A Vertebrate-type Ferredoxin Domain in the Na⁺-translocating NADH Dehydrogenase from Vibrio cholerae*

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The Na⁺-translocating NADH:quinone oxidoreductase from Vibrio cholerae contains a single Fe-S cluster localized in subunit NqrF. Here we study the electronic properties of the Fe-S center in a truncated version of the NqrF subunit comprising only its ferredoxin-like Fe-S domain. Mössbauer spectroscopy of the Fe-S domain in the oxidized state is consistent with a binuclear Fe-S cluster with tetrahedral sulfur coordination by the cysteine residues Cys79, Cys86, Cys87, and Cys111. Important sequence motifs surrounding these cysteines are conserved in the Fe-S domain and in vertebrate-type ferredoxins. The magnetic circular dichroism spectra of the photochemically reduced Fe-S domain exhibit a striking similarity to the magnetic circular dichroism spectra of vertebrate-type ferredoxins required for the in vivo assembly of iron-sulfur clusters. This study reveals a novel function for vertebrate-type [2Fe-2S] clusters as redox cofactors in respiratory dehydrogenases.

Iron-sulfur proteins are present in all domains of living organisms where they exhibit diverse functions like electron transport, catalysis, and sensing in regulatory processes (1). In some NADH-oxidizing, respiratory complexes, Fe-S centers accept electrons from flavin cofactors in an overall exergonic reaction that results in the reduction of quinone. This electron transfer reaction drives the uphill transport of protons or Na⁺ across the inner membrane of mitochondria or bacteria. The NADH:quinone oxidoreductase (Na⁺-NQR)³ from the human pathogen Vibrio cholerae maintains an electrochemical Na⁺ gradient across the inner bacterial membrane, which strongly influences the production of virulence factors (2). The Na⁺-NQR consists of six subunits, NqrA-F, and contains one Fe-S center, two covalently bound FMNs, one non-covalently bound FAD, one riboflavin, and ubiquinone-8 as prosthetic groups (3–7). The NqrF subunit of the Na⁺-NQR complex is anchored to the inner membrane and displays a clearly defined domain structure. The N-terminal Fe-S domain harbors the [2Fe-2S] cluster, while the binding sites for the non-covalently bound FAD and NADH are located in the C-terminal domain of NqrF. The initial oxidation of NADH by the NqrF subunit results in the two-electron reduction of the FAD followed by one-electron transfer steps to the [2Fe-2S] cluster in the Fe-S domain (7). Here we study the electronic properties of the [2Fe-2S] cluster in the isolated Fe-S domain of NqrF. A comparison of its amino acid sequence with sequences of [2Fe-2S] ferredoxins from vertebrates and plants reveals that the Fe-S domain is related to ferredoxins of the vertebrate-type family. Vertebrate-type ferredoxins are soluble redox carriers that accept electrons from specific NADH:ferredoxin reductases and deliver them to enzymatic systems catalyzing the hydroxylation of various compounds like steroids or camphor (8). The Fe-S domain exhibits highest sequence similarity to vertebrate-type ferredoxins required for the in vivo assembly of iron-sulfur clusters (ISC-type ferredoxins) (9). This is further supported by Mössbauer and magnetic circular dichroism (MCD) spectra of the Fe-S domain, which are reminiscent to ISC-type ferredoxins. Our finding that a vertebrate-type [2Fe-2S] cluster is an intrinsic redox cofactor of the Na⁺-translocating NADH dehydrogenase adds a novel function in respiration to this class of Fe-S centers.

MATERIALS AND METHODS

Preparation of the Fe-S Domain—The Fe-S domain of the Na⁺-NQR comprises the amino acids Met¹-Phe⁴⁴ of subunit NqrF devoid of the hydrophobic residues Val¹-Ahr¹, which are likely to anchor the NqrF subunit to the membrane. The molecular mass of the Fe-S domain including its N-terminal polyhistidine tag is 17,873 Da. Following NifS-mediated in vitro reconstitution of the Fe-S cluster (10), the Fe-S domain was purified by nickel-nitritolriatic acid affinity chromatography to remove precipitated iron sulfides and the cysteine desulfurase, Nifs (7).

