The Deactive Form of Respiratory Complex I from Mammalian Mitochondria Is a Na\(^+\)/H\(^+\) Antiporter\(^*\)\(^{\text{S}}\)

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Background: Mammalian complex I spontaneously forms a deactive state when it is not turning over.

Results: Deactive complex I is a Na\(^+\)/H\(^+\) antiporter.

Conclusion: In deactive complex I the ion transfer domain is functionally disconnected from the redox domain; the antiporter-like subunits assert their independent function.

Significance: Na\(^+\)/H\(^+\) exchange by deactive complex I may influence the outcome of ischemia-reperfusion.

In mitochondria, complex I (NADH:ubiquinone oxidoreductase) uses the redox potential energy from NADH oxidation by ubiquinone to transport protons across the inner membrane, contributing to the proton-motive force. However, in some prokaryotes, complex I may transport sodium ions instead, and three subunits in the membrane domain of complex I are closely related to subunits from the Mrp family of Na\(^+\)/H\(^+\) antiporters. Here, we define the relationship between complex I from Bos taurus heart mitochondria, a close model for the human enzyme, and sodium ion transport across the mitochondrial inner membrane. In accord with current consensus, we exclude the possibility of redox-coupled Na\(^+\) transport by B. taurus complex I. Instead, we show that the “deactive” form of complex I, which is formed spontaneously when enzyme turnover is precluded by lack of substrates, is a Na\(^+\)/H\(^+\) antiporter. The antiporter activity is abolished upon reactivation by the addition of substrates and by the complex I inhibitor rotenone. It is specific for Na\(^+\) over K\(^+\), and it is not exhibited by complex I from the yeast Yarrowia lipolytica, which thus has a less extensive deactive transition. We propose that the functional connection between the redox and transporter modules of complex I is broken in the deactive state, allowing the transport module to assert its independent properties. The deactive state of complex I is formed during hypoxia, when respiratory chain turnover is slowed, and may contribute to determining the outcome of ischemia-reperfusion injury.

In mitochondria, complex I (NADH:ubiquinone oxidoreductase) (1) uses potential energy from the oxidation of NADH by ubiquinone (\(\Delta G\)) to translocate protons across the mitochondrial inner membrane, contributing to the proton-motive force (\(\Delta p\)) that is used for ATP synthesis and transport processes. Complex I has an unusual L-shaped structure (Fig. 1A); the redox reaction is contained in the hydrophilic domain that extends into the mitochondrial matrix, and proton translocation in the membrane-bound domain. Recent structural data from two prokaryotic complexes I (2) has revealed that the membrane-bound ND2, ND4, and ND5 subunits form three related structural units linked by a lateral helix from ND5; together they form the part of the membrane domain farthest from the hydrophilic domain. ND2, ND4, and ND5 are related to two subunits from the Mrp family of Na\(^+\)/H\(^+\) antiporters (3, 4) (see supplemental Fig. 1 and Fig. 1B), suggesting that they share a common ancestor and that their mechanisms of ion translocation are related. Together, these observations suggest that, for energy transduction by complex I, redox-coupled conformational changes drive proton transfer through the three antiporter-like subunits.

Proton transfer is firmly established as the basis for energy conversion in mitochondria, but some bacteria use sodium ion transfer instead. For example, some organisms use Na\(^+\)-coupled ATP synthases, and only minor adjustments to the residues of the ion-binding site are required to alter the specificity (5). Redox-driven Na\(^+\) translocation has been proposed also for complex I. The complexes I from Klebsiella pneumoniae (6) and Escherichia coli (7) have been proposed to be primary Na\(^+\) pumps (transport Na\(^+\) out of the cytoplasm), those from Rhodothermus marinus and Escherichia coli have been proposed to transport protons out of and Na\(^+\) into the cytoplasm together (4, 8, 9), and E. coli complex I has also been reported to be a simple H\(^+\)-coupled enzyme (10).

