Mössbauer Spectroscopy on Respiratory Complex I: The Iron–Sulfur Cluster Ensemble in the NADH-Reduced Enzyme Is Partially Oxidized

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ABSTRACT: In mitochondria, complex I (NADH:quinone oxidoreductase) couples electron transfer to proton translocation across an energy-transducing membrane. It contains a flavin mononucleotide to oxidize NADH, and an unusually long series of iron–sulfur (FeS) clusters that transfer the electrons to quinone. Understanding electron transfer in complex I requires spectroscopic and structural data to be combined to reveal the properties of individual clusters and of the ensemble. EPR studies on complex I from Bos taurus have established that five clusters (positions 1, 2, 3, 5, and 7 along the seven-cluster chain extending from the flavin) are (at least partially) reduced by NADH. The other three clusters, positions 4 and 6 plus a cluster on the other side of the flavin, are not observed in EPR spectra from the NADH-reduced enzyme: they may remain oxidized, have unusual or coupled spin states, or their EPR signals may be too fast relaxing. Here, we use Mössbauer spectroscopy on $^{57}$Fe-labeled complex I from the mitochondria of Yarrowia lipolytica to show that the cluster ensemble is only partially reduced in the NADH-reduced enzyme. The three EPR-silent clusters are oxidized, and only the terminal 4Fe cluster (position 7) is fully reduced. Together with the EPR analyses, our results reveal an alternating profile of higher and lower potential clusters between the two active sites in complex I; they are not consistent with the consensus picture of a set of isopotential clusters. The implications for intramolecular electron transfer along the extended chain of cofactors in complex I are discussed.

Mitochondrial complex I comprises two domains: a hydrophobic domain that is embedded in the inner membrane and a hydrophilic domain that protrudes into the matrix. NADH is oxidized by a flavin mononucleotide cofactor in the hydrophobic domain, and the electrons are then passed along a “chain” of iron–sulfur (FeS) clusters to the ubiquinone binding site, located close to the interface with the hydrophilic domain. All complexes I contain eight conserved FeS clusters: two [2Fe–2S] clusters and six [4Fe–4S] clusters. An additional [4Fe–4S] cluster is present in a small number of prokaryotes, but not in any known mitochondrial complex I, so it is not discussed further here. The eight conserved clusters are ligated by a set of conserved sequence motifs; they have been defined structurally in the hydrophilic domain of Thermus thermophilus complex I and observed also in an electron density map of complex I from Yarrowia lipolytica.

The FeS clusters in the complexes I from a number of species have been characterized extensively by X-band EPR spectroscopy. Oxidized [2Fe–2S] and [4Fe–4S] clusters are diamagnetic, and so no signals are observed in the EPR spectrum of oxidized complex I. When the enzyme is reduced by NADH, the signals from five reduced clusters are observed. The five signals observed in spectra from the mitochondrial enzymes are named N1b, N2, N3, N4, and N5; how to assign them to the eight now structurally defined clusters has been much debated and only became clear recently. Signal N1b is a slow-relaxing signal from the [2Fe–2S] cluster in the 75 kDa subunit (assigned because the same signal is exhibited by overexpressed 75 kDa subunit homologues). Here, we use the nomenclature from complex I from Bos taurus, the enzyme best studied by EPR; the cluster with signal N1b is named 2Fe[75]. The remaining four signals are from faster-relaxing [4Fe–4S] clusters. Interactions between the cluster that exhibits signal N3 and the flavosemiquinone radical, and the cluster that exhibits signal N2 and the ubisemiquinone radical, showed that N3 is from the [4Fe–4S] cluster in the 51 kDa subunit, 4Fe[51], and N2 is from the [4Fe–4S] cluster in the PSST subunit, 4Fe[PS]. Signal N5 is from the 75 kDa subunit and N4 is from the TYKY subunit because overexpressed homologues of the 75 kDa subunit exhibit signal N5, but not signal N4; the results from site-directed mutagenesis were used to assign N5 to the all-cysteine ligated cluster, 4Fe[75]C. Recently, signal N4 was assigned to the “first” cluster in TYKY, 4Fe[TY]1, using double electron electron resonance. The FeS clusters in complex I and the EPR signals (exhibited by the NADH-
FeS clusters in mitochondrial complex I and their corresponding EPR signals. The FeS cluster arrangement is from the structure of the hydrophilic arm of complex I from T. thermophila; the “N7” cluster (non-conserved) has been deleted. The clusters are named according to their cluster type and subunit location in B. taurus complex I (black), and the EPR signals (N1b, N2, N3, N4, and N5, red) that are exhibited by the NADH-reduced mitochondrial enzyme are indicated next to the clusters that they have been assigned to. Clusters in gray do not contribute to the EPR spectrum of NADH-reduced mitochondrial complex I. The distances between the clusters are the distances between the centers of the two closest atoms.

