

The Redox-Bohr Group Associated with Iron-Sulfur Cluster N2 of Complex I*

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Proton pumping respiratory complex I (NADH:ubiquinone oxidoreductase) is a major component of the oxidative phosphorylation system in mitochondria and many bacteria. In mammalian cells it provides 40% of the proton motive force needed to make ATP. Defects in this giant and most complicated membrane-bound enzyme cause numerous human disorders. Yet the mechanism of complex I is still elusive. A group exhibiting redox-linked protonation that is associated with iron-sulfur cluster N2 of complex I has been proposed to act as a central component of the proton pumping machinery. Here we show that a histidine in the 49-kDa subunit that resides near iron-sulfur cluster N2 confers this redox-Bohr effect. Mutating this residue to methionine in complex I from *Yarrowia lipolytica* resulted in a marked shift of the redox midpoint potential of iron-sulfur cluster N2 to the negative and abolished the redox-Bohr effect. However, the mutation did not significantly affect the catalytic activity of complex I and protons were pumped with an unchanged stoichiometry of $4\text{H}^+/2\text{e}^-$. This finding has significant implications on the discussion about possible proton pumping mechanism for complex I.

In mammalian cells mitochondrial complex I (NADH:ubiquinone oxidoreductase) provides 40% of the proton motive force needed to make ATP. Defects in this giant and most complicated membrane-bound enzyme (1) cause numerous human disorders (2). Very recently, the x-ray structure of the peripheral arm of complex I from *Thermus thermophilus* has been solved (3). However, it is still unclear how complex I couples electron transfer from NADH to ubiquinone with the vectorial translocation of four protons across the bioenergetic membrane (4, 5). Numerous hypothetical mechanisms have been proposed for this energy conversion over the years (6–9). None of the known redox groups of complex I (FMN and 8–9 iron-sulfur clusters) resides in the membrane domain (3, 10). The Fe_4S_4 cluster N2 is the last component in a “wire” of seven iron-sulfur clusters connecting FMN, the immediate oxidant for NADH, and substrate ubiquinone (3, 11). While all other

clusters in this chain have a redox midpoint potential of around -250 mV , iron-sulfur cluster N2 has a significantly more positive E_{m7} of typically -150 mV (12, 13). Moreover, the redox midpoint potential of cluster N2 exhibits pronounced pH dependence between pH 6 and 8 (12). Based on this “redox-Bohr” effect (14) and the comparably more positive potential, cluster N2 has been implicated as a key component of the proton pump (6, 15).

The structural model for the ubiquinone reducing catalytic core of complex I (1, 16) that had been deduced from distinct homologies between [NiFe] hydrogenase and complex I (17, 18) was now confirmed by the structure of the peripheral arm of complex I (3). Like the proximal iron-sulfur cluster of hydrogenase, iron-sulfur cluster N2 is at the interface between two subunits, the PSST subunit and the 49-kDa subunit (Fig. 1). In water-soluble [NiFe] hydrogenases a conserved histidine of the large subunit forms a hydrogen bond to the iron-sulfur cluster ligated to the small subunit (19). Remarkably, a fully conserved histidine is also found at the corresponding position in the 49-kDa subunit of complex I. Mutating this histidine (His^{226}) to methionine in the 49-kDa subunit of complex I from the strictly aerobic yeast *Yarrowia lipolytica* shifts the EPR spectrum of reduced iron sulfur cluster N2 to slightly lower field values but does not significantly affect the catalytic activity of complex I (20). Here we show that residue His^{226} in the 49-kDa subunit of complex I from *Y. lipolytica* is the redox-Bohr group associated with iron-sulfur cluster N2 but is not involved in the proton pumping mechanism of complex I.

EXPERIMENTAL PROCEDURES

Isolation of Mitochondrial Membranes—Mitochondrial membranes were prepared from *Y. lipolytica* wild-type strain and mutant H226M (20) as described previously (21, 22).

