The Ferredoxin:NAD⁺ Oxidoreductase (Rnf) from the Acetogen Acetobacterium woodii Requires Na⁺ and Is Reversibly Coupled to the Membrane Potential*

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Background: Ferredoxin:NAD⁺-oxidoreductases (Rnf) found in many bacteria are novel ion-translocating electron transport chains.

Results: A Na⁺ requirement for the reaction and its reversible coupling to the transmembrane Na⁺ gradient are demonstrated.

Conclusion: Na⁺ is the coupling ion. Rnf not only generates a Na⁺ potential but also uses it to drive the reverse reaction.

Significance: Evidence for a function of Rnf in ferredoxin reduction is provided.

The anaerobic acetogenic bacterium Acetobacterium woodii has a novel Na⁺-translocating electron transport chain that couples electron transfer from reduced ferredoxin to NAD⁺ with the generation of a primary electrochemical Na⁺ potential across its cytoplasmic membrane. In previous assays in which Ti³⁺ was used to reduce ferredoxin, Na⁺ transport was observed, but not a Na⁺ dependence of the electron transfer reaction. Here, we describe a new biological reduction system for ferredoxin in which ferredoxin is reduced with CO, catalyzed by the purified acetyl-CoA synthase/CO dehydrogenase from A. woodii. Using CO-reduced ferredoxin, NAD⁺ reduction was highly specific and strictly dependent on ferredoxin and occurred at a rate of 50 milliunits/mg of protein. Most important, this assay revealed for the first time a strict Na⁺ dependence of this electron transfer reaction. The $K_m$ was 0.2 mM. Na⁺ could be partly substituted by Li⁺. Na⁺ dependence was observed at neutral and acidic pH values, indicating the exclusive use of Na⁺ as a coupling ion. Electron transport from reduced ferredoxin to NAD⁺ was coupled to electrogenic Na⁺ transport, indicating the generation of $\Delta\mu_{\text{Na}⁺}$. Vice versa, endergonic ferredoxin reduction with NADH as reductant was possible, but only in the presence of $\Delta\mu_{\text{Na}⁺}$, and was accompanied by Na⁺ efflux out of the vesicles. This is consistent with the hypothesis that Rnf also catalyzes ferredoxin reduction at the expense of an electrochemical Na⁺ gradient. The physiological significance of this finding is discussed.

Growth and acetate formation as carried out by the acetogenic bacterium Acetobacterium woodii are strictly sodium ion-dependent (1). Sodium ions are essential for ATP synthesis, rotation of the flagellar motor, and accumulation of nutrients that are taken up by secondary transport systems (2–5). Acetogenesis as carried out by resting cells is coupled to the generation of a transmembrane electrochemical Na⁺ gradient across the cytoplasmic membrane that is the driving force for the above-mentioned endergonic membrane processes. The nature of the enzyme(s) that generates $\Delta\mu_{\text{Na}⁺}$ has been obscure for decades, but we recently demonstrated a ferredoxin:NAD⁺ oxidoreductase activity at the cytoplasmic membrane of A. woodii (6). This enzyme is part of the electron transport chain leading from, for example, hydrogen to carbon dioxide or caffeate as electron acceptor. The enzyme was partially purified, and polypeptides in the preparation were identified to be encoded by rnf genes (7) that were hypothesized before in Rhodobacter capsulatus to encode a membrane-bound, ion-motive electron transport system (8). Rnf complexes are now widely believed to be of central importance for the bioenergetics of anaerobic bacteria (and some archaea), and very often, they are the only ion-motive electron transport chain present in these anaerobes (9). Inspection of the genome sequence of A. woodii did not reveal any candidate apart from the Rnf cluster that could potentially link acetogenesis or caffeate respiration to the generation of $\Delta\mu_{\text{Na}⁺}$ (10). Experiments using inverted membrane vesicles clearly revealed that the ferredoxin:NAD⁺ oxidoreductase is coupled to Na⁺ transport (11). However, the interpretation of these data as evidence for the use of Na⁺ as coupling ion was not unambiguous because a Na⁺ dependence of NAD⁺ reduction was not observed, and it could not be excluded that Na⁺ transport is only a side reaction of the enzyme. We have followed up this central question and present here an enzyme assay that enabled us to demonstrate a strict Na⁺ dependence of Rnf-catalyzed electron transport. Moreover, electron transport was coupled to the generation of $\Delta\mu_{\text{Na}⁺}$ that, vice versa, drove endergonic electron flow from NADH to ferredoxin.