Figure 1.

| Reduced mitochondrial enzymes | N1b, N2, N3, N4, and N5 | N1a, N1b, N3, N4, and N5 |
|------------------------------|-------------------------|-------------------------|

Here, we use Mössbauer spectroscopy to define the proportion of reduced and oxidized clusters in mitochondrial complex I from Y. lipolytica (a eukaryotic model enzyme with very similar spectroscopic properties to the B. taurus enzyme) cultured on iron-57 and reduced by NADH. The Mössbauer technique is sensitive to all the FeS clusters present, regardless of their oxidation states, and thus offers a direct route to determine the proportion of oxidized and reduced clusters.

**EXPERIMENTAL PROCEDURES**

Preparation of Isotopically Labeled Yarrowia lipolytica Complex I. Samples of 56Fe complex I were prepared from Y. lipolytica strain GB10 as described previously. 56Fe complex I were prepared following the same procedure, except that the cells were grown in synthetic medium (pH 5.5) containing 0.69% yeast nitrogen base without iron (ForMedium, UK), 4% glucose, 0.7% sodium glutamate, 0.08% Complete Supplement Mixture (ForMedium), and 650 µg L−1 57FeSO4. 57FeSO4 was prepared by dissolving 57Fe (>97% purity, CKGas Ltd.) in 300 mM H2SO4 overnight, in contact with a Pt wire to catalyze H+ reduction; the solution was corrected to pH 5.5 with 1 M Tris-Cl (pH 7.5), and the iron concentration was determined using ferrene. 35, 36 The buffer used in the final step of the preparation was 20 mM 3-morpholinopropionate-1-sulfonic acid (MOPS), pH 7.5, 150 mM NaCl, and 0.03% n-dodecyl-β-D-maltopyranoside (DDM); all samples were prepared in this buffer solution. The yield of wet cells was ~10 g L−1 using the synthetic 57Fe medium (compared to ~35 g L−1 from the standard medium). The 57Fe- and 56Fe-containing enzymes displayed the same banding pattern in SDS PAGE, the same elution volume in gel filtration, and the same specific NADH:hexaammineruthenium(III) oxidoreductase activity (~70 µmol NADH min−1 mg protein−1) in 100 µM NADH and 3.5 mM HAR, pH 7.5, 32 °C).

**EPR Spectroscopy**. Complex I EPR samples (56Fe and 57Fe, final concentration ~15 µg protein mL−1) were reduced by 50 mM NADH (Sigma-Aldrich) in an N2-containing anaerobic glovebox (Beltec Technology, UK) and frozen immediately. No signals were observed unless NADH was added. Spectra were recorded on a Bruker EMX X-band
spectrometer using an ER419HS cavity, maintained at low temperature by an ESR900 continuous-flow liquid helium cryostat (Oxford Instruments, UK); the sample temperature was measured with a calibrated Cernox resistor (Lake Shore Cryotronics Inc., Westerville, OH). Spin quantitation of N2 was carried out by double integration of the N2 signal recorded at 15 K, by comparison to a 1 mM Cu(II) standard.37

Mössbauer Spectroscopy. The oxidized Mössbauer sample contained ~16 (mg protein) mL–1 of 57Fe complex I (~0.46 mM 57Fe) and was frozen “as prepared”. Reduced complex I Mössbauer samples (final concentration ~24 (mg protein) mL–1, ~0.73 mM 57Fe) were prepared by adding 50 mM NADH to the enzyme in the anaerobic glovebox, and frozen immediately. Mössbauer spectra were recorded on a spectrometer with alternating constant acceleration. The minimum experimental line width was 0.24 mm/s (full width at half-height). The sample temperature was maintained constant in either an Oxford Instruments Variox or an Oxford Instruments Mössbauer-Spectromag cryostat with split-pair magnet system; the latter was used for measurements with applied fields up to 7 T, with the field at the sample orientated perpendicular to the γ-beam. The γ-source (57Co/Rh, 1.8 GBq) was kept at room temperature. By using a re-entrant bore tube, the source was positioned inside the gap of the magnet coils at a position with zero field. Isomer shifts are quoted relative to iron metal at 300 K. Zero-field spectra were fitted using quadrupole doublets, and applied field measurements were simulated with a spin Hamiltonian program based on the usual nuclear Hamiltonian.36 In both cases the line shapes were Voigt profiles, calculated using the complex error function with the rational approximation of Hui et al.39,40 The Lorentzian contributions were fixed to the natural line width of 57Fe spectra (0.2 mm/s).