Isolation of Intact Mitochondria—Intact mitochondria from *Y. lipolytica* were prepared essentially by the enzymatic digestion method described in Ref. 23 with minor modifications. *Y. lipolytica* cells were harvested at early logarithmic stage (OD $\sim 3-4$), washed twice in ice-cold water, resuspended (0.1 g wet cells/ml) at room temperature in 50 mM Tris-HCl buffer (pH 8.6) supplemented with 5 mM dithiothreitol and incubated for 10 min, diluted with water, and washed twice again. After the last centrifugation the weakened cells were resuspended (0.1 g wet cells/ml) in 1.2 M sorbitol and 10 mM Hepes-KOH (pH 7.5) and 3–4 mg/ml zymolyase 20T (from *Arthrobacter luteus*, ICN Biomedicals) was added to digest the cell wall. The formation of spheroplasts was monitored spectrophotometrically, and usually 10–15 min of incubation at 30°C was enough to complete the digestion. After that, 0.2 mM Pefablock SC was added, the

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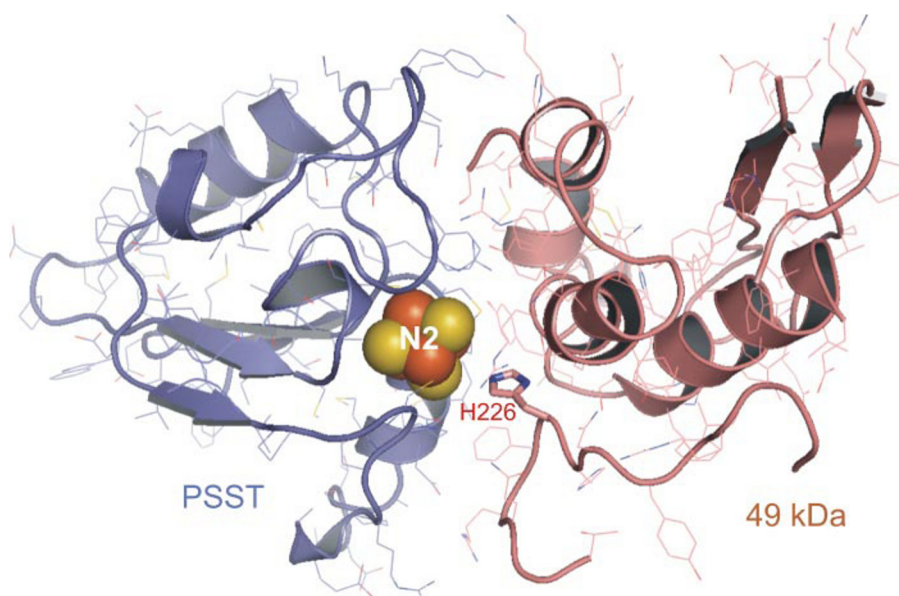


FIGURE 1. Structure of the interface between the 49-kDa and PSST subunits near iron-sulfur cluster N2. The figure was made using the coordinates of the peripheral arm of *T. thermophilus* complex I (2FUG; Ref. 3). Cluster N2 is ligated by subunit PSST (NQ06). The histidine side chain identified as the redox-Bohr group associated with iron-sulfur cluster N2 of complex I (His²²⁶ of the 49-kDa subunit in *Y. lipolytica*) is shown as a stick model. In *T. thermophilus* this residue corresponds to His¹⁶⁹. The figure was prepared using PyMOL v0.99.

spheroplast suspension was rapidly cooled and centrifuged at $500\text{--}600 \times g$ for 10 min, and the pellet was resuspended and washed twice in the same buffer containing 4 mg/ml fatty acid-free BSA.³ The pellet of the last centrifugation was resuspended in grinding buffer (20 mM Tris-HCl (pH 7.3), 0.4 M mannitol, 0.5 mM EDTA, and 4 mg/ml BSA, fatty acid-free). Spheroplasts were disrupted by 20 gentle strokes in a loosely fitting Dounce homogenizer. The suspension was diluted twice with isolation buffer (20 mM Tris-HCl (pH 7.3), 0.6 M mannitol, 0.5 mM EDTA, and 4 mg/ml BSA) and centrifuged at $2000 \times g$ for 10 min. The supernatant was collected and centrifuged once more at $7000 \times g$ for 20 min, and the pellet was resuspended with a smaller volume of isolation buffer and centrifuged again. The mitochondria were resuspended in $500\text{--}700 \mu\text{l}$ of isolation buffer and used immediately.

