EXAFS reveals a structural zinc binding site in the bovine NADH-Q oxidoreductase

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Abstract The metal content of bovine NADH-Q oxidoreductase determined by inductively-coupled plasma atomic-emission spectroscopy reveals the presence of about one atom of zinc per molecule of flavin mononucleotide. We applied Zn K-edge extended X-ray absorption fine structure spectroscopy (EXAFS) to investigate the local structure of the bound zinc ion and to identify the nature of the coordinating residues. The EXAFS spectrum is consistent with a structured zinc binding site. By combining information from first-shell analysis and from metalloprotein data bases putative binding clusters have been built and fitted to the experimental spectrum using ab initio simulations. The best fitting binding cluster is formed by two histidine and two cysteine residues arranged in a tetrahedral geometry.

Keywords: NADH-Q oxidoreductase; EXAFS; Zinc binding site

1. Introduction

The NADH-quinone oxidoreductase (complex I) plays a central role in energy transduction, coupling electron transfer from NADH to ubiquinone with proton pumping across the inner mitochondrial membrane in eukaryotic organisms or across the cytoplasmic membrane in prokaryotes. Mitochondrial complex I is one of the largest membrane-bound protein assemblies, being formed by 14 central subunits and up to 32 accessory subunits, and including as cofactors a non-covalently bound flavin mononucleotide (FMN) molecule, two binuclear and six tetranuclear iron–sulfur clusters [1,2].

Due to this high complexity, the information on its structure and mechanism of action is still limited. Low resolution electron microscopy analyses [3–10] show that complex I is structurally organized in two major domains: a membrane spanning segment and a peripheral arm, which protrudes into the mitochondrial matrix (or the bacterial cytosol). Sazanov and Hinchliffe reported the crystal structure of the hydrophilic domain of complex I from the bacterium Thermus (T) thermophilus at 3.3 Å resolution [11]. Within this segment, the iron–sulfur clusters are arranged in a “wire” that covers a distance of 84 Å, leading from the NADH oxidation site to the membrane domain, where quinone reduction takes place.

The activity of complex I is inhibited at the level of the quinone site by a large number of compounds, being rotenone the most commonly used [12]. The inhibitory effect of divalent cations on the catalytic activity has been reported. Within all the metals tested, Zn\textsuperscript{2+} is the most effective one [13]. Micromolar concentrations of Zn\textsuperscript{2+} are known to inhibit the respiratory chain [14] also at the level of complex III and complex IV [15,16]. The inhibitory Zn binding site in complex III has been identified by integrating X-ray diffraction (XRD) structural information [17] with extended X-ray absorption spectroscopy (EXAFS) [18]. Several Zn binding sites, characterized by a different affinity, have been recently located by XRD in crystals of the bovine cytochrome c oxidase [19]. In this enzyme EXAFS analysis allowed us to define the coordinating residues and the local structure of a high-affinity inhibitory Zn site, putatively located on the D-channel pathway [20]. In the bovine cytochrome c oxidase, however, a constitutive, tightly bound, Zn ion is also present. As a consequence, EXAFS analysis of the inhibitory, exogenous Zn binding site, required a differential approach, in which Zn–protein complexes, characterized by different Zn/enzyme stoichiometries were examined [20].

In the present paper, we provide evidence that the bovine complex I purified following standard procedures, also retains a tightly bound Zn ion. By using EXAFS spectroscopy, we determined its coordinating residues and local geometry, which are typical of a constitutive, endogenous metal site. Beside its structural interest per se, the characterization of this site opens the way to the study of additional inhibitory Zn binding sites, which can now be carried out by using the differential EXAFS approach already exploited in the case of bovine cytochrome c oxidase.

2. Materials and methods

NADH-quinone oxidoreductase was purified from bovine heart mitochondria according to Hatefi [21]. The complex I concentration

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Abbreviations: EXAFS, extended X-ray absorption fine structure spectroscopy; XRD, X-ray diffraction; ICP-AES, inductively-coupled plasma atomic-emission spectroscopy; PVA, polyvinyl alcohol

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was estimated on the basis of FMN content, determined spectrophotometrically after extraction of the cofactor from the complex by heating in boiling water for 3 min and spinning down of the denatured proteins. An oxidized–reduced extinction coefficient of 9.8 mM$^{-1}$ cm$^{-1}$ at 450 nm was used. The metal content of different preparations was measured by inductively-coupled plasma atomic-emission spectroscopy (ICP-AES) after resuspension in buffer TRIS 20 mM, dodecyl maltoside 0.1%, pH 7.3 to FMN concentrations ranging from 0.4 and 0.9 μM. The specific NADH–quinone oxidoreductase activity of our preparations amounted to $3.0 \pm 0.4 \mu l$ NADH min$^{-1}$ mg$^{-1}$.