## RESULTS

EPR Spectroscopy of 57Fe-Containing Complex I. Figure 2 compares X-band EPR spectra from NADH-reduced samples of Y. lipolytica complex I containing either 56Fe or 57Fe. The two sets of spectra are very similar and typical of spectra from mitochondrial complex I. By comparison with the extensively characterized spectra of bovine complex I, the N1b, N2, N3, N4, and N5 signals are readily identified41 (the most clearly apparent features from each signal are indicated in Figure 2). The spectral features of the 57Fe-containing complex I are broadened, relative to those from the 56Fe-containing enzyme. The broadening is particularly evident for signal N1b (resolved most clearly at 12 and 25 K), and close inspection reveals that the N1b g⊥ signal is split into an apparent triplet, with relative intensities of ~1:2:1. The splitting can be attributed to hyperfine interactions with two I = 1/2 57Fe nuclei (56Fe has I = 0), and it is fully consistent with the assignment of signal N1b to a [2Fe–2S]4+ cluster with localized valences. In this case, the cluster ground state with S = 1/2 results from strong antiferromagnetic coupling between an FeIII center with spin S1 = 5/2 and an FeII center with S2 = 4/2.42 The local hyperfine coupling tensors (referring to S1 and S2) may be denoted A1(FeIII) and A1(FeII). Spin projection considerations43 then yield effective hyperfine coupling tensors (referring to the total spin, S) of a1(FeIII) = 7/3 A1(FeIII) and a1(FeII) = –4/3 A1(FeII).44 Based on this model, the resolved hyperfine splitting of g⊥(N1b) could be readily simulated with the coupling constants a1(FeIII) = –43 MHz and a1(FeII) = +35 MHz, determined from applied-field Mössbauer measurements on the [2Fe–2S]4+ clusters of putidaredoxin45 and the Rieske protein.46 As indicated by the stick spectrum included in Figure 2, the hyperfine pattern is actually a quartet (not a triplet) because a1(FeIII) and a1(FeII) do not coincide. Incidentally, the intensity ratio of the apparent line pattern is consistent with a high level of isotopic labeling with 57Fe, because there are no obvious contributions from nonlabeled (single line) or singly labeled (double lines) species. The broadening of the other signals in Figure 2, which are from reduced [4Fe–4S] clusters, results from unresolved hyperfine interactions with the 57Fe nuclei (reduced [4Fe–4S] clusters exhibit both ferromagnetic and antiferromagnetic coupling to also achieve S = 1/2 spin states). Finally, similar EPR spectra have been reported previously for the FeS clusters in complex I in submitochondrial particles from the fungus Candida utilis, grown in the presence of 57Fe.47 Signal N1b was resolved into a triplet, and its g⊥ signal was characterized by an apparent hyperfine splitting of ~1.2 mT (the value determined here is ~1.5 mT). In C. utilis, the signals from the [2Fe–2S] cluster in succinate dehydrogenase and the Rieske cluster in the cytochrome bc1 complex were split similarly by hyperfine interactions, reiterating that the behavior of signal N1b is not unusual in this respect.

Mössbauer Spectra from Oxidized 57Fe-Containing Complex I. Figure 3 shows the zero-field Mössbauer spectra of oxidized 57Fe complex I, recorded at 80 K. The oxidized enzyme does not exhibit any EPR signals, so each molecule should contain two [2Fe–2S] and six [4Fe–4S] oxidized clusters. Accordingly, Figure 3 shows a relatively simple quadrupole pattern without paramagnetic splitting, which is fit by summing two doublet subspectra to reproduce the slight asymmetry that is apparent in the peak intensities (see Table 1). Subspectrum 1 (green) has a low isomer shift (δ
Figure 3. Zero-field Mössbauer spectrum recorded at 80 K on oxidized complex I from Y. lipolytica. The data were fit by two quadrupole doublets with Voigtian line shapes (subspectrum 1, oxidized [2Fe–2S], in green; subspectrum 2, oxidized [4Fe–4S], in blue; sum of subspectra 1 and 2 in red), with the parameters given in Table 1.