Here, we use three preparations of tightly coupled membrane vesicles to define the ion translocation reactions of mitochondrial complex I (CI); \(^2\) proteoliposomes (PLs) containing CI from Bos taurus (Bt) or the yeast Yarrowia lipolytica (Yl) and submitochondrial particles (SMPs) from B. taurus heart. PLs are simpler than SMPs because they contain only CI, but SMPs are more versatile because other respiratory enzymes can be used to set \(\Delta p\) and reduce the quinone pool. In accord with the well-established consensus, our results exclude the possibility of

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\(^{\text{2}}\) The abbreviations used are: CI, complex I; ACMA, 9-amino-6-chloro-2-methoxyacridine; Bt, B. taurus; Ch\(^-\), choline; DQ, decylubiquinone; EIPA, ethyl-isopropyl amiloride; NEM, N-ethyl maleimide; PL, proteoliposome; SMP, submitochondrial particle; Yl, Y. lipolytica.
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![Diagram of complex I](attachment:complexI.png)

**FIGURE 1. Structural information about complex I, taken from 3M9S.pdb (Thermus thermophilus complex I), 319P.vdb (T. thermophilus hydrophilic domain), and 3RKO.pdb (six subunits of the E. coli hydrophilic domain) (2, 57). A, complex I is an L-shaped enzyme, with a large hydrophilic domain containing the flavin mononucleotide, iron-sulfur (FeS) clusters, and the binding site for the ubiquinone headgroup. The membrane domain contains several subunits; the structure of subunit ND1 has been determined only at low resolution, and two possible positions for helix I of ND3 are shown, from the low resolution structure of the intact enzyme and the structure is shown from the top. B, the structure of E. coli NuoM (ND4; the 14 core helices of ND2 and ND5 have the same structure) (2). The transmembrane helices are in light blue; fully conserved residues between all of the antiporter-like subunits of B. taurus and E. coli and MrpA and MrpD from B. subtilis are shown in purple (see supplemental Fig. 1). Contact points with the lateral helix of ND5 are highlighted in green, and the loops in the two broken helices in red. On the right, the same structure is shown from the top.

In preparations containing the "deactive" form of BtCI, the active/deactive status was confirmed by treating the PLs with 1 mM N-ethylmaleimide (NEM) for 25 min; following treatment, the deactive CI is catalytically inactive, whereas catalysis by the active CI is unaffected by the NEM (22).

**Preparation of CI, PLs, and SMPs—BtCI and YICl were prepared as described previously (16–19) in 20 mM Tris-Cl (pH 7.4), 150 mM NaCl, 10% (w/v) ethylene glycol, 0.02% (w/v) n-dodecyl-β-d-maltoside (Anatrace), and 20 mM Na-MOPS (pH 7.2), 150 mM NaCl, 0.05% (w/v) n-dodecyl-β-d-maltoside, respectively. To prepare BtPLs, a 1:2:1 (w/w) mixture of bovine heart phosphatidylcholine, phosphatidylethanolamine, and cardiolipin (Avanti Polar Lipids Inc.) in chloroform was dried under N\(_2\) and then homogenized to 10 mg ml\(^{-1}\) in 10 mM Tris-Cl, pH 7.5, 50 mM NaCl (unless otherwise stated) and extruded through a 0.1-μm Whatman Nuclepore track-etched membrane. The preformed liposomes were partially solubilized (20) using 1.2% n-octyl-β-d-glucoside (Anatrace), and the solution was sonicated to clarify it. Then, 0.15–0.25 mg ml\(^{-1}\) BtCI (10–15 mg ml\(^{-1}\) stock solution) was added, the mixture was incubated for 10 min on ice, and prewashed SM-2 Bio-Beads (Bio-Rad) were added to remove the detergents (21) (50 μl of wet Bio-Beads per ml every 30 min for 4 h). The Bio-Beads were removed, and the BtPLs were collected by centrifugation (70,000 × g for 30 min) and resuspended. YIFls were prepared similarly using soybean asolectin (Sigma-Aldrich) (18), with enzyme that had not undergone the final gel filtration step of the purification (this enzyme contains low levels of cytochrome c oxidase). Following reconstitution, the complex I in PLs was predominantly deactive (11, 12); when required, PLs (~20 mg ml\(^{-1}\)) were activated by incubating them on ice in 800 μM NADH and 800 μM decylubiquinone (DQ) for 10 min (until the NADH was consumed), and then the active PLs were collected by centrifugation, resuspended, and used immediately. For CI in PLs, the deactive status was confirmed by treating the PLs with 1 mM N-ethylmaleimide (NEM) for 25 min; following treatment, the deactive CI is catalytically inactive, whereas catalysis by the active CI is unaffected by the NEM (22).

