FOR THE RECORD

Redox-dependent structural changes in archaeal and bacterial Rieske-type [2Fe-2S] clusters

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Abstract

Proteins containing Rieske-type [2Fe-2S] clusters play important roles in many biological electron transfer reactions. Typically, [2Fe-2S] clusters are not directly involved in the catalytic transformation of substrate, but rather supply electrons to the active site. We report herein X-ray absorption spectroscopic (XAS) data that directly demonstrate an average increase in the iron–histidine bond length of at least 0.1 Å upon reduction of two distantly related Rieske-type clusters in archaeal Rieske ferredoxin from *Sulfolobus solfataricus* strain P-1 and bacterial anthranilate dioxygenases from *Acinetobacter* sp. strain ADP1. This localized redox-dependent structural change may fine tune the protein–protein interaction (in the case of ARF) or the interdomain interaction (in AntDO) to facilitate rapid electron transfer between a lower potential Rieske-type cluster and its redox partners, thereby regulating overall oxygenase reactions in the cells.

Keywords: Anthranilate dioxygenase (AntDO); archaeal Rieske ferredoxin (ARF); iron–sulfur cluster; reduction potential; Rieske-type ferredoxin; X-ray absorption fine structure; X-ray absorption spectroscopy

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Proteins containing Rieske-type [2Fe-2S] clusters provide critical electron transfer reactions (Mason and Cammack 1992; Trumpower and Gennis 1994; Link 1999; Berry et al. 2000) in biological pathways. Rieske-type [2Fe-2S] clusters differ from the classical plant-type ferredoxin (Fd) [2Fe-2S] clusters in the nature of the amino acid ligands to the cluster. Although plant-type Fd [2Fe-2S] clusters are ligated by four cysteine residues, Rieske-type [2Fe-2S] clusters have a mixed coordination environment involving two histidines to

one iron and two cysteines to the other iron. The cluster coordination has been firmly established by recent X-ray crystal structures of the Rieske iron–sulfur protein domains from the bovine mitochondrial *bc_L* complex (Iwata et al. 1996), the spinach chloroplast *b₆f* complex (Carrell et al. 1997), bacterial naphthalene dioxygenase (NDO) (Kauppi et al. 1998), and bacterial Rieske-type Fd (BphF) (Colbert et al. 2000). The di-histidine-coordinated iron becomes a localized ferrous site upon reduction of the cluster with the di-cysteine-coordinated iron remaining ferric (Fee et al. 1984; Cline et al. 1985; Kuila and Fee 1986; Gurbiel et al. 1996).

Two different types of Rieske clusters have been observed in proteins: one with higher reduction potentials ($E_m = +150$ to $+490$ mV) in cytochrome *bc_L/b₆f* complexes

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of the aerobic respiratory chain and photosynthesis, and the other with lower reduction potentials ($E_m = -150$ to -50 mV, NHE) in a diverse group of bacterial multicomponent terminal oxygenases and soluble Rieske-type ferredoxins (Kuila and Fee 1986; Link et al. 1992; Mason and Cammack 1992; Riedel et al. 1995; Link et al. 1996; Brugna et al. 1999). The degree to which structural variations in the vicinity of the clusters contribute to the functional properties is still a matter of intense debate, although correlations among reduction potentials, variations of hydrogen-bond networks (Denke et al. 1998; Schröter et al. 1998; Guergova-Kuras et al. 2000), and polypeptide dipoles in the vicinity of the Rieske-type clusters (Colbert et al. 2000) have been proposed. The midpoint reduction potentials of the clusters in archaeal Rieske-type ferredoxin (ARF) from *Sulfolobus solfataricus* strain P-1, a small, soluble, thermophilic protein of unknown function, and bacterial *Acinetobacter* sp. strain ADP1 anthranilate dioxygenase (AntDO), which catalyzes the conversion of anthranilate to catechol in the β -ketoadipate pathway for biodegradation of aromatic compounds (Eby et al. 2001), are examples of Rieske-type clusters that belong to the lower reduction potential class. The midpoint redox potentials of the clusters in ARF and AntDO are -155 mV (T. Iwasaki, A. Kounosu, N. Kurosawa, T. Imai, A. Urishiyama, unpubl.) and approximately -125 mV (estimated from redox potential of 2-halobenzoate 1,2-dioxygenase) (Correll et al. 1992; Riedel et al. 1995; Coulter et al. 1999; Eby et al. 2001), respectively. We report herein X-ray absorption spectroscopic (XAS) data that directly demonstrate an increase in the iron–histidine bond length of at least 0.1 Å upon reduction of the Rieske-type cluster, suggesting a functional role in biocatalysis for these clusters.