Table 1. Mössbauer Parameters Obtained for the Subspectra Exhibited by Complex I at 80 K (Oxidized) and 160 K (Reduced)

| sample         | subspecies       | δ (mm/s) | ΔE_Q (mm/s) | Γ_U (mm/s) | rel int (%) |
|----------------|------------------|----------|-------------|------------|-------------|
| oxidized (80 K)| 1. [2Fe–2S]²⁺ (Fe⁺) | 0.25     | 0.51        | 0.40       | 14.3        |
|                | 2. [4Fe–4S]²⁻ (Fe⁺) | 0.43     | 0.93        | 0.45       | 85.7        |
| reduced (160 K)| 1. [2Fe–2S]³⁺⁻¹⁺ (Fe⁺) | 0.23     | 0.51        | 0.40       | 13.1        |
|                | 2. [4Fe–4S]²⁺⁻¹⁻ (Fe⁺⁺) | 0.41     | 0.92        | 0.34       | 44.5        |
|                | 3. [2Fe–2S]³⁻⁻¹⁻ (Fe⁺⁺) | 0.72     | 3.40        | 0.10       | 12.²        |
|                | 4. [4Fe–4S]²⁻⁻¹⁻⁻¹⁻ (Fe⁺⁺⁺) | 0.44     | 0.74        | 0.33       | 20.6        |
|                | 5. [4Fe–4S]³⁻⁻¹⁻⁻¹⁻ (Fe⁺⁺⁺) | 0.53     | 1.13        | 0.33       | 20.6        |

* Gaussian contribution to the Voigt line width; the Lorentzian contribution was the natural line width of 0.2 mm/s. 

Figure 4. Magnetic Mössbauer spectrum of oxidized complex I from Y. lipolytica. The spectrum was recorded at 4.2 K with a field of 7 T applied perpendicular to the γ-rays. The red line is the sum of two subspectra simulated using the usual nuclear Hamiltonian for S = 0 with parameters similar to those in Table 1; slight differences allow for the difference in temperature (subspectrum 1, oxidized [2Fe–2S], in green; δ = 0.27 mm/s, ΔE_Q = 0.51 mm/s, η = 0.1 (η represents the asymmetry of the electric field gradient), 14.3%; subspectrum 2, oxidized [4Fe–4S], in blue: δ = 0.46 mm/s, ΔE_Q = 1.1 mm/s, η = 0.6, 85.7%; sum of subspectra 1 and 2 in red).

Mössbauer Spectra from NADH-Reduced ⁵⁷Fe-Containing Complex I. First, we consider the two [2Fe–2S] clusters. Signal N1b is clearly observed in EPR spectra from NADH-reduced Y. lipolytica complex I (see Figure 2), and as described above, it is from cluster 2Fe[7S] (see Figure 1). There are no additional signals from reduced 2Fe clusters in the spectra shown in Figure 2; in particular, signal N1a (described above), which would be exhibited by cluster 2Fe[24] if it was reduced, is absent. Furthermore, in NADH-reduced B. taurus complex I, which has been characterized extensively by EPR, signal N1b is substoichiometric; it increases in intensity more than 3-fold when the enzyme is reduced to −1 V⁸ and has a low signal amplitude both in continuous-wave X-band and echo-detected spectra.⁹⁰ On the basis of these results, we estimate that cluster 2Fe[7S] is 1/3 reduced by NADH in B. taurus complex I, and because of the high similarity of the B. taurus and Y. lipolytica enzymes and their EPR spectra, we expect signal N1b to be similarly substoichiometric in NADH-reduced Y. lipolytica complex I. Consequently, we expect the two [2Fe–

