while the other (SQ_{Ns}) is insensitive to ΔP [4]. Although their sensitivity to piericidin A is almost the same, their sensitivity to rotenone inhibition is considerably different. These differences were exploited using tightly coupled bovine heart submitochondrial particles with a high respiratory control ratio (>8). We determined the distance between SQ_{Nf} and iron–sulfur cluster N2 on the basis of their direct spin–spin interaction analysis [5]. We have extended using the reconstituted bovine heart complex I proteoliposomes which shows a respiratory control ratio >5 [6]. High frequency (33.9 GHz) Q-band EPR spectra of individual SQ_{Nf} and SQ_{Ns} molecules appear to favor our two-semiquinone model complex I proton pumping mechanism.

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6L4

Initial electron transfer steps in complex I

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NADH:ubiquinone oxidoreductase (complex 1) from *Escherichia coli* is a membrane-bound proton pump with an overall stoichiometry of 3 or $4H^+/2e$. The enzyme is composed of 13 subunits that are arranged in an L-shape. The peripheral arm (seven subunits) is located in the cytoplasm and harbors the FMN and all iron–sulfur centers. The seven other hydrophobic subunits are located in the membrane. Three of them are homologues of Na^+/H^+ antiporters and contain possible proton transfer pathways. A possible fourth proton translocation path lies at the interface of NuoN, K, J and A. The precise location of the quinone binding site is unknown; it is assumed to be at the membrane-interface, but might in fact be located up to 30 Å away in the peripheral arm at a distance of ~12 Å from the most distal iron–sulfur center N2.

The *E. coli* complex I contains a total of nine iron–sulfur centers and an FMN redox group that serves as the direct oxidant of NADH. A linear electron transfer chain is made up by the sequence FMN:N3:N1b:N4:N5:N6a:N6b:N2:Q where edge-to-edge distances are maximally 14 Å (N5:N6a). Center N7 lies outside the main electron transfer pathway (20.5 Å) and is not reduced by NADH. Center N1a (with the lowest Em ~ −330 mV, the other centers at −220–−270 mV, N2 at −160 mV at pH 6, as in this work) lies at a dead-end side path, but is reducible by NADH via FMN.

The large spatial separation between the redox centers and the membrane arm suggests a proton-pumping mechanism driven solely by protein conformational changes; however, the finding of a semiquinone responding to the proton-motive force suggests that quinone oxidoreduction forms part of the energy transducing mechanism and further suggests a quinone location at the membrane interface.

In order to study the functional link between electron transfer and energy transduction we have performed microsecond freeze-quench kinetic analyses in which oxidized enzyme was reacted with NADH and the reaction monitored by UV–vis and EPR spectroscopies.

Our results indicate a rapid (<100 μs) reduction of FMN simultaneous with the disappearance of a chromophore absorbing at 416 nm followed by disappearance (130 μs) of another chromophore at 471 nm. These events occur before any of the EPR detectable iron–sulfur centers are reduced (N2 ~ 300 μs, N1a ~ 1.2 ms, N1b/N4/(N3) in ~1.8 ms). Electron tunneling and other specific mechanistic aspects are discussed.

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6L5

On the mechanistic stoichiometry of proton translocation by respiratory Complex I

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The proton translocation stoichiometry of Complex I has long been thought to be 4 $H^+/2e^-$ based on experiments in which proton translocation has been traditionally measured in the absence of a significant counteracting proton-motive force. The fact that the membrane domain of Complex I contains three (and not four) homologous subunits that are also homologues of certain bacterial Na^+/H^+ antiporters [1] raises some doubts against this stoichiometry, which may therefore need to be reassessed. Based on the redox potential drop across Complex I, the measured proton-motive force in State 4 mitochondria, and basic thermodynamic principles, the $H^+/2e^-$ stoichiometry must be lower than 4 [2]. However, in the phosphorylating State 3 the proton-motive force is lowered enough to allow for a stoichiometry of 4. An independent measure of the $H^+/2e^-$ ratio under phosphorylating State 3 conditions is therefore important, and may be obtained from the relationship $H^+/2e^- = H^+/ATP \times ATP/2e^-$. On that basis, the measured $ATP/2e^-$ ratio for a defined span of the respiratory chain yields a measure of the $H^+/2e^-$ ratio of that span, provided that we know the H^+/ATP ratio of ATP synthesis. Recent work by Watt et al. [3] has shown that the H^+/ATP ratio is 8/3 for ATP synthase in animal mitochondria. Conversion to extramitochondrially produced ATP requires import of one more proton, so the effective extramitochondrial H^+/ATP ratio is 3.67. Using this value, and dependable $ATP/2e^-$ ratios reported by Hinkle et al. [4], we obtain an $H^+/2e^-$ ratio of 2.9 for Complex I [2], which has significant implications on possible proton-pumping mechanisms.

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6P1

Site-directed mutagenesis of the NADH binding site of prokaryotic Complex I (NADH:ubiquinone oxidoreductase) affects generation of reactive oxygen species

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