Results

Fe XAS for both ARF and AntDO show the expected shift in edge position to lower energy upon reduction of the Rieske-type $[2\text{Fe-2S}]$ cluster (Fig. 1, inset). For Rieske-type ferredoxins, the extended X-ray absorption fine structure (EXAFS) is a measure of the average coordination environment of both iron atoms in the cluster. One iron has a coordination sphere including four sulfur atoms and a distant iron, while the other iron has a sphere that includes two sulfur atoms, two nitrogen atoms, and a distant iron. Thus, the *average* coordination environment is three sulfur atoms, one nitrogen atom, and a distant iron. The EXAFS data (Table 1) for oxidized ARF (AntDO) are best fit assuming three sulfur atom scatterers at 2.20 Å (2.21 Å), *one nitrogen atom at* 1.95 Å (1.97 Å), and one iron scatterer at 2.67 Å (2.64 Å). The EXAFS data for reduced ARF (AntDO) are best fit assuming three sulfur atom scatterers at 2.24 Å (2.21 Å), *one nitrogen atom at* 2.15 Å (2.15 Å), and an iron scatterer at 2.69 Å (2.62 Å). The outer-shell atoms of the

Fig. 1. EXAFS (top) and sulfur phase-corrected Fourier transforms (bottom, over $k = 2$ – 13.5 $\text{\AA}^{-1}$) of oxidized (a) and reduced (b) ARF (solid) and AntDO (dashed). The vertical line in the Fourier transform is positioned to illustrate the shift to longer distance of the shoulder of the first shell peak, which represents the Fe- N_{imid} scattering shell. X-ray absorption Fe K edge spectra (bottom, inset) for oxidized (a) and reduced (b) ARF (solid) and AntDO (dashed).

histidine–imidazole ligand can also be incorporated into all fits, with reasonable Debye-Waller factor values. However, inclusion of these atoms contributes minimally to the fit and results in no significant improvement of the goodness-of-fit value.

The distance of the Fe- N_{imid} shell in oxidized ARF and AntDO EXAFS is better defined than that of the Fe- N_{imid} shell in the reduced proteins. The shorter Fe- N_{imid} distances in the oxidized clusters are evidenced by the low- R shoulder on the Fe EXAFS FT first-shell peaks (Fig. 1, bottom). The uncertainty in this distance in the reduced forms is a result of the lack of resolution between the Fe-S and Fe- N_{imid} distances. Nonetheless, the comparison between improvement in goodness-of-fit value and Fe- N_{imid} distance clearly demonstrates a lengthening of the Fe- N_{imid} shell in the reduced form and precludes the presence of a short (1.96 Å) Fe- N_{imid} bond length (Fig. 2). Curve fitting of the reduced Fe EXAFS data is not significantly worsened by omitting

Table 1. Curve fitting results for Fe EXAFS^a

Sample filename (<i>k</i> range) $\Delta k^3 \chi$	Fit	Shell	R_{as} (Å)	σ_{as}^2 (Å ²)	f^b
ARF (oxid) FFO0A (2–13.5 Å ⁻¹) $\Delta k^3 \chi = 13.39$	1	Fe-S ₃	2.20	0.0050	0.064
		Fe-Fe	2.67	0.0060	
		Fe-N	1.95	0.0010	
	2	Fe-S ₃	2.18	0.0055	0.082
		Fe-Fe	2.67	0.0058	
ARF (red) FFR0A (2–13.5 Å ⁻¹) $\Delta k^3 \chi = 12.58$	3	Fe-S ₃	2.24	0.0052	0.059
		Fe-Fe	2.69	0.0049	
		Fe-N	2.15	0.0067	
	4	Fe-S ₃	2.24	0.0049	0.063
		Fe-Fe	2.68	0.0050	
AntDO (oxid) AFORA (2–13.5 Å ⁻¹) $\Delta k^3 \chi = 15.07$	5	Fe-S ₃	2.21	0.0044	0.083
		Fe-N	1.97	0.0027	
		Fe-Fe	2.64	0.0049	
	6	Fe-S ₃	2.20	0.0045	
		Fe-Fe	2.64	0.0051	
AntDO (red) AFRRA (2–13.5 Å ⁻¹) $\Delta k^3 \chi = 14.60$	7	Fe-S ₃	2.21	0.0028	0.070
		Fe-N	2.15	0.0028	
		Fe-Fe	2.62	0.0039	
	8	Fe-S ₃	2.22	0.0052	0.072
		Fe-Fe	2.62	0.0038	

^a Shell is the type and number of scatterers per metal. R_{as} is the metal-scatterer distance. σ_{as}^2 is a mean square deviation in R_{as} .