perfectly equivalent (Γ_U = 0.44 mm/s in a preliminary fit using Lorentzian line shapes). Therefore, to describe the broadening effect, we used Voigt line shapes, which comprise Gaussian distributions of Lorentzian line shapes, throughout this work.⁹⁰ Note also that one of the [4Fe–4S] clusters in complex I has an unusual 3Cys1His ligation. Previously, a similar [4Fe–4S]²⁺ cluster in 4-hydroxybutyryl-CoA dehydratase⁵⁹ was shown to exhibit an entirely symmetrical Mössbauer spectrum, matching the spectra of canonical 4Cys-ligated clusters.⁷⁴ Therefore, although the resolution of our spectra is not sufficient to rule out a unique subspectrum from this single Fe subsite, we do not consider the 3Cys1His cluster separately. Finally, the applied-field measurement shown in Figure 4 could be reasonably well simulated using S = 0 and the two subspectra from Figure 3, revealing no evidence for an appreciable amount of any paramagnetic cluster.
The subspectra from the oxidized \([2Fe−2S]\) and \([4Fe−4S]\) clusters, subpectra 1 (green) and 2 (blue), respectively, correspond closely to those from the oxidized sample. Subspectrum 1 represents purely ferric species, so it was constrained to account for 13.1% of the total iron (1.67 \([2Fe−2S]\) clusters, plus the ferric substate of 0.33 reduced \([2Fe−2S]\) clusters; see below). A distinct subspectrum for purely \(Fe^{2+}\) species (subspectra 3, cyan), a unique indicator for reduced \([2Fe−2S]\) clusters with spin \(S = 1/2\), is poorly resolved from the background. It is included only because the N1b signal is present in the EPR spectrum. Subspectrum 3 clearly corresponds to less than one iron out of 28 (1/28 = 3.6%), and it is included in Figure 5 at \(1\%\), based on our estimate (described above) that only \(1/3\) of the N1b clusters are reduced. The spectrum from the reduced \([4Fe−4S]\) clusters exhibits slightly higher isomer shifts and quadrupole splitting than exhibited by the oxidized clusters,\(^{49,50}\) and we introduced two “phenomenological” subspectra in the fit, subspectra 4 and 5 (dark and light pink), to account for distinguishable \(Fe^{2.5+}Fe^{2.5+}\) and \(Fe^{2+}Fe^{2+}\) pairs, respectively,\(^{50}\) but without further distinction of individual clusters (as expected, subspectra 2 and 4 are similar). To fit the data in Figure 5, the relative contributions from the oxidized and reduced \([4Fe−4S]\) clusters were allowed to vary. In the best fit (Table 1) the relative intensities are 44.5% of the total iron for \([4Fe−4S]\) and 41.2% for \([4Fe−4S]\); on average, 3.1 oxidized clusters and 2.9 reduced clusters. The best fit using only Lorentzian line shapes gave very similar results: 3.0 oxidized and 3.0 reduced clusters. However, the signal-to-noise level in the data suggests that it is not appropriate to consider only a single, unique fit. Therefore, fits to the data using different intensity ratios for subspectra 2 and (4 + 5) were optimized and evaluated. Consequently, we found that the data are consistent with 3.1 ± 0.5 oxidized \([4Fe−4S]\) clusters and 2.9 ± 0.5 reduced \([4Fe−4S]\) clusters in the NADH-reduced sample.

The 0.1 and 7 T low-temperature measurements on NADH-reduced complex I shown in Figure 6 are consistent with the overall assignment of oxidized and reduced species described above. The paramagnetic \([4Fe−4S]\) clusters (dark and light pink traces) show sizable paramagnetic splitting due to the presence of induced static internal fields at the iron substates, whereas the diamagnetic \([2Fe−2S]\) and \([4Fe−4S]\) clusters are virtually unaffected in the weak-field condition (Figure 6A1, green and blue traces) (the subspectrometric \([2Fe−2S]\) contribution was not included in the simulations). With the strong applied field (7 T, Figure 6A2) the diamagnetic clusters also show magnetic splitting, due to the nuclear Zeeman effect, although, importantly, their contribution does not fully overlap those of the paramagnetic reduced clusters (note the line at ca. \(+3\ \text{mm/s})\). Subspectrum 5 (light pink) originates from the \(Fe^{2+}Fe^{2+}\) sites of the \([4Fe−4S]\) clusters, which are coupled antiparallel to their respective cluster spins to give positive effective a values and large magnetic splitting. The fact that the distinct feature of subspectrum 5 at ca. \(+3\ \text{mm/s}) does not increase its relative intensity under high field conditions (along with the high-quality overall fit to the high-field spectrum) rules out spin-coupling between reduced paramagnetic clusters as an alternative explanation for the diamagnetic behavior of some of the iron–sulfur clusters in NADH-reduced complex I. (There are no direct bonds (mono- or diatomic bridges) between the clusters, so long-range exchange coupling that is strong enough to “survive” the strong 7 T field can be excluded unambiguously.) Thus, the spectra in Figure 6 confirm that...
conjunction with the EPR analyses described above, our data suggest that, in the NADH-reduced enzyme, cluster 2Fe[24] is fully oxidized and cluster 2Fe[75] is partially reduced, cluster 4Fe[PS] (N2) is fully reduced and clusters 4Fe[75]H and 4Fe[TY]2 are fully oxidized, and clusters 4Fe[51], 4Fe[75]C, and 4Fe[TY]1 are (on average) 2/3 reduced each.