BtSMPs were prepared as described previously, in 10 mM Tris-SO\(_4\) (pH 7.5) and 250 mM sucrose (23), except the NADH incubation step was omitted. To deactivate the complex I, they were incubated at 37 °C for 20 min; to activate it, ~10 mg ml\(^{-1}\) BtSMPs were incubated in 1 mM NADH at 4 °C for 20 min and used immediately (11, 12). The active/deactive status of BtSMPs was confirmed by measuring the reverse electron transfer activity; deactive BtSMPs exhibit less than 10% of the reverse electron transfer activity of active BtSMPs.

**Spectroscopic Assays—** Unless otherwise stated, SMP assays were in 10 mM Tris-SO\(_4\) (pH 7.5) and 250 mM sucrose, and PL assays were in 5 mM Tris-SO\(_4\) (pH 7.5) and 20 mM KCl, at 32 °C. NADH:O\(_2\) and NADH:DQ oxidoreduction were followed at 340–380 nm (ε\(_{\text{NADH}}\) = 4.81 mm\(^{-1}\) cm\(^{-1}\)), and succinate:O\(_2\) oxidoreduction (in 20 mM succinate) was followed with a Clark electrode. Reverse electron transfer (succinate:NAD\(^+\) oxidoreduction driven by the ∆p from ATP hydrolysis) by SMPs...
was measured in 10 mM succinate, 1 mM NAD\textsuperscript{+}, 1 mM ATP-MgSO\textsubscript{4}, and 400 \muM KCN (23). 10 \muM \textit{mL}\textsuperscript{-1} gramicidin (a mixture of A, B, C, and D, Sigma-Aldrich) or 25 \muM \textit{mL}\textsuperscript{-1} alamethicin (\textit{Trichoderma viride}, Sigma-Aldrich) was used to dissipate \Delta\textit{p}. Complex I inhibitors (24, 25) were added at concentrations sufficient for >95\% inhibition of the NADH:O\textsubscript{2} or NADH:DQ activity: 50 \muM capsaicin, 100 \muM 2-decyl-4-quinazolinyl amine (from Dr. V. Zickermann, Frankfurt, Germany) (26), 30 \muM ethyl-isopropyl amiloride (EIPA) (10, 27–29), 150 \muM fenpyrocymate (30), 30 \muM \textit{Δlac-acetogenin} (from Prof. H. Miyoshi, Kyoto, Japan, compound 7 in Ref. 31), 5 \muM 1-methyl-4-phenyl-pyridinium, 25 \muM palmitic acid (32), 1 \muM piericidin A, 200 \muM \textit{mL}\textsuperscript{-1} of iron, prepared from Sigma-Aldrich iron AA/ICP calibration standard) in the internal volumes of the PL preparations were determined by including iron nitrate (10 \muM \textit{mL}\textsuperscript{-1}) of iron, prepared from the Sigma-Aldrich iron AA/ICP calibration standard) in the reconstitution. The PLs were washed three times by centrifugation and resuspension in iron-free buffer, and then the total internal volumes were determined by measuring the iron contents (19) of matched pairs of samples prepared with and without additional iron.

### RESULTS

**Characterization of the PLs and SMPS—Comprehensive characterizations of the three preparations of coupled vesicles used here (BtPLs, YfPLs and BtSMPS) are summarized in Table 1. Each preparation displays high catalytic activity (NADH:DQ oxidoiodation for PLs (16, 38) or NADH:O\textsubscript{2} oxidoiodation for SMPS (involving complexes I, III, and IV of the respiratory chain, with endogenous ubiquinone-10 as an intermediate)) that is inhibited by piericidin A, a canonical complex I inhibitor. Gramicidin, an ionophore that collapses \Delta\textit{p}, stimulates catalysis significantly, showing that \Delta\textit{p} is substantial (see below also). Finally, average hydrodynamic radii and total internal volume measurements showed that each particle contains at least 100 correctly oriented enzyme molecules, allowing them to be treated as homogeneous populations.