**Experimental Procedures**

*Growth Conditions*—For preparation of membranes and purification of acetyl-CoA synthase/CO dehydrogenase (Acs/
Correlation of CODH,

A. woodii (DSM 1030) was grown at 30 °C on 20 mM fructose under anaerobic conditions as described previously (2). For preparation of vesicles, A. woodii was grown on 20 mM fructose as described (11) in 20-liter flasks (Glasgerätebau Ochs, Bovenden-Lengern, Germany).

Preparation of Membranes—Membranes of fructose-grown cells were prepared as described previously (6). The cell-free extract was separated into cytoplasmic and membrane fractions by ultracentrifugation at 150,000 × g for 2 h at 4 °C. The resulting sediment was washed once with membrane buffer (50 mM Tris-HCl (pH 8.5) containing 20 mM MgSO4, 20% glycerol, 2 mM dithioerythritol (DTE) as a reducing agent, and 4 μM resazurin as a redox indicator).

Preparation of Vesicles—Vesicles were prepared as described (11) but with slight modifications. Protoplasts were harvested anaerobically by centrifugation at 6250 × g for 20 min at 4 °C and washed with vesicle buffer (50 mM Tris-HCl (pH 8) containing 25 mM MgSO4, 420 mM sucrose, 8 mM DTE, and 4 μM resazurin). After washing the protoplasts, they were resuspended in a total volume of 10 ml of vesicle buffer. These protoplasts were passed through a French pressure cell at 41 mega-pascals (MPa) and centrifuged three times at 4500 × g for 35 min at 4 °C. The resulting supernatant was centrifuged further by ultracentrifugation at 120,000 × g for 40 min at 4 °C. The pellet was washed with vesicle buffer and centrifuged again. The resulting pellet was resuspended in the same buffer in a volume of 5 ml.

Purification of Acs/CODH—For purification of Acs/CODH, A. woodii (DSM 1030) was grown at 30 °C under anaerobic conditions in 20-liter flasks using 20 mM fructose to an A560 of ~2.0 as described previously (2). The cytoplasmic fraction was prepared as described previously (12). The cytoplasmic fraction containing ~1000 mg of protein was applied to Q-Sepharose high performance column (2.6 × 5 cm) equilibrated with buffer A (25 mM Tris-HCl, 20 mM MgSO4, 150 mM NaCl, and 20% glycerol (pH 7.5)). Protein was eluted with a 170-ml linear gradient of 150–400 mM NaCl in buffer A. Methyl viologen-dependent CO dehydrogenase activity eluted at ~350 mM NaCl. Ammonium sulfate (0.8 M) was added to the pooled fractions, and these were loaded onto a phenyl-Sepharose high performance column (1.6 × 10 cm) equilibrated with buffer B (25 mM Tris-HCl, 20 mM MgSO4, 0.8 M (NH4)2SO4, and 20% glycerol (pH 7.5)). Protein was eluted with a 160-ml linear gradient from 0.8 to 0 M (NH4)2SO4 in buffer B. Acs/CODH activity eluted in a peak at ~0.4 M (NH4)2SO4. Pooled fractions were concentrated by ultrafiltration in 50-kDa Vivaspin tubes (Sartorius Stedim Biotech GmbH, Göttingen, Germany), applied to a Sephacryl S-300 high resolution column (1.6 × 60 cm) equilibrated with buffer C (25 mM Tris-HCl, 20 mM MgSO4, and 20% glycerol (pH 7.5)), and eluted with buffer C at a flow rate of 0.8 ml/min. Acs/CODH activity eluted after 54 ml. The size of the protein was determined using a high molecular weight calibration kit (GE Healthcare) with conalbumin, aldolase, ferritin, and thyroglobulin as size standards run under identical conditions as Acs/CODH on a Sephacryl S-300 column. The enzyme was stable for weeks when stored at 4 °C.