**Discussion**

Reduction of the FeS Clusters in Mitochondrial Complex I by NADH. The Mössbauer analyses presented here demonstrate clearly that only a subset of the clusters in mitochondrial complex I are reduced in the presence of NADH: the three clusters that are not represented in EPR spectra of NADH-reduced mitochondrial complex I are oxidized. Together, the EPR and Mössbauer results suggest that the FeS clusters in mitochondrial complex I can be classified as "high" or "low" potential clusters: the high potential clusters are mostly (or entirely) reduced in NADH (4Fe[51], 4Fe[75]C, 4Fe[TY]1, and 4Fe[PS]), see Figure 1), and the low potential clusters are mostly (or entirely) oxidized (2Fe[24], 2Fe[75], 4Fe[75]H, and 4Fe[TY]2). The high and low potential clusters alternate along the cluster chain from the flavin to the ubiquinone-binding site (see Figure 7).

The potential of 4Fe[PS] (N2) is higher than the potentials of the other clusters (see above), so it is very probably fully reduced in NADH, accounting for one of the ~3 reduced clusters. If the 4Fe[51] (N3), 4Fe[75]C (N5), and 4Fe[TY]1 (N4) clusters were fully reduced also, then there would be four reduced 4Fe clusters present in NADH-reduced complex I— but the Mössbauer data presented here are not consistent with four reduced 4Fe clusters. The substoichiometric reduction of 4Fe[51] (N3), 4Fe[75]C (N5), and 4Fe[TY]1 (N4) by NADH is supported by the relatively low signal amplitudes of N3 and N4 and their increased intensity when the enzyme is reduced to −1 V.8,9 The behavior of N5 is more complicated because its temperature dependence varies with the level of enzyme reduction.8 Conversely, as described above, redox titrations provided potentials of around −0.25 V for all these three clusters, so they should, in principle, all be fully reduced in NADH (approximately −0.4 V). We propose the following explanation for the discrepancy. The redox-titration data (signal amplitude vs set potential) were fit using the Nernst equation or evaluated qualitatively for the midpoint potential of the titration, but only limited low potential data were available or included in the fitting, and the stoichiometries of the reduced clusters were not measured (only assumed).22,23,25 Thus, it is likely that clusters 4Fe[51], 4Fe[75]C, and 4Fe[TY]1 do not display perfect Nernstian behavior, especially at low potential— their EPR signal intensities vary over a wider range of potential than expected, most likely because of interactions between the reduced clusters. Non-Nernstian behavior for some of the FeS clusters in complex I from *E. coli* has been described previously.26 However, at relatively high potentials (where the probability of multiple reduced clusters in the same molecule is low) the reduction potentials provide a reasonable picture of the thermodynamics of cluster reduction. For this reason we have used the "consensus" reduction potentials of −0.25 V for 4Fe[51], 4Fe[75]C, and 4Fe[TY]1 in the kinetic simulations described below. Finally, we note that we cannot absolutely exclude the possibility that a small subset of the complex I molecules present are unable to react with NADH (and so remain fully oxidized). However, the flavin is present stoichiometrically (flavin:protein ratio ~ 1:1); spin quantitation

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Figure 6. Magnetic Mössbauer spectra of complex I from *Y. lipolytica* reduced by NADH, recorded at 4.2 K with 0.1 and 7 T fields applied perpendicular to the γ-rays. (A) Spectra recorded with 0.1 T (A1) and 7 T (A2) applied fields. The blue and green traces for the oxidized [2Fe−2S] and [4Fe−4S] clusters are simulated with S = 0, using the parameters from the zero-field measurement (Figure 5 and Table 1), whereas the traces for the reduced [4Fe−4S] clusters (dark and light pink) are obtained using S = 1/2 and “typical” magnetic hyperfine coupling tensors adapted from the magnetic Mössbauer spectrum of a bacterial ferredoxin: a/ggβFe = (−22, −23.4, −19.4) T for Fe2.5+Fe2.5+, and a/ggβS = (+17.2, +7.6, +8.7) T for Fe2.5+Fe2.5+Fe2.5+Fe2.5+. The simulations are not intended to be unique, but to present a picture to support the quantification of the contributions from the diamagnetic and paramagnetic subspectra. The substoichiometric contribution from the Fe7 subsite of [2Fe−2S]++ clusters was neglected, and the relative intensities of the other contributions were taken from the fit to the zero-field data. (B) The residual spectrum from (A1) after subtraction of the diamagnetic subspectra (the green and blue traces); the dark and light pink traces are the simulations of the paramagnetic contributions presented in (A1).
of signal N2, using a Cu(II) standard, gave results which were consistent with the stoichiometric reduction of the 4Fe[PS] (N2) cluster (N2:protein ratio ~1:1); and EPR samples prepared in high DDM concentrations did not display decreased signal intensities.