**Confirmation That Mitochondrial CI Catalyzes Only Redox-driven Proton Translocation—ACMA is a fluorescent dye that is used widely to demonstrate \Delta\textit{p} formation across vesicular membranes; when ACMA is taken up by activated vesicles its fluorescence is quenched, most likely by dimerization and excimer formation (39). Fig. 2A shows that adding NADH and DQ to BtPLs, and NADH to BtSMPS, rapidly quenches the ACMA fluorescence; the effect is reversed when \Delta\textit{p} is dissipated by gramicidin or when proton translocation is inhibited by rotenone. Note that a membrane-permeant charge-compensating ion is required to dissipate \Delta\textit{ψ} (so that \Delta\textit{ψ} \rightarrow 0 and \Delta\textit{pH} \rightarrow \Delta\textit{p}). Thus, for BtSMPS, chloride or nitrate was added to the external solution (the membrane is readily permeable to both (23, 33,
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![FIGURE 2. Mitochondrial complex I is a redox-coupled proton pump.](image)

For example, if Na\textsuperscript{+} is co-translocated with the H\textsuperscript{+}, then adding Na\textsuperscript{+} to the external solution would create a ΔNa\textsuperscript{+} that favors NAD\textsuperscript{H} oxidation over NAD\textsuperscript{+} reduction (an apparent uncoupling) and the apparent Δp would decrease. Fig. 2B shows that the external Na\textsuperscript{+}, K\textsuperscript{+}, or Ch\textsuperscript{+} concentration does not affect the apparent Δp (the rate of ATP hydrolysis was not affected), so mitochondrial CI catalyzes only redox-coupled H\textsuperscript{+} transfer.

**ACMA Fluorescence Experiments Indicate That Deactive BtCI Catalyzes Na\textsuperscript{+}/H\textsuperscript{+} Exchange**—In Experiment A (Fig. 3), PLs loaded with Na\textsuperscript{+} (formed in high [Na\textsuperscript{+}] buffer) were placed into low Na\textsuperscript{+} buffer (with the same pH and ionic strength, balanced with Ch\textsuperscript{+}). If CI acts as an antiporter then Na\textsuperscript{+} from inside are exchanged for H\textsuperscript{+} from outside, and the intravesicular pH decreases (ΔNa\textsuperscript{+} is converted to ΔpH). It is not possible to control the Na\textsuperscript{+} concentration in the lumen of SMPs (see supplemental Information), so Experiment A is only possible for PLs. In Experiment B, SMPs were loaded with protons by succinate:O\textsubscript{2} oxidoreduction (independently of CI) to drive proton uptake. Then, succinate oxidation was inhibited, and Na\textsuperscript{+} was added to the external solution. If CI acts as an antiporter, then H\textsuperscript{+} from inside are exchanged for Na\textsuperscript{+} from outside, and the intravesicular pH rises (ΔpH is converted to ΔNa\textsuperscript{+}). It is not possible to load the lumen of PLs with sufficient H\textsuperscript{+} for this experiment without using CI itself, so Experiment B is only possible for SMPs. Ideally, both the intravesicular H\textsuperscript{+} and Na\textsuperscript{+} concentrations should be monitored during these experiments. However, despite extensive trials (see supplemental Information), we were unable to incorporate H\textsuperscript{+} - and Na\textsuperscript{+} -specific probes into the luminal volumes of either PLs or SMPs while retaining the catalytic activity of CI. Therefore, we used ACMA to monitor the formation or dissipation of ΔpH (with Δψ collapsed to zero using a permeant ion).