For EXAFS measurements the complex I suspensions were supplemented with 2.5% w/v polyvinyl alcohol (PVA) (Fluka, Buchs, Switzerland, $M_w = 130000$) and dehydrated under N$_2$ flow. By this procedure the enzyme is embedded at high concentration in dry PVA films, which have also the advantage of stability and ease of handling [22].

EXAFS measurements were performed at the BM8 “GILDA” beam-line of the European Synchrotron Radiation Facility (ESRF) in Grenoble using a Si(311) double crystal monochromator employing beam-line of the European Synchrotron Radiation Facility (ESRF). The photon flux was of the order of $5 \times 10^{10}$ photons per second and the spot size was $\sim 1 \times 1$ mm$^2$. Data were collected at room temperature using a 13-element hyper-pure Ge detector equipped with fast digital electronics with a peaking time equal to 1 μs. The analyzed spectrum was obtained from the average of three scans for a total integration time of 60 s/prompt.

Data analysis was performed using the IFEFFIT code [24] as implemented in the ATHENA and ARTEMIS packages [25] and adopting the strategy described in [18]. Theoretical amplitude and phase shift functions were calculated using the ab initio code FEFF 8.2 [26]. Scattering potentials were calculated by overlapping the free atom densities in the muffin tin approximation and then using the partially non-local form for the exchange potentials within a self-consistent field.

3. Results and discussion

Evaluation of the iron content in four independent enzyme preparations by ICP-AES yielded Fe/FMN ratios varying from $16 \pm 2$ to $20 \pm 2$. These values are in line with previous estimates obtained in preparations purified according to the same procedure, in which iron contents ranging from 15 to 19 iron atoms per FMN group were found [21]. From XRD data of the hydrophilic arm of the complex [11], which contains all the iron–sulfur centers, two binuclear Fe$_2$S$_2$ and six tetranuclear Fe$_4$S$_4$ clusters have been identified for a total of 28 Fe atoms per complex. We have no unique explanation for the systematically lower iron stoichiometry found by analytical methods in the isolated preparations; we can hypothesize that this discrepancy reflects the loss of one or more subunits during the resuspension of the enzyme for ICP-AES analysis or, more likely, a systematic underestimate of Fe by ICP-AES in a complex characterized by a very high density of Fe cofactors. Evaluation of the iron content in four independent enzyme preparations amounted to $3.0 \pm 0.4 \mu l$ NADH min$^{-1}$ mg$^{-1}$.

To assess the tightness of the stoichiometric Zn binding to complex I, we exposed a fresh preparation of the complex to the action of Chelex resin for 20 h at 4 °C in batch mode (0.4 g resin in 10 ml of a 0.5 μM complex I suspension). Following this treatment, the Fe/Zn ratio of the suspension was tested by ICP-AES and compared with that of a non-exposed control. A maximum 20% decrease was found upon Chelex treatment, indicating a high-affinity Zn binding site.

The presence of an endogenous, high-affinity, Zn$^{2+}$ binding site in complex I is corroborated by the observation of a well structured EXAFS signal, symptomatic of a highly ordered local structure around the Zn atom. In Fig. 1 we show the $k^3$ weighted EXAFS function (panel A) and its Fourier transform (panel B) obtained from a PVA-embedded complex I sample characterized by a Zn/FMN ratio of 0.8 ± 0.2. In the Fourier transform (Fig. 1B) we observe two clearly distinct peaks centered at approximately 1.6 Å and 1.9 Å that, after corrections for the phase factor, give a distance of about 2.0 Å and 2.3 Å, respectively. When the contributions of more distant atoms are considered, a triple peak in the 2.5–4.0 Å range appears, characteristic of histidine residue [27].

Inspection of Zn$^{2+}$ target distances reported in datasets of high quality protein crystal structures (metal coordination sites in proteins, http://tanna.bch.ed.ac.uk/) shows typical Zn–O/N

![Fig. 1. (A) Experimental $k^3$ weighted EXAFS function (continuous line) obtained in a complex I sample characterized by a Zn/FMN stoichiometry of 0.8 ± 0.2. The dotted line represents the calculated best fitting EXAFS function corresponding to a cluster formed by two histidines and two cysteines. (B) Amplitude of the Fourier transform (FT) of the $k^3$ weighted EXAFS function.](image-url)
and Zn–S coordination distances of 2.00 Å and 2.31 Å, respectively. Such a difference is consistent with the markedly different radii of the O/N and S atoms, and agrees with the separation of the two peaks detected in the FT.

First-shell analysis confirms the presence of a mixture of N/O and S atoms and a total coordination number of four. Since the Zn–N/O and Zn–S EXAFS oscillations are nearly out of phase, it is difficult to determine the relative number of sulfur and nitrogen scatterers from a first-shell analysis, as already observed by Clark-Baldwin et al. [28].