Much less is known about the low potential clusters in mitochondrial complex I. Our Mössbauer data support the proposal (described above) that cluster 2Fe[75] (N1b) is only partly reduced in NADH-reduced mitochondrial complex I. If 2Fe[75] (N1b) is 1/3 reduced in NADH (at −0.4 V), then its apparent reduction potential is −0.42 V. Furthermore, our Mössbauer data confirm that cluster 2Fe[24] (N1a) is not reduced by NADH in mitochondrial complex I. The reason why 2Fe[24] remains oxidized in the intact enzyme but can be reduced readily in overexpressed subunits and in the flavoprotein subcomplex (described above) remains unclear. It is possible that the reduction of 2Fe[24] is limited kinetically. Finally, there is no evidence, from either EPR or Mössbauer analyses, for the reduction of clusters 4Fe[75]H and 4Fe[TY]2 in any NADH-reduced complex I, although additional spectral features (suggestive of interactions between adjacent reduced clusters) were observed in B. taurus complex I at −1 V. As an estimate, if 4Fe[75]H and 4Fe[TY]2 are 5% reduced in NADH (at −0.4 V), then their apparent reduction potentials are −0.48 V.

Effect of the Ensemble on Cluster Reduction. The reduction potential profile for the seven FeS clusters between the flavin and the ubiquinone binding site in complex I, constructed using the potentials described above, is shown in black in Figure 7. The profile reveals an apparent pattern of alternating higher and lower potential clusters. However, it is clear that electrostatic interactions between adjacent clusters may influence their apparent reduction potentials in redox titrations, and attempts have been made to use calculations, based on the structure of the hydrophilic arm of T. thermophilus complex I, to deconvolute the intrinsic cluster potentials from redox titration data. For example, the linearized Poisson–Boltzmann equation was used to calculate the expected shift in apparent reduction potential of each cluster upon the reduction of each of the other clusters, to define a set of pairwise interactions. However, the calculated values depend heavily upon the modeling procedure and the value chosen for the protein dielectric (ε), which is poorly represented by a single macroscopic parameter. With ε = 20, individual reduction potentials were calculated to shift by up to 60 mV upon reduction of both adjacent clusters.

The profile shown in blue in Figure 7 shows the “dampening” effect of removing the ε = 20 electrostatic interactions between adjacent clusters from the potential profile deduced above: the interactions exaggerate existing, intrinsic potential differences by shifting the lower potential clusters yet lower. With ε = 4 the electrostatic interactions are much larger, and individual potentials are calculated to shift by up to 0.23 V when both adjacent clusters are reduced. Consequently, the reduction potential profile is flattened significantly when the ε = 4 interactions are subtracted (shown in red in Figure 7), and provided that the terminal 4Fe[PS] cluster acts as a high potential “anchor”, the interactions alone are sufficient to produce an alternating profile (like the black profile shown in Figure 7) from a set of intrinsically isopotential clusters. Currently, we do not know which of the three profiles in Figure 7 best represents the free energy profile for transfer of a single electron through complex I. We note only that EPR spectra from subcomplex Iλ, the hydropophilic domain of complex I produced by fragmentation of the enzyme close to the 4Fe[PS] cluster, are identical to those from intact complex I, except that N2 is decreased or missing (no additional signals are present). Similarly, no additional EPR signals were observed when several residues around cluster 4Fe[PS] in Y. lipolytica complex I were mutated, resulting in the loss of signal N2. As the high-potential 4Fe[PS] “anchor” cluster is missing in both cases, these observations argue against electrostatic interactions as the only reason why the 2Fe[75], 4Fe[75]H, and 4Fe[TY]2 clusters have such low apparent reduction potentials. Thus, it is most likely that electrostatic interactions only exaggerate intrinsic potential differences between the clusters, favoring the ε = 20 (blue) profile as the most representative possibility included in Figure 7. Finally, alternating profiles are exhibited by redox titrations of a number of enzymes, including succinate dehydrogenase and fumarate reductase, NiFe hydrogenase, and the photosynthetic reaction center. In particular, the electrostatic interactions between the hemes in the reaction center have been characterized and their effects on the potential profile and rates of electron transfer described.