During initial trials with BtPLs and YPLs in Experiment A, and BtSMPs in Experiment B, it was noted that the YPLs behaved more reproducibly than the BtPLs or BtSMPs; for BtCI, different preparations presented very different levels of apparent antiporter activity. Subsequent investigation revealed that BtCI, in both PLs and SMPs, was present as a mixture of deactive and active forms (11, 12), whereas YPLs were essentially all deactive (as judged by their NEM sensitivity) (41). Therefore, in all the experiments described below, the deactive/

![FIGURE 3. Schematic representation of two experiments that reveal the Na\textsuperscript{+}/H\textsuperscript{+} antiporter activity of complex I. The diagrams represent a typical complex I PL (see data in Table 1); the complex at the top has been magnified. A, Na\textsuperscript{+}-loaded vesicles are placed into a low [Na\textsuperscript{+}] buffer. If complex I is a Na\textsuperscript{+}/H\textsuperscript{+} antiporter, then Na\textsuperscript{+} efflux drives H\textsuperscript{+} uptake and the pH of the lumen rapidly decreases, forming ΔpH. B, the vesicles have been loaded with protons to create ΔpH. The driving force for H\textsuperscript{+} efflux is high, but uncatalyzed H\textsuperscript{+} efflux is slow; rapid H\textsuperscript{+} efflux is observed if it is coupled to Na\textsuperscript{+} uptake by complex I (the pH of the lumen rapidly increases, and ΔpH collapses).
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For Experiment B (Fig. 5A), succinate was added to BtSMPs, to use succinate:O\(_2\) oxidoreduction to create ΔpH (the protons are translocated by complexes III and IV) and quench the ACMA fluorescence (Cl\(^-\), a permeant anion, ensures that Δψ is converted to ΔpH). Then, cyanide was added (to inhibit catalysis by complex IV and so stop proton translocation), along with 10 mM Na\(^+\) (ΔpH collapses when an antiporter is present as H\(^+\) efflux is coupled to Na\(^+\) uptake). For deactive BtSMPs, the fluorescence recovers immediately (as ΔpH collapses); it recovers much more slowly in the presence of rotenone, or for active BtSMPs. Note that deactive and active BtSMPs exhibit the same respiratory control ratio for succinate:O\(_2\) oxidoreduction (1.6 ± 0.25), so the deactive particles are not uncoupled. In addition, when cycles of activation and deactivation were applied to BtSMPs, the results from Experiment B alternated accordingly, although accompanied by a gradual loss of succinate:O\(_2\) oxidoreduction activity and/or coupling. Fig. 5B shows equivalent experiments using K\(^+\) instead of Na\(^+\); the data are consistent with the antiporter activity being specific to Na\(^+\).

Finally, the small apparent “burst” of recovery in Fig. 5A upon the addition of Na\(^+\) and the “dip” in Fig. 5B upon the addition of K\(^+\) are experiment-independent, ion-dependent artifacts of the ACMA system. In fact, otherwise identical experiments with...
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A: Bt-PLs D + Na⁺
+ Caps.
+ Fenp.
+ Acetol.
+ MPP⁺
+ Pier.
+ Rot.
+ Stig.
+ NEM

B: PLs + Na⁺
Bt-A
Bt-D
Yt-A
Yt-D

C: Bt-SMPs
D
D + Rot.
A
Δ
D + Rot.
A
D
D + Rot.
A

FIGURE 6. Characteristics of the antiporter reactions in PLs and BtSMPs. A, BtPLs, with deactive CI, were tested using Experiment A in the presence of various inhibitors. A high value for the normalized fluorescence quench (100% fluorescence quench = fluorescence in the presence of monensin) 75 s after the PLs were added indicates high antiporter activity (Fig. 4). Caps.: 50 μM capsaicin; Fenp.: 150 μM fenpyroximate; Acetol.: 30 μM Δlac-acetogenin; MPP⁺, 5 mM 1-methyl-4-phenyl-pyridinium; Pier.: 1 μM piericidin A; Ranol.: 200 μM ranolazine; Rot.: 200 mM rotenone; Stig.: 250 μM stigmatellin. NEM indicates that the BtPLs were preincubated in 1 mM NEM for 25 min and then washed and collected by centrifugation. B, BtPLs and YtPLs were tested using Experiment A following activation (-A) or deactivation (-D). C, BtSMPs, with active or deactive CI, in the presence or absence of 200 mM rotenone (Rot.), were tested for M⁺/H⁺ antiporter activity (M⁺ = Li⁺, Na⁺, K⁺, or Rb⁺). A high 1/t½ (where t½ is the time taken for the fluorescence to recover to half its full value upon the addition of M⁺) indicates significant antiporter activity (Fig. 5).