Spectroscopy—For Mössbauer spectroscopy, the NifS-mediated reconstitution of the Fe-S cluster of the Fe-S domain (7) was carried out with the ⁵⁷Fe-enriched Mohr’s salt, (NH₄)₂⁵⁷FeSO₄·6H₂O, as source of redox-stable ferrous ions. The salt was obtained from metallic iron foil (95% ⁵⁷Fe) by adding a stoichiometric amount of 1 M H₂SO₄ to 1.88 × 10⁻⁹ mol of metallic ⁵⁷Fe. The solution was heated until the iron foil was completely dissolved. The volume was kept constant by adding distilled water to compensate for evaporation. Subsequently, the solution was concentrated at 80 °C until a thin crystal film appeared on its surface. Likewise, 1.88 × 10⁻⁹ mol of (NH₄)₂⁵⁷FeSO₄·6H₂O formed overnight at room temperature. The mother liquor was removed and the dried crystals were stored in the anaerobic chamber until use. Mössbauer data were recorded with a spectrometer of the alternating constant-acceleration type equipped with a Variox Cryostat (Oxford Instruments). The minimum experi-
mental line width was 0.24 mm s⁻¹ (full width at half-height). The 57Co/Rh source (1.8 GBq) was positioned at room temperature inside a vacuum-jacketed cryostat capable of fields up to 11 T and temperatures down to 1.8 K.

**Visible absorption and EPR spectra of the Fe-S domain of the NqrF subunit from V. cholerae**

The Fe-S domain (Ala25–Asn140) of the NqrF subunit was determined by the microbiuret method (14) and with representatives of vertebrate- and plant-type ferredoxins with special focus on the arrangement of the cysteine residues Cys70, Cys76, Cys79, and Cys111, which ligate the Fe-S cluster (16). The alignment was created with Clustal W and was adjusted considering structural elements. The Fe-S domain (Ala25–Asn140) of the NqrF subunit from *V. cholerae* (NqrF_VIBCH, Q9X4Q8) was aligned with the putative NqrF subunit from *Chlamydia pneumoniae* (NqrF_CHLPN, 15214181) and with representatives of vertebrate- and plant-type ferredoxins with special focus on the arrangement of the cysteine residues Cys70, Cys76, Cys79, and Cys111, which ligate the Fe-S cluster (16). The alignment was created with Clustal W and was adjusted considering structural elements.

**RESULTS AND DISCUSSION**

Visible absorption and EPR spectra of the Fe-S domain of the NqrF subunit are reminiscent of [2Fe-2S] ferredoxins of the vertebrate type, with a maximum around 540 nm in the visible spectrum and a nearly axial EPR signal with *g*₁~ = 2.020 and *g*₂~ = 1.938 in the reduced state. The EPR spectrum of the Fe-S domain of the NqrF subunit is indistinguishable from the spectrum of the NqrF subunit, indicating a very similar structural environment of the cluster in the two proteins (7). Axial *S* = 1/2 EPR signals are typical of [2Fe-2S] centers in vertebrate-type ferredoxins (15), while [2Fe-2S] centers in plant-type ferredoxins exhibit rhombic *S* = 1/2 resonances. Sequence comparisons were performed with the aim to assign the Fe-S domain to known classes of ferredoxins. The NqrF subunit is highly conserved among *Na*⁺-[NQR of different organisms, especially among *Vibrio* sp. (identity 90%). Including NqrF of *Chlamydia sp.*, which form a group apart from other identified NqrF sequences, reduces the consensus to 36% identity (Fig. 1). The [2Fe-2S] cluster in NqrF is coordinated by the conserved residues RLXN (identity 21%) and the ISC-type ferredoxin Yah1p (S0006173). The highest similarity of the Fe-S domain to a biological 5-deazaflavin/oxalate system generates electrons at very low potentials of 650 mV (11) required for the reduction of the Fe-S domain. MCD spectra were obtained at liquid He temperatures (1.8–650 mV) on a Jasco J-715 (200–1060 nm) with an extended S-20 and S-1 photomultiplier tube (Hammamatsu). The J-500C spectrometer was equipped with an Oxford Instruments SM-11 T superconducting magnet/cryostat capable of fields up to 11 T and temperatures down to 1.5 K.