Rates of Electron Transfer along the Cluster Chain. Dutton and co-workers have described a simple approach to predicting the rates of intramolecular electron transfer in proteins. Thus, we used Dutton’s model for electron transfer in complex I to predict the time required for two electrons to transfer from the flavin to a bound quinone along the chain of FeS clusters. We used the same parameters as described previously, except that we altered the reduction potentials of the seven clusters in the chain, to evaluate the three potential energy profiles of Figure 7. With the flattest reduction potential profile (ε = 4, red profile in Figure 7) it takes 0.42 ms for ubiquinol to be formed in 50% of the

![Figure 7. Three possible reduction potential profiles for electron transfer through the FeS clusters in complex I.](image-url)
population of complex I molecules considered (the half-transfer time is 0.42 ms). The time required is slightly longer than the time required by the profile used by Dutton and co-workers because of the 0.05 V more-negative potential of cluster 4Fe[75]H and because the potential of 4Fe[PS] (N2) was set to ~0.15 V, instead of ~0.1 V (with ~0.1 V a significant fraction of the population remains in the (semiquinone + reduced N2) state, rather than going on to form ubiquinol); raising the potential of 4Fe[75]H from ~0.3 to ~0.25 V provides a half-transfer time of 0.16 ms. With the intermediate profile (ε = 20, blue in Figure 7) the calculated half-transfer time is 7.9 ms, and with the most strongly alternating profile (ε → ∞, black in Figure 7) it is 36 ms.

There have been few attempts to determine the rate of electron transfer along the chain of clusters in complex I experimentally. Most notably, a fast freeze–quench procedure has been used to attempt to monitor electron transfer in E. coli complex I. However, as revealed by the seminal experiments of DeVault and Chance, freezing a sample in liquid nitrogen cannot be relied on to prevent electron transfer between the clusters, and in fact, in the complex I experiments, the electrons are always observed on the highest potential clusters available to them, with the rate at which pairs of electrons enter the chain being limited first by NADH binding/hydride transfer and then by NAD+ dissociation. Alternatively, complex I turnover (NADH:ubiquinone oxidoreduction) is a well-defined reaction that occurs at up to ~200 s⁻¹ (~400 electrons transferred down the chain per second) in isolated complex I. Consequently, the maximum possible half-transfer time is 1.7 ms (if, which is highly unlikely, intramolecular electron transfer is fully rate limiting).

Comparison of the experimentally determined limit of 1.7 ms with the half-transfer times calculated using Dutton’s model suggests that neither the ε → ∞ nor the ε = 20 profiles shown in Figure 7 are able to support fast enough electron transfer. Only the ε = 4 profile leads to a rate of electron transfer that is fast enough to support the observed rate of catalysis, but the relevance of this profile has been questioned above. Alternative explanations for the mismatch in rates are that the electron transfer model applied here is too simple to describe the system adequately, or that the standard parameters used in the model are not appropriate for complex I (for example, the reorganization energy used, 0.7 eV, may be too high for an FeS cluster). Recently, there have been two attempts to define the rates of individual transfer steps in complex I using “atomistic” approaches. These approaches highlight the importance of specific residues, particularly aromatic residues, in the intercluster regions, but the rates calculated for individual transfer steps are, in fact, lower than those predicted by Dutton’s empirical approach.

The Mössbauer data presented here define the pattern of oxidized and reduced clusters in NADH-reduced mitochondrial complex I, and set limits on the apparent reduction potentials of the clusters in redox titrations. The results highlight the need to consider how the apparent potentials reflect both the intrinsic cluster potentials and the electrostatic interactions between clusters; both factors need to be better understood before models to calculate rates of electron transfer can be further implemented and evaluated. Thus, a complete understanding of how electrons transfer along extended cofactor chains in redox enzymes will require an integrated thermodynamic and kinetic approach that focuses on the cofactors as an ensemble rather than as a collection of individual sites.

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