Li⁺, Na⁺, K⁺, or Rb⁺ revealed an individual “signature” for each ion; we are currently unable to explain these effects.

Fig. 6 shows a selection of results that have been quantified using the normalized fluorescence intensity 75 s after the PLs were added (Fig. 4, Experiment A, expressed as the percentage of the normalized fluorescence that was quenched) or the time taken for the normalized fluorescence intensity to recover to 50% (t½) (Fig. 5, Experiment B, expressed as 1/t½). In each case, high values indicate significant antiporter activity. Fig. 6A shows the results from testing several complex I inhibitors in Experiment A (see “Experimental Procedures”). Capsaicin, the Δlac-acetogenin, 1-methyl-4-phenyl-pyridinium, piericidin A, ranolazine, and stigmatellin had no effect on the antiporter activity, whereas fenpyroximate (as well as rotenone) inhibited it. However, fenpyroximate was atypical because the slow initial rate of quenching eventually begins to increase; this was not observed for rotenone. EIPA is a canonical Na⁺/H⁺ antiporter inhibitor (42) that has been used to study proton translocation by complex I (10, 27–29), and palmitic acid has been linked to the deactive-active transition in complex I (32). Both inhibitors abolished ACMA fluorescence quenching by deactive BtPLs, but no quenching was observed when monensin was added, and further investigation revealed that they are both uncouplers (see also Ref. 43). It was not possible to identify any concentrations (from 5 pm to 5 mM) that inhibited but did not uncouple; EIPA and palmitic acid should not be used to study ion translocation by complex I. Similarly, 2-decyl-4-quinazolinyl amine is both a complex I inhibitor (26) and an uncoupler. In Experiment B, inhibition and uncoupling oppose each other, and inhibition of the antiporter activity by 2-decyl-4-quinazolinyl amine could be observed at low concentrations (100 nM).

Fig. 6A shows that treating deactive BtPLs with NEM, as required to derivatize the widely conserved cysteine in ND3 that is accessible only in the deactive enzyme (22) (Fig. 1A), inhibits the antiporter activity. Note that it was necessary to remove the NEM before the assay to avoid artifacts in the ACMA measurement; this extra experimental step, and the effects of small amounts of DMSO, resulted in greater variation in the results. Qualitatively similar results were obtained from SMPs, except that side reactions of NEM with complex II compromised the formation of ΔpH, decreasing the intensity of the ACMA response. In separate experiments, ATP hydrolysis was used to create ΔpH and inhibited by adenosine 5’-(β,γ-imido)-triphosphate (44); ATP hydrolysis was also affected by NEM, but a large enough ΔpH could be formed to confirm that the antiporter activity of complex I is sensitive to NEM. Fig. 6B shows that YtPLs do not display any Na⁺/H⁺ antiporter activity, irrespective of attempts to convert the Y. lipolytica enzyme between the deactive and active forms. Finally, Fig. 6C compares the results from Experiment B with Li⁺, Na⁺, K⁺, and Rb⁺, confirming that antiporter activity is Na⁺-specific.

Vesicle-swelling Experiments Confirm That Deactive BtCI Catalyzes Na⁺/H⁺ Exchange—Mitochondria-swelling experiments in isotonic media have been used to characterize ion movement across the inner mitochondrial membrane (45–47); ions accumulating in the matrix cause water uptake (both a cation and an anion must be taken up to balance the charge), and the mitochondria swell as the inner membrane unfolds. Here, we used an analogous approach to monitor ion uptake by BtSMPs; changes in the hydrodynamic radii (the “radii”) were monitored by dynamic light scattering. Thus, sodium acetate was added to the external buffer solution; when an antiporter is present, Na⁺ uptake coupled to H⁺ efflux creates an “inverted” ΔpH (alkali inside) which drives the uptake of neutral acetic acid and the net uptake of sodium acetate, driving water uptake and swelling.