Redox Titration of Iron-Sulfur Cluster N2—Redox titrations of mitochondrial membranes from parental strain and mutant H226M were performed under anaerobic conditions in the presence of redox mediators essentially as described previously (13). Membranes were diluted in buffer containing sodium acetate, Mes, Mops, Tris, glycine each at 30 mM, 100 mM NaCl, and 1 mM EDTA. The solution was adjusted to the desired pH value at a protein concentration of 25–30 mg/ml. The following redox mediator dyes were added at final concentrations of 50 μM , *N,N,N',N'*-tetramethylphenylene diamine, phenazine methosulfate, methylene blue, menadione, resorufin, indigo-trisulfonate, 1,2-naphthoquinone, 2-hydroxy-1,4-naphthoquinone, phenosafranine, benzyl viologen, and methyl viologen. The membrane suspension was poised at appropriate potential values by addition of small amounts of 50 mM dithionite solution. Aliquots were anaerobically transferred into EPR tubes,

shock frozen in cold isopentane/methyl cyclohexane (5:1) and stored in liquid nitrogen until measurement. The degree of cluster N2 reduction was monitored by EPR spectroscopy at 12 K. N2 signal intensities were determined after baseline correction and subtraction of high potential components (subtraction of an EPR spectrum obtained at higher redox potential without any detectable N2 signal). Fitting N2 signal intensities to the standard Nernst equation resulted in the given E_m values. To check reversibility, after some titrations the membrane suspension was incubated in the presence of oxygen, and the slow autoxidation process was followed by taking several EPR samples. The cluster N2 EPR intensities of these samples at a given potential closely matched those obtained during reductive titration of the solution.

Determination of Proton-pumping Stoichiometries—Proton pumping of different segments of the respiratory chain in intact mitochondria from *Y. lipolytica* was measured at 30 °C as intra-mitochondrial alkalization using the pH indicator neutral red (80 μM , 529–475 nm). The buffer was as follows: 0.6 M mannitol, 0.5 mM EDTA, 4 mg/ml BSA, 60 mM Tris-HCl (pH 7.6). Consecutively, appropriate substrates, 40 mM KCl, 1 μM valinomycin, and 2 mM cyanide were added, and oxidant pulses were applied by the addition of small amounts of electron acceptors to initiate redox-dependent proton translocation from the matrix. Matrix NADH was generated through reduction by malate dehydrogenase and the pyruvate dehydrogenase complex by adding malate/pyruvate to the mitochondria.

RESULTS

The Midpoint Potential of Iron-Sulfur Cluster N2 Is pH-dependent—Similar to complex I from other sources (24) the N2 midpoint potential of *Y. lipolytica* complex I at pH 7 is -140 mV (Fig. 2; Ref. 13). To determine the redox-Bohr effect of iron-sulfur cluster N2 in *Y. lipolytica* mitochondrial membranes we measured its midpoint potential in the range between pH 5 and 9 (Fig. 2B). Due to some instability of the cluster at extreme pH values, it was difficult to reliably quantify the degree of reduction in particular at more positive redox potentials. Therefore, the E_m values determined at pH 5 and 9 were not used for calculating the pH profile of iron-sulfur cluster N2. The pH dependence of the midpoint potential of iron-sulfur cluster N2 could be described by assuming a single protonated group. The fitted curve had a maximal slope of -36 mV/pH, and pK values of 5.7 for the oxidized state and 7.3 for the reduced state were calculated (Fig. 2B). These pK values fit well to a histidine side chain as the protonated group involved.

³ The abbreviations used are: BSA, bovine serum albumin; Mes, 4-morpholineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid.

