (Dimroth & Thomer, 1986). Cleavage of the α subunit between the carboxyltransferase and biotin binding domains by limited proteolysis with trypsin (Schwarz et al., 1988), however, abolished the activity of Na^+ translocation.

Inhibition of Na^+ translocation was also observed after treatment of the enzyme with $Hg(NO_3)_2$. This compound abolishes the oxaloacetate decarboxylase activity by specific binding to the α subunit (Dimroth & Thomer, 1992). In summary, these results indicate that the translocation of Na^+ ions involves the carboxylation/decarboxylation of the biotin prosthetic group, but occurs independent of the oxaloacetate/pyruvate interconversion by the carboxyltransferase subunit. Nevertheless, the structural integrity of the α subunit appears to be of essence for Na^+ translocation, because neither the biotin binding domain obtained by proteolysis nor the α subunit modified by bound Hg^{2+} ions was capable of catalyzing this activity.

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