Measurement of Rnf Activity—All measurements were performed at 30 °C in anaerobic cuvettes (Glasgerätebau Ochs) sealed by rubber stoppers with 1 ml of 20 mM Tris (sodium-free)-HCl (pH 7.7) containing 2 mM DTE and 2 μM resazurin in 3-fold distilled water. Measurement of CO-mediated ferredoxin:NAD+ oxidoreductase activity was performed at a pressure of 0.5 × 105 Pa of CO with 30 μg/ml Acs/CODH, 30 μM ferredoxin, and 150 μg/ml washed membranes. Ferredoxin was purified from Clostridium pasteurianum as described (13). The reaction was started by the addition of NaCl. Formation of NADH was measured photometrically at 340 nm (εNADH = 6.2 mM−1 cm−1). NaCl was added as indicated. For determination of electron transfer from reduced ferredoxin to NaCl, membrane vesicles were added at a concentration of 50 μg/ml ETH2120, 3,3’4,4’-tetrachlorosalicylanilide, valinomycin, and monensin were added at a concentration of 10 μM each. The potassium concentration inside the vesicles in the assay with valinomycin and potassium was 50 mM. Measurement of titanium citrate-mediated ferredoxin:NAD+ oxidoreductase activity was performed in a N2 atmosphere as described for the Acs/CODH-mediated assay, except that Acs/CODH was substituted with 5 mM titanium citrate, prepared according to Zehnder and Wuhrmann (14), and sodium salts were substituted with potassium salts. Electron transfer from reduced methyl viologen to NADH was measured at 340 nm as described for the CO-mediated ferredoxin:NAD+ oxidoreductase activity measurement, except that ferredoxin was substituted with 10 μM methyl viologen. Electron transfer from NADH to ferredoxin was measured in 100 mM Tris (pH 7.5), 5 mM MgCl2, 2 mM NaCl, 30 μM ferredoxin, 2 mM DTE, and 2 μM resazurin in a nitrogen atmosphere with 50 μg/ml washed membrane vesicles. ATP was added at a concentration of 2 mM. The reaction was started by the addition of 500 μM NADH. ETH2120 was used at a concentration of 10 μM.

Measurement of Acs/CODH Activity—Measurements were performed at 30 °C in anaerobic cuvettes filled with 1 ml of buffer containing 100 mM HEPES/NaOH (pH 7.0), 2 mM DTE, and 2 μM resazurin at a gas phase of 100% CO with an overpressure of 1.1 × 105 Pa. For CO-dependent reduction of methyl viologen (10 mM), activity was measured at 604 nm (ε = 13.9 mM−1 cm−1). Reduction of ferredoxin (20 μM) was assayed at 430 nm (ε = 13.1 mM−1 cm−1).

Measurement of Na+ Translocation—The experiments for Rnf-mediated Na+ import into the inverted membrane vesicles were performed under anaerobic conditions in 20 mM Tris (sodium-free)-HCl (pH 7.7) containing 2 mM DTE and 4 μM resazurin in 3-fold distilled water at 30 °C as described previously (2, 11). In 3.5-ml glass vials, 1 ml of buffer was supplemented with 30 μM ferredoxin and 3 mg/ml vesicles. The vials were pressurized with an atmosphere of CO or N2 (0.5 × 105 Pa). NaCl was added at the concentrations indicated; the contaminating amount of Na+ was 65 μM. Radioactive 22Na (carrier-free) was added to a final concentration of 0.5 μCi/ml. The sample was incubated for 20 min at 30 °C to ensure equilibration of 22Na before the reaction was started. Acs/CODH (30 μg/ml) or titanium citrate (5 mM) was added before the reaction.