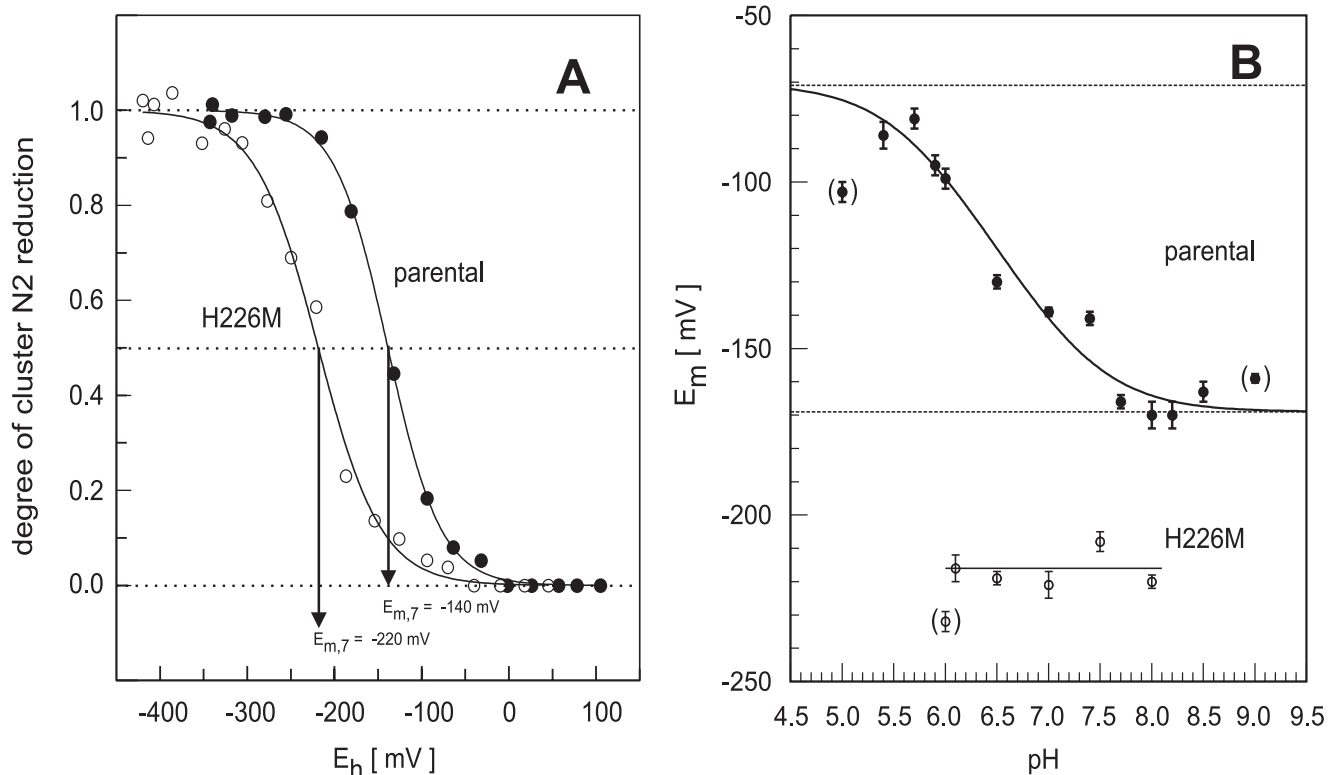


FIGURE 2. pH dependence of the redox midpoint potential of iron-sulfur cluster N2. *A*, redox titrations of mitochondrial membranes from parental strain and mutant H226M at pH 7. *B*, pH dependence of the redox midpoint potential of cluster N2 in mitochondrial membranes prepared from parental strain (filled circles) and mutant H226M (open circles). Error bars in *B* represent standard deviations obtained upon fitting individual titration curves according to the standard Nernst equation. The overall experimental error of the midpoint potentials resulting from calculating difference spectra and determining N2 signal intensities is estimated to add up to about ± 10 mV. At extreme pH values instability of iron-sulfur clusters was observed. Therefore, data points at pH 5 and 9 were omitted from the numerical fit of the parental strain data. The fitted line has a maximal slope of -36 mV per pH unit with $pK_{ox} = 5.7 \pm 0.3$ and $pK_{red} = 7.3 \pm 0.2$ for oxidized and reduced cluster N2, respectively. The midpoint potential of the cluster N2 redox couple was $E_{m(acid)} = -70$ mV for the protonated form and $E_{m(base)} = -170$ mV for the deprotonated form. In membranes from mutant H226M, the pH dependence of E_m was completely abolished and the midpoint potential was shifted down to a pH independent value of -216 ± 10 mV. Complex I in mutant H226M seemed more sensitive to extreme pH values; the E_m value measured at pH 6 was clearly offset and not used in the calculation of the average E_m value.