Deactive BtSMPs were placed into 250 mM sucrose and 10 mM Tris-SO₄ pH 7.5, and their average radius was measured to be 145 ± 3 nm. When 250 mM sodium acetate was added to the external buffer (at constant sucrose and buffer concentrations), the radius increased significantly to 190 ± 15 nm; measurements were taken over 10 min from the addition of the sodium acetate (as the BtSMPs continue to swell until they burst). In equivalent experiments with active BtSMPs or with lithium acetate or potassium acetate, no significant changes in radius were observed (the variation was <5 nm). The results are consistent with Na⁺/H⁺ exchange by deactive complex I only. However, experiments to test the effects of rotenone were
unsuccessful because rotenone alone caused a significant (20 ± 5 nm) increase in the radius of both deactive and active BtSMPs.

**DISCUSSION**

**Monitoring Redox-driven Ion Translocation by Complex I**—In our initial experiments, we used the ΔpH-sensitive dye ACMA to reconfirm that mitochondrial complex I catalyzes redox-linked H⁺ translocation, and a thermodynamic balance experiment to exclude the possibility of additional redox-linked Na⁺ translocation. To monitor H⁺ and Na⁺ transfer directly, in real time, we investigated several pH- and Na⁺-sensitive dyes, but were unable to identify any that did not inhibit catalysis and that could be incorporated into the luminal volumes of coupled vesicles (see supplemental information). Thus, to monitor Na⁺/H⁺ exchange, we designed experiments that relied upon the formation or dissipation of ΔpH monitored by using ACMA. ACMA was compatible with both PLs and SMPs, but its response is only semiquantitative; its fluorescence is affected by many molecules used here (notably DQ, ethanol, and M⁺ (where M⁺ = Li⁺, Na⁺, K⁺, or Rb⁺)), the level of quenching observed is subject to the size and concentration of the vesicles, the enzyme activity/concentration, and the lipid composition, and equal ΔpH values created differently (using different pH buffers, different NaCl concentrations with monensin or valinomycin + carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) give different responses. Therefore, we compared only “like-with-like,” employed exhaustive sets of controls, and supported our conclusions by vesicle-swelling experiments, a completely different measure of ion transport. We note that Na⁺ translocation by several prokaryotic complexes I has been studied previously by using radioactive 23Na, atomic absorption spectroscopy, or 23Na NMR with a shift reagent in the external solution to follow Na⁺ uptake into vesicles directly (6–9). However, these are not real-time methods (so short-lived ion gradients established during catalysis may relax before the measurement). Furthermore, the conclusions of studies of redox-driven ion translocation by *E. coli* complex I using these methods have disagreed completely; they have proposed that Na⁺ transfer occurs in opposite directions (7, 9).

**The Na⁺/H⁺ Antiporter Activity of Deactive *B. taurus Complex I***—Our results show that deactive BtCI is a Na⁺/H⁺ antiporter; it exchanges Na⁺ and H⁺ across a membrane to balance the ion- and proton-motive forces. Importantly, Na⁺/H⁺ exchange is independent of energy transduction; it halts when the enzyme is reactivated by redox-linked H⁺ translocation. Together with the sequence similarity of the complex I ND2, ND4, and ND5 subunits with the Mrp antiporter subunits MrpA and MrpD (see supplemental Fig. 1 and Fig. 1B), the ability of the complex I ND2, ND4, and ND5 subunits to rescue Mrp antiporter subunit knockouts in *Bacillus subtilis* (3, 4), and the Na⁺/H⁺ antiporter activity of an overexpressed truncated form of ND5 (27), our results suggest that one or more of ND2, ND4, and ND5 retain an inherent antiporter capability that is exhibited in the deactive enzyme. We propose that in deactive BtCI, the functional connection between the redox and antiporter modules (Fig. 1) is broken; energy-transducing catalysis abolishes the antiporter activity by initiating their reconnection, whereupon the redox domain “harnesses” the antiporter domain for proton translocation. It is further possible that the H⁺ transfer-ready states created by the redox reaction and by Na⁺ transfer resemble one another. Interestingly, rotenone binds more strongly to active BtCI than to the deactive enzyme, and it partially prevents and reverses deactivation (48). Thus, rotenone does not inhibit the antiporter activity of deactive complex I directly; it abolishes it by functionally reconnecting the antiporter domain to the redox domain. The antiporter domain is brought under control and can no longer freewheel. Our proposal is consistent with the location of the rotenone-binding site in complex I, at the base of the redox domain, not in the antiporter-like subunits (49).