The abbreviations used are: Acs/CODH, acetyl-CoA synthase/CO dehydrogenase; DTE, dithioerythritol; Pa, pascals.
Rnf from A. woodii

start, which was done by the addition of NAD\(^+\) (concentrations as indicated). If CO-reduced methyl viologen was tested as electron donor, ferredoxin was substituted with 1 mM methyl viologen. The experiments for Rnf-mediated export of Na\(^+\) were performed as described above in buffer containing 100 mM Tris (pH 7.5), 5 mM MgCl\(_2\), 2 mM NaCl, 100 \(\mu\)M ferredoxin, 2 mM DTE, and 2 \(\mu\)M resazurin in a nitrogen atmosphere. ATP was added at a concentration of 2 mM. Sodium export was induced by the addition of 5 mM NADH.

**Results**

**Determining the Na\(^+\) Concentration**—The Na\(^+\) concentration in the buffer was determined with an Orion 84-11 ROSS sodium electrode (Thermo Electron Corp., Witchford, United Kingdom) as described (15).

**Analytical Methods**—Protein concentration was measured according to Bradford (16). Proteins were separated under denaturing conditions on 12% polyacrylamide gels (17) or under native conditions using clear native PAGE according to Wittig et al. (18) with a gradient of 5–11% polyacrylamide. Proteins were stained with Coomassie Brilliant Blue G-250.

**Results**

**Establishing a CO-dependent Enzyme Assay for Rnf**—The ferredoxin:NAD\(^+\) oxidoreductase activity is difficult to assay because reduction of the electron donor ferredoxin (\(E_0^-\) \(\sim\) 500 mV) requires electrons of very low redox potential. In the assay that was used previously (6, 11), these were provided by Ti\(^{3+}\) (\(E_0^-\) \(\sim\) -480 mV) (15). However, Ti\(^{3+}\) is a very strong and unspecific reducing agent. For example, it is able to reduce NAD\(^+\) directly in a pH-dependent manner (19). This is the reason that the assay for ferredoxin:NAD\(^+\) oxidoreductase is usually done at pH 6. However, this assay did not reveal a Na\(^+\) dependence of ferredoxin-dependent NAD\(^+\) reduction.

An alternative biological reducing agent is carbon monoxide (\(E_0^-\)\(_{\text{CO/CO}_2}\) \(\sim\) -520 mV), which is oxidized to carbon dioxide by Acs/CODH from A. woodii. The electron acceptor in this reaction is ferredoxin (20). Acs/CODH was purified from a cell-free extract of A. woodii grown on fructose by chromatography on Q-Sepharose, phenyl-Sepharose, and Sephacryl S-300. This procedure led to an enrichment of 9-fold with a yield of 5%. The preparation contained primarily proteins of \(\sim\) 63 and 78 kDa as derived from denaturing gel electrophoresis (Fig. 1A), which correspond in mass to the AcsA and AcsB subunits of Acs/CODH encoded by the genome of A. woodii. Separation by native gel electrophoresis revealed one major protein complex with an apparent mass of \(\sim\) 350 kDa (Fig. 1B), which fitted well with the values obtained by calibrated gel filtration (Fig. 1C).

The purified Acs/CODH catalyzed CO oxidation with methyl viologen (70 units/mg) or ferredoxin (11 units/mg) from C. pasteurianum as an electron acceptor.

Next, we tested whether the purified Acs/CODH was able to reduce NAD\(^+\) directly. As this was not the case (Fig. 2), we added washed membranes from A. woodii. This also did not result in NAD\(^+\) reduction, showing that the preparation did not contain ferredoxin and that there was no other entry port for electrons into the membrane. After the addition of ferredoxin, NAD\(^+\) was reduced at a rate of 50 million units/mg of protein. This activity was strictly dependent on the presence of ferredoxin, CO dehydrogenase, CO, and membranes.