His²²⁶ Is the Redox-Bohr Group Associated with Iron-Sulfur Cluster N2—We have shown previously that the mutation of histidine 226 in the 49-kDa subunit to methionine has little effect on steady-state activity and inhibitor sensitivity of complex I (20). However, when we determined the redox midpoint potential of complex I from mutant H226M at pH 7, we found that it was shifted to the negative from -140 mV to -220 mV (Fig. 2A). More importantly, the E_m value did not change significantly from pH 6 to 8, indicating that the mutation had completely abolished the pH dependence of this redox group (Fig. 2B). This indicated that the redox-Bohr group associated with iron-sulfur cluster N2 of complex I is the conserved histidine at position 226 of the 49-kDa subunit. It should be noted that complex I from the mutant was somewhat more sensitive to extreme pH values limiting the range where the redox-midpoint potential could be determined.

His²²⁶ Is Not Part of the Proton Pump—If the redox-Bohr effect of cluster N2 was part of the proton-translocating machinery of complex I, abolishing the pH dependence of the midpoint potential should impair proton pumping. Therefore, we determined the H^+/e^- stoichiometry for complex I in intact mitochondria from the parental strain and from mutant H226M using the oxidant pulse technique (25): pulses of oxidant were added to coupled mitochondria in the presence of

excess reductant. Proton extrusion was monitored using the pH indicator neutral red. By choosing appropriate reductants and oxidants, the proton pumping activity of individual complexes or a larger segment of the respiratory chain could be monitored (Fig. 3). The response was calibrated internally (4) by measuring proton pumping of respiratory complex III (cytochrome bc_1 complex) that has a well established pumping stoichiometry of $1 H^+/e^-$. When we probed the segment comprising complexes I and III in the parental strain by malate/pyruvate and ferricyanide, proton pumping per added oxidation equivalent was about three times higher than for complex III alone that was probed separately using ubiquinol and ferricyanide (Fig. 3A). With five different batches of mitochondria an overall stoichiometry of $2.8 \pm 0.1 H^+/e^-$ for complexes I + III was determined. When complex I alone was probed with malate/pyruvate and ubiquinone the measured stoichiometry was $1.7 \pm 0.1 H^+/e^-$ ($n = 3$). This indicated that, like its counterpart from bovine heart (5), complex I from *Y. lipolytica* pumped four H^+ per NADH oxidized ($2 e^-$) across the inner mitochondrial membrane. With mutant H226M we determined stoichiometries of $2.8 \pm 0.1 H^+/e^-$ ($n = 5$) for complexes I + III and $1.9 \pm 0.1 H^+/e^-$ ($n = 2$) for complex I alone (Fig. 3B). Moreover, mitochondria from parental and mutant strain exhibited almost identical respiration rates and synthesized the same