**Analytical Methods**—Protein was determined by the microbiuret method (12) preceded by trichloroacetic acid precipitation. Bovine serum albumin served as standard. The concentration of the Fe-S domain was determined by the microbiuret method standardized by UV spectrophotometry using the theoretical extinction coefficient at 280 nm, 6290 M⁻¹ cm⁻¹, of the colorless Fe-S domain devoid of its Fe-S cluster. Iron was determined colorimetrically by the 3-(2-pyridyl)-5,6-bis(5-sulfo-2-furyl)-1,2,4-triazinedisodium salt trihydrate (ferene) complex (13). For the determination of acid-labile sulfur the methylene blue method (14) was applied.
from *Saccharomyces cerevisiae* (19, 20) and ferredoxin IV from *Azotobacter vinelandii* (21, 22) (Fig. 1). Yah1p and FdIV are essential components of the ISC assembly machinery, a biochemical pathway for the formation of iron-sulfur proteins in eukaryotes and bacteria (9, 23).

Mössbauer spectroscopy is a powerful tool to determine the number of ions per cluster, its oxidation state, and the type of ligands to the Fe atoms (24). The zero-field Mössbauer spectrum of the oxidized Fe-S domain of Na⁺-NQR recorded at 80 K shows an asymmetric doublet as depicted in Fig. 2. The spectrum could be deconvoluted into three symmetric quadrupole doublets with Lorentzian line shape. The major component, with 80% relative intensity, has small quadrupole splitting $\Delta E_Q = 0.61$ mm/s and characteristic low isomer shift $\delta = 0.283$ mm/s, which is typical of ferric iron with tetrahedral sulfur coordination (18, 25, 26). Since the subspectrum does not show any indications of paramagnetic broadening, and moreover, the Fe-S domain in the as isolated state is EPR-silent, the major contribution of the Mössbauer subspectrum can be assigned to a diamagnetic Fe(III)-Fe(III) pair due to the presence of binuclear [2Fe-2S]²⁺ clusters. This is further supported by the content of Fe and S²⁻ (1.61 ± 0.28 and 3.13 ± 0.11 mol/mol of Fe-S domain, respectively). Two further subcomponents are found in the Mössbauer spectrum with the following simulation parameters: $\Delta E_Q = 1.142$ mm/s and $\delta = 0.436$ mm/s, weight 8% (component 2) and $\Delta E_Q = 2.469$ mm/s and $\delta = 1.342$ mm/s, weight 12% (component 3). The distinct high isomer shift of component 3 is typical of hexa-coordinated Fe(II) with "hard" donor ligands. We assign it to remaining Fe(II) ions in the solution (hex-aquo complex or non-specifically bound iron), originating from (NH₄)₂SO₄·6 H₂O, the iron source in the reconstitution assay. The Mössbauer parameters of the minor component 2 resemble those of the delocalized mixed valence Fe(2.5)/Fe(2.5) pair of a cubane 4Fe-4S cluster (1). However, the component cannot be clearly discriminated from high spin Fe(III) precipitates due to the presence of some non-specifically bound iron oxide or hydroxide.

To further compare the [2Fe-2S] cluster in the Fe-S domain with other ferredoxins, we applied variable temperature magnetic circular dichroism (VTMCD) spectroscopy, a complementary approach to EPR for investigating the electronic properties of paramagnetic iron-sulfur clusters. In particular, VTMCD spectroscopy allows to distinguish between vertebrate- and plant-type [2Fe-2S]⁺ centers and provides a sensitive probe of the electronic structure of clusters with paramagnetic ground states (27). Each electronic transition from a spin degenerate ground state gives rise to positive or negative absorption-shaped MCD bands that increase in intensity with decreasing temperature indicating C-term behavior. The concentration of the Fe-S domain was 1.1 mM.

**Figure 2.** Zero-field Mössbauer spectrum of the Fe-S domain of the Na⁺-translocating NADH:quinone oxidoreductase. The Mössbauer spectrum of the Fe-S domain (0.4 mm) was recorded at zero magnetic field at 80 K. × marks indicate the experimental values. The solid line is a theoretical spectrum that represents the sum of the simulated spectra of three components. The major component (80% weight) is assigned to a diamagnetic [2Fe-2S]²⁺ cluster with $\Delta E_Q = 0.613$ mm/s and $\delta = 0.283$ mm/s.

**Figure 3.** Magnetic circular dichroism spectra of the [2Fe-2S]⁺ cluster in the photochemically reduced Fe-S domain of the Na⁺-translocating NADH:quinone oxidoreductase. The spectra were recorded at 5.2 K (dashed line) and 1.6 K (solid line) with an applied field of 5 T. All bands increase in intensity with decreasing temperature indicating C-term behavior. The concentration of the Fe-S domain was 1.1 mm.
Fe-S Cluster of the Sodium Pump from V. cholerae