**Sodium Ion Dependence of Ferredoxin:NAD\(^+\) Oxidoreductase Activity**—With Acs/CODH and CO as the reduction system for ferredoxin, we reinvestigated the effect of Na\(^+\) on ferredoxin-dependent NAD\(^+\) reduction. As shown in Fig. 3, there was only low activity in the absence of added NaCl; the Na\(^+\) concentration in this assay due to contamination was 65 \(\mu\)M. However, upon addition of NaCl, electron transfer and thus NAD\(^+\) reduction were restored in a Michaelis-Menten-type manner. Half-maximal activity was at 200 \(\mu\)M, and saturation was at 3 mM. Li\(^+\) could partly substitute for Na\(^+\), but K\(^+\) only to a small extent. Other sodium salts such as sodium glutamate and sodium bromide were as effective as NaCl, but not the corresponding potassium salts. These data demonstrate a strict Na\(^+\) dependence of ferredoxin:NAD\(^+\) oxidoreductase activity.

The ferredoxin:NAD\(^+\) oxidoreductase activity had a pH optimum at 7–8 and declined sharply below or above the optimum. When the enzyme was tested for its Na\(^+\) dependence at
low pH (i.e. high proton concentrations), the results showed that the activity at low salt was similar at pH 6 and 7.7. The addition of NaCl led to the same Michaelis-Menten-type kinetics with a $K_m$ of 201 ± 30 μM and a $V_{max}$ of 50 milliunits/mg at pH 7.7 and 30 μM ferredoxin. At pH 6, the $K_m$ was 155 ± 39 μM, and the $V_{max}$ was 8 milliunits/mg at 30 μM ferredoxin. These analyses argue against a promiscuity of the enzyme toward Na⁺ and H⁺, as recently observed for other Na⁺-translocating enzymes such as ATP synthase (21) or the flagellar motor (22).

Different Electron Transfer Activities and Their Na⁺ Dependence—To dissect electron transfer and Na⁺ transport, we analyzed the effect of Na⁺ on the different activities catalyzed by Rnf. Again, Na⁺ stimulated NAD⁺ reduction by a factor of 5 (activity increased from ~10 to 50 milliunits/mg after the addition of NaCl) when CO-reduced ferredoxin was used as reductant. When Ti³⁺ was added as reductant for ferredoxin, the NAD⁺ reduction rate was 65 milliunits/mg without salt, but the activity was stimulated by only a factor of 2 with 20 mM NaCl. There was even less stimulation when the reducing agent Ti³⁺ was used in the absence of ferredoxin (activity increased from 35 to 50 milliunits/mg). These data confirm our data from a previous study in which we could not unequivocally demonstrate a Na⁺ dependence of NAD⁺ reduction driven by Ti³⁺-reduced ferredoxin (6). In addition, they also demonstrate a ferredoxin-independent entry port(s) into the electron transport chain for electrons coming from Ti³⁺.

Reduced methyl viologen has a redox potential $E_0'$ of ~450 mV, so we tested whether methyl viologen reduced by Acs/CODH is used as an electron donor for NAD⁺ reduction. Indeed, NAD⁺ was reduced at a rate of 1 unit/mg, but this activity was independent of Na⁺ and also not coupled to Na⁺ transport.

Comparison of Na⁺ Transport Coupled to Different Electron Transfer Activities—As observed before, electron transfer from Ti³⁺-reduced ferredoxin in one-time-washed vesicles was coupled to Na⁺ transport into the lumen of the vesicles. However, when Ti³⁺ was used in the absence of added ferredoxin, the same Na⁺ transport was observed (Fig. 4A). This is in accordance with previous data showing that Na⁺ translocation with Ti³⁺ as reductant was ferredoxin-dependent only if the vesicles were washed three times (11). In contrast, when CO-reduced ferredoxin was used as reductant, there was a clear dependence of the rate and the final accumulation factor on the ferredoxin concentration, although the vesicles were washed only once (Fig. 4B). These data are in accord with the previous measurements and the hypothesis that there is a ferredoxin-independent entry port of electrons derived from Ti³⁺ into the Rnf complex.