Redox-Bohr Group of Complex I

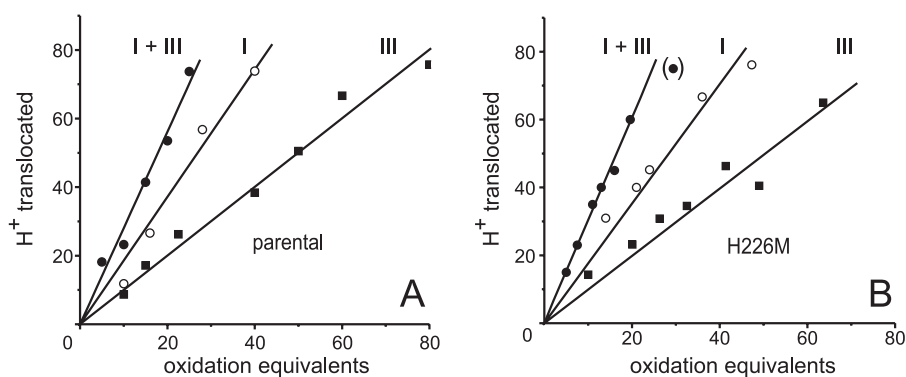


FIGURE 3. Proton pumping stoichiometries. Proton pumping by complex I + complex III (closed circles) was assayed by adding 10 mM malate/10 mM pyruvate to generate NADH in the mitochondrial matrix and by applying oxidant pulses of 5–25 μM ferricyanide. For complex III alone (closed squares), 200 μM NADH (in the presence of 5 μM decylquinazolineamine to inhibit complex I activity) were added to generate ubiquinol via the external alternative NADH-dehydrogenase (21); oxidant pulses were applied by 5–80 μM ferricyanide. For complex I alone (open circles) 10 mM malate/10 mM pyruvate were used as reductant, and oxidant pulses were applied as 5–20 μM ubiquinone-1. The number of translocated protons was calibrated internally using the neutral red response of complex III (filled squares) for which the pumping stoichiometry is known to be $1 \text{ H}^+/\text{e}^-$. A, parental strain mitochondria (3.0 mg/ml total protein; phosphate to oxygen ratio = 2.6 ± 0.1 on malate/pyruvate); B, mutant H226M mitochondria (2.8 mg/ml total protein; phosphate to oxygen ratio = 2.5 ± 0.2 on malate/pyruvate).

amount of ATP per oxygen consumed. This also indicated that proton pumping was not at all affected by the mutation.

DISCUSSION

Our data indicate that residue His²²⁶ of the 49-kDa subunit of *Y. lipolytica* complex I is the protonated group that is responsible for the pH dependence of the redox midpoint potential of iron-sulfur cluster N2. This is consistent with the fact that in the structure of the peripheral arm of *T. thermophilus* complex I the corresponding histidine is found within 4 Å of iron-sulfur cluster N2 (3). In earlier studies, we had changed this histidine to alanine, glutamine, or cysteine. These mutations also had only moderate effects on the catalytic activity of complex I, but the EPR signal of iron-sulfur cluster N2 was markedly reduced (16, 20).

It is quite remarkable that removing the redox-Bohr group of iron-sulfur cluster N2 and thereby abolishing the pH dependence of its redox midpoint potential and shifting E_{m7} by -80 mV had no effect on the proton pumping capacity of complex I. Because such dramatic changes in the physicochemical properties of iron-sulfur cluster N2 had no functional consequences, one can effectively rule out any specific role for this redox center within the energy converting mechanism of complex I other than serving as the electron donor for ubiquinone.

In mutant H226M, electrons that are fed into complex I from NADH stay on a very negative potential until they reach ubiquinone. The unexpected observation that this does not affect proton pumping by complex I strongly suggests that the energy conversion driving proton translocation across the membrane is immediately linked to the redox chemistry of ubiquinone but is not associated to one of the electron transfers upstream. As implicated in several proposed mechanisms for complex I (6, 7, 26), redox-linked protonations and deprotonations of ubiquinone intermediates could be directly employed to drive vectorial proton translocation. In more recent models combinations of direct and conformational coupling mechanisms have been

suggested to account for the observed pumping stoichiometry of complex I (7, 8). In fact, biochemical (28) and structural evidence (29) for redox-linked conformational changes of complex I has been presented. However, the spatial separation of all redox centers from the membrane domain (3) seems to exclude any direct link between redox chemistry and vectorial proton translocation as defined by Peter Mitchell's "redox-loop" concept (30). Instead, coupling exclusively by long range conformational changes seems likely. Therefore, we propose that, although the redox transitions of ubiquinone do generate the driving force for proton pumping, protonation of the substrate upon reduction is a purely scalar reaction. Thus, the protons involved in quinone chem-

istry are not those that are translocated across the membrane. Rather we suggest that stepwise reduction of ubiquinone at the interface between the 49-kDa and PSST subunit triggers conformational changes that are transmitted to subunits of the membrane arm acting as the actual proton-pumping devices. The most likely candidates for this task are the mitochondrially encoded subunits ND2, ND4, and ND5 because they exhibit homology to bacterial Na^+/H^+ antiporters (27, 31).

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