Antiporter activity was observed here only in deactive BtCI, and not in YCl, despite the fact that the ND3 cysteine in “as prepared” YClI could be derivatized by NEM, a key diagnostic criterion for the deactive enzyme. Derivatizing the ND3 cysteine with NEM in BtCI inhibits the antiporter activity, further confirming the link between the active/deactive status of the enzyme and the antiporter activity. Clear differences between the active/deactive transitions in BtCI and YCI have been noted previously; for example, the apparent active/deactive transition is much less temperature-dependent in YCI (50). Thus, we propose that the deactive and active states in BtCI differ more extensively than they do in YCI, with only the transition in BtCI progressing to the functional disconnection of the redox and transport domains. It is possible that the truncation of ND2 in BtCI (Fig. 1A) is one determinant of the extent of the transition (51); the widely conserved cysteine in ND3 that is accessible to NEM only in the deactive enzyme is in a loop close to the three (truncated) helices (2, 22). Finally, we note that increased ACMA quenching was observed during catalysis by *E. coli* complex I PLs upon the addition of either extra NaCl or ETH-157, a sodium ionophore (although no antiporter activity was observed in an experiment similar to that shown in Fig. 4) (10). This result was used to suggest “secondary antiporter activity,” but no readily permeant ion was present, so it may arise from enhanced ion transfer that relaxes Δψ and facilitates ΔpH formation instead.

**A Physiological Role for the Antiporter Activity of Deactive Complex I?**—The deactive transition of complex I may contribute to determining the outcome of ischemia-reperfusion. Partially inhibiting the respiratory chain during ischemia, for example by inhibiting complex I, protects against mitochondrial damage and myocardial injury (52). Decreased respiratory chain turnover also favors the deactivation of complex I, making it susceptible to thiol reagents, such as S-nitrosothiols, that “lock” it in the deactive state (14) until the thiol pools recover. Thus, mitochondrially targeted S-nitrosothiols protect against ischemia-reperfusion injury (53). Our observation that deactive complex I is a Na⁺/H⁺ antiporter suggests an additional role for it during ischemia-reperfusion. Under normal conditions, transport cycles in the mitochondrial inner membrane, comprising Na⁺/H⁺ exchange, Na⁺/Ca²⁺ exchange, and Ca²⁺ uniport, contribute to cellular Ca²⁺ homeostasis (54). During ischemia, the intracellular Ca²⁺, Na⁺, and H⁺ concentrations rise, and Ca²⁺ accumulates in the mitochondrial matrix, contributing to opening the permeability transition pore (54, 55). It is possible that Na⁺/H⁺ exchange by deactive complex I influ-
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ences Ca²⁺ accumulation by altering the ion transport properties of the inner membrane. Furthermore, Na⁺/H⁺ exchange across the inner membrane of mammalian mitochondria was identified more than 40 years ago by mitochondria-swelling experiments (45–47), but the protein responsible has not been identified (56). In our experiments, complex I is the dominant Na⁺/H⁺ exchanger, and a clear preference for Na⁺ over K⁺ was also observed in nonenergized mitochondria, consistent with a significant contribution from deactive complex I under these conditions. Finally, inhibiting Na⁺/H⁺ exchange across the plasma cell membrane, to attenuate the rise in intracellular Na⁺, is a well developed strategy for limiting ischemia-reperfusion injury (55). EIPA is one of the 5-N-substituted amiloride family of Na⁺/H⁺ exchange inhibitors, and ranolazine, in clinical use for angina, is proposed to be a Na⁺/H⁺ exchange inhibitor and also a complex I inhibitor (33). Here, we found no evidence that either compound inhibits Na⁺/H⁺ exchange by deactive complex I (although EIPA is an efficient uncoupler of Δp). Thus, the response of deactive complex I to pharmacological agents is clearly distinct from that of the plasma membrane exchangers, inviting the prospect of targeting them individually.

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