The Magnitude of the Na⁺ Gradient Depends on the NAD⁺ Concentration—NAD⁺ is the electron acceptor of the electron transport chain. NAD⁺ reduction was dependent on the concentration of the electron acceptor, and the dependence followed Michaelis-Menten kinetics. The apparent $K_m$ for NAD⁺ at a ferredoxin concentration of 30 μM was 85 ± 10 μM. Interestingly, the magnitude of Na⁺ accumulation was dependent on the NAD⁺ concentration. After a 10-min incubation, it was 4-fold at 0.2 mM NAD⁺ but increased to 11-fold at 4 mM NAD⁺ (Fig. 5). This is consistent with the $\Delta G'$ of the reaction being the limiting factor for $\Delta \mu_{\text{Na}^+}$ generation. An increase in NAD⁺/NADH ratio shifts the redox potential of the acceptor couple to more positive values and increases $\Delta G$.

Rnf-catalyzed Na⁺ Transport Is Electrogenic—After having established a sodium ion dependence of the enzymatic reaction and a dependence of the magnitude of the Na⁺ gradient on the electron acceptor concentration, we asked whether the Na⁺ transport is electrogenic. Reduced ferredoxin:NAD⁺-oxidoreductase activity at inverted membrane vesicles (100 ± 9%) was not stimulated by the protonophore 3,3',4',5-tetrachlorosalicylanilide but was stimulated by the sodium ionophore ETH2120 (155 ± 12%) and monensin (138 ± 15%), as well as by valinomycin in the presence of potassium (128 ± 20%). This is
consistent with the generation of an electrical field during Na\(^+\) transport.

**A Sodium Ion Gradient Drives NADH-dependent Ferredoxin Reduction**—The experiments described so far are in full accord with the use of Na\(^+\) to couple the redox reaction to the electrical field across the membrane. Next, we determined whether the reaction is reversible. Therefore, a transmembrane Na\(^+\) gradient was generated by ATP hydrolysis, catalyzed by the Na\(^+\) F\(_{1}\)F\(_{0}\)-ATP synthase from *A. woodii*. ATP hydrolysis generated a much larger Na\(^+\) gradient than ferredoxin-dependent NAD\(^+\) reduction, as expected from the larger \(\Delta G\) of the reaction. When NADH was added to the assay in the steady state of ATP hydrolysis, the Na\(^+\) content of the vesicles dropped from 36 to 6 nmol of Na\(^+\)/mg (Fig. 6). This decrease was observed only in the presence of oxidized ferredoxin. The ferredoxin dependence indicated Na\(^+\) eflux from the inverted membrane vesicles coupled to ferredoxin reduction with NADH as reductant. This was indeed observed. When vesicles were incubated with ferredoxin and the membrane was energized by Na\(^+\) translocation coupled to ATP hydrolysis, ferredoxin was reduced (Fig. 7). Ferredoxin reduction was dependent on ATP and a transmembrane Na\(^+\) gradient, as evident from the observed inhibition of ferredoxin reduction by the sodium ionophore ETH2120.