In order to associate each first ligand with one or more amino acid residues we make the following considerations. The presence of sulfur points with high probability to cysteine residues, because the methionine residue appears with a much lower frequency, i.e. only two times (PDB entry code: 1qq9, 1b1b), in the full list of zinc coordination groups reported in http://tanna.bch.ed.ac.uk/. The presence of N/O can be associated, in principle, with a number of aminoacid residues, i.e His, Lys, Asp, Glu, Asn and Gln. However, the frequency reported in the data bank strongly privileges the presence of His, Asp and Glu and the FT of the experimental data show the presence of at least one histidine residue. Moreover a large number of ab initio simulations (not shown) indicate that, when a carboxylate residue is inserted in a tetrahedral zinc binding site, the corresponding amplitude of the FT of the simulated EXAFS should exhibit a prominent contribution in the 2.6–3.2 Å region which is not observed in the experimental data. In view of the above arguments, we are left with three main possibilities for the binding cluster: 1Cys 3His, 2Cys 2His and 3Cys 1His.

The three binding clusters were built in a tetrahedral geometry [29] following the criteria described in [18]. For all the clusters, histidine residues were built in N\textsubscript{2} protein coordination mode, being this conformation the most commonly found in crystal structure [29]. Final fitting was performed directly in k space (with a k weight of 3), in the range 2.0–12.5 Å\textsuperscript{-1}, using all paths involving up to five scattering processes with an effective path length ≤5 Å. For each amino acid we have considered as degrees of freedom the distance of the first neighbour from zinc and an angular parameter (see Fig. 2B). As angular parameters we chose: a bending angle for the His residues (β in Fig. 2B, accounting for rotation of the imidazole ring around an axis through the nitrogen bound to the metal, and perpendicular to the imidazole plane) and the Zn–S\textsubscript{C}–C\textsubscript{S} angle of cysteine (α in Fig. 2B).

The Debye–Waller (DW) factors were parametrized with analytical expressions of the form:

\[
\sigma^2(R, T) = \sigma^2(R_0, T) + A_i(T)\Delta R_i + B_i(T)\Delta R_i^2
\]

where \( T \) is the absolute temperature, \( R_0 \) is the equilibrium distance for the atom found out by density functional theory (DFT) calculations, \( \Delta R_i \) is the variation between the equilibrium and EXAFS calculated distance for atom \( i \) and \( \sigma^2(R_0, T) \) is the second order polynomial functions derived from DFT phonon normal modes calculations [30,31].

We found that the cluster which minimizes both the \( R \) factor and the reduced-\( x^2 \) is formed by two histidine and two cysteine residues (see Fig. 2A). The correspondent best fitting structural and disorder parameters are reported in Table 1. When comparing the three models on the basis on the reduced-\( x^2 \) statistics [18] it appears that the best fitting model is significantly better. In particular cluster 2His 2Cys is statistically better than 1His 3Cys within the confidence interval of 2σ and better than cluster 3His 1Cys within 1σ.

Tetrahedral zinc binding clusters formed by two histidine and two cysteine residues are observed in a large number of well-structured protein regions, where the bridges formed between the metal ion and its coordinating residues contribute significantly to the stability of the protein.

It is a fact that no Zn\textsuperscript{2+} was seen in the high resolution structure of the water soluble part of \( T. thermophilus \) complex I. The presence of an endogenous zinc site in the bovine structure implies therefore that: (a) Zn binding residues, i.e. cysteine and histidine, which are conserved in the bovine subunits are absent in the crystalized portion of \( T. thermophilus \) complex I (subunits Nqo1–6 and Nqo9) and (b) the residues involved in zinc binding are localized in subunits out of the seven crystallized ones or in polypeptides not belonging to the fourteen bacterial complex I central subunits.

We checked for conserved cysteine and histidine in the mammalian subunits (\textit{Box Taurus}, \textit{Homo sapiens} and \textit{Mus musculus}) not present in the correspondent \( T. thermophilus \)
crystallized ones and found out that several putative Zn-binding residues are localized in all the seven proteins tested. Analogously, several conserved cysteine and histidine residues are found in the other, not crystallized, central subunits. The occurrence of these conserved residues is consistent with our finding of a constitutive Zn binding site in the bovine complex, not seen in the X-ray structure of the crystallized part of the T. Thermophilus enzyme. In the absence of crystallographic information, such a high number of putative zinc binding residues makes impossible the localization of the metal site. We also notice that all the histidine and cysteine residues of the accessory subunits are in principle putative ligands.

In conclusion, our data indicate the presence in the bovine complex I of one zinc binding site showing a coordination typical of endogenous, constitutive zinc ions playing a structural role.

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