**DISCUSSION**

*A. woodii* is one of the rare organisms that rely only on a sodium ion potential for cellular bioenergetics: its F\(_{1}\)F\(_{0}\)-ATP synthase is strictly sodium ion-dependent (3, 23–25), its flagellar motor is powered by a sodium-motive force (4), and it grows in the absence of a transmembrane electrochemical proton gradient, indicating that the essential secondary transporters are also Na\(^+\)-coupled. A search for the enzyme that couples metabolism to the generation of the electrochemical sodium ion potential revealed that none of the enzymes that catalyze carbon flow in the Wood-Ljungdahl pathway are membrane-bound, excluding their role as Na\(^+\) pumps. This was recently corroborated by analyzing the proteins predicted from the genome sequence (10). Instead, we found a membrane-bound...
electron transfer chain that transfers electrons from reduced ferredoxin to NAD\(^+\) (6). Ferredoxin has a low redox potential \(E_{0}^\prime \approx -500 \text{ mV}\), and its reduction with H\(_2\) \((E_{0}^\prime = -414 \text{ mm})\) as reductant is catalyzed by a soluble iron-only hydrogenase that uses the exergonic electron transfer from H\(_2\) to NAD\(^+\) as reductant is catalyzed by a soluble iron-only hydrogenase. This is in contrast to other Na\(^+\) pumps, in which the enzymatic activity/rotation was found for methyl viologen:NAD\(^+\) oxidoreductase from \(A. \text{ woodii}\) as catalyst for ferredoxin reduction overcomes these problems. CO can be provided in large excess over NAD\(^+\), and electron transfer to NAD\(^+\) is strictly dependent on ferredoxin; thus, there is only one entry point for electrons into the Rnf complex. Using this assay, we observed a ferredoxin-dependent, highly reproducible NAD\(^+\) reduction; enhanced Na\(^+\) transport, which also leads to higher accumulation factors; and most importantly, a clear Na\(^+\) dependence of the ferredoxin:NAD\(^+\) oxidoreductase activity. These data are in line with the hypothesis that the ferredoxin:NAD\(^+\) oxidoreductase from \(A. \text{ woodii}\) is a Na\(^+\) pump. Evidence for additional H\(^+\) transport was not found, which is in agreement with previous data that demonstrate a tight coupling between acetogenesis and ATP synthesis via a sodium ion potential (1). \(\Delta \mu_{\text{H}^+}\)-driven ATP synthesis was excluded in that study.

The Rnf complex of \(A. \text{ woodii}\) has six subunits (Fig. 8). RnfB has a predicted polyferredoxin and is hypothesized to be the ferredoxin-binding site and RnfC to be the NAD\(^+\)-binding site. RnfG has only one transmembrane helix, which leaves RnfD (with its covalently bound flavin), RnfE, and RnfA as likely candidates for the Na\(^+\)-translocating subunit(s). It is interesting to note that Ti\(^{3+}\) can reduce NAD\(^+\), but this activity is hardly Na\(^+\)-dependent, indicating more than one entry point for Ti\(^{3+}\)-based electrons. This would then also argue for a Na\(^+\) site that is “after” the Na\(^+\) translocation site. The highest enzymatic activity was found for methyl viologen:NAD\(^+\)-oxidoreductase activity, but this activity was Na\(^+\)-independent and also not accompanied by Na\(^+\) transport. These data are consistent with the hypothesis that these activities are mediated by subcomplexes not harboring the Na\(^+\) translocation site. Possible candidates would be RnfC, RnfG, and RnfB.

As demonstrated here, Rnf activity at inverted membrane vesicles is stimulated by sodium ionophores. However, stimulation is weak, indicating a weak coupling. The redox potential difference between reduced ferredoxin and NAD\(^+\) is small, leading to a \(\Delta G_{0}'\) of the reaction of only \(-34.7 \text{ kJ/mol}\). During the course of the reaction, NAD\(^+\) is reduced, and the energetics becomes even worse through an increase in the NADH concen-
cation of the reaction. Increasing the NAD\(^+\) concentration shifts the \(\Delta G'\) of the reaction to more negative values, thus leading to higher Na\(^+\) gradients. However, they are still much smaller compared with ATP hydrolysis as driving force for Na\(^+\) export (\(\Delta G' = -190 \text{ mV}\)). Moreover, the activity of the Rnf complex is in the milliunits/mg range, whereas ATP hydrolysis is in the units/mg range. Both low activities and small \(\Delta G\) values may explain why we were not able to detect the generation of \(\Delta \Psi\) or ATP synthesis so far. However, we were able to reverse the reaction: a Na\(^+\) gradient established by ATP hydrolysis drove ferredoxin reduction. This not only demonstrates the metabolism of low potential substrates such as lactate (the reaction: a Na\(^+\)\() may explain why we were not able to detect the generation of complexin in the milliunits/mg range, whereas ATP hydrolysis.

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