^b f' is defined as:

$$f' = \frac{\left\{ \sum_i [k^3(\chi_i^{obs} - \chi_i^{calc})]^2 / N \right\}^{1/2}}{[(k^3\chi^{obs})_{max} - (k^3\chi^{obs})_{min}]}$$

the Fe-N_{imid} shell (compare Fits 3 and 4 or 7 and 8; Table 1). However, based on ENDOR studies of other lower potential Rieske-type clusters (Gurbiel et al. 1996; Link 1999), it seems more likely that the histidine ligands remain bound to the ferrous iron. The NDO crystal structure of predominantly reduced Rieske-type cluster also shows that both histidine-Nδs are bound to the cluster (Karlsson et al. 2000).

Discussion

The XAS results indicate that the Fe-S bond distances vary only slightly upon reduction of the cluster, which is in agreement with a previous report (Tsang et al. 1989). The Fe-N_{imid} bond distance, however, has increased by at least 0.1 Å upon reduction in both ARF and AntDO. Such a significant increase in Fe-N_{imid} bond distance has not been recorded for any type of Rieske Fe-S cluster (Tsang et al. 1989).

Evidence does exist, however, for redox-dependent, protein-based structural changes associated with biological [2Fe-2S] clusters with complete cysteinyl ligation. For example, NMR analysis of human ferredoxin (Xia et al. 1998),

and X-ray crystallographic analysis of *Anabaena* ferredoxin (Morales et al. 1999) indicate significant structural rearrangement between the oxidized and reduced states. *Anabaena* ferredoxin undergoes a peptide conformational change upon reduction that leads to a [2Fe-2S] cluster environment very similar to that in *Burkholderia cepacia* phthalate dioxygenase reductase (PDR) (Morales et al. 1999).

In the Rieske domain of NDO, which is homologous to bacterial and archaeal Rieske-type ferredoxins, one of the histidine ligands to the cluster is connected to the neighboring subunit via a hydrogen bond network (Kauppi et al. 1998), implying a specific protein-mediated electron and/or proton transfer pathway between the Rieske-type cluster and the mononuclear active site. This pathway in NDO has been further explored using single turnover studies with an enzyme that contained a Rieske cluster in the oxidized or reduced states (Wolfe et al. 2001). Surprisingly, the oxidation state of the Rieske cluster was a determining factor, along with the presence of substrate, in O₂ activation at the mononuclear site. The mechanism for regulation of activation at the mononuclear site might well be the lengthening of the Fe-N_{imid} bond that is observed upon reduction of the two distantly related Rieske-type clusters of ARF and AntDO. This structural change is most likely to be an inherent property of the Rieske-type cluster, at least for those with the lower reduction potentials. The localized redox-dependent structural change may fine tune the protein-protein interaction (in the case of ARF) or the interdomain interaction (in AntDO) to facilitate rapid electron transfer, as well as O₂ binding, thereby regulating overall oxygenase activity.

Fig. 2. Comparison of the goodness-of-fit value (f') with the Fe-N_{imid} bond distance for oxidized (dashed) and reduced (solid) ARF (open circles) and AntDO (filled circles) in fits where the Fe-N_{imid} distance was constrained to the specified value and the Fe-N_{imid} Debye-Waller factor was constrained to be 0.0020 Å². Lines connect the points for visual comparison.

The scale of redox-dependent structural changes of the cluster, ca. 0.17 Å for the Fe-N_{imid} bond, is such that the changes could easily evade detection by crystallographic techniques, which often have a lower precision in metal–ligand bond distances (Kauppi et al. 1998). XAS has been used in only two cases to analyze the iron coordination environment of a Rieske cluster (Powers et al. 1989; Tsang et al. 1989), neither of which quantitatively describes the contribution of the histidine ligands to the iron EXAFS for the cluster.

The XAS results presented here provide the first direct evidence that the coordination sphere of a Rieske-type [2Fe-2S] cluster varies significantly between oxidation states. These structural changes may be limited to Rieske-type clusters that play specific catalytic roles in biology and not applicable to the entire class of iron–sulfur cluster proteins. Nonetheless, this work provides some of the first structural evidence in support of the hypothesis that oxidation state-mediated structural changes of iron–sulfur centers play a critical role in regulating enzymatic catalysis.

Materials and methods

The *arf* gene coding for hypothetical archaeal Rieske-type ferredoxin (ORF c06009) of the hyperthermoacidophilic archaeon *S. solfataricus* P-1 (DSM 1616T) was cloned and sequenced (the DDBJ accession number, AB047031), and its gene product overproduced as a hexa-histidine-tagged protein in *Escherichia coli* strain BL21 CodonPlus (DE3)-RIL strain (Stratagene) and purified to homogeneity. The recombinant protein has been characterized by circular dichroism, electron paramagnetic resonance, resonance Raman, and potentiometric analyses (T. Iwasaki, A. Kounosu, N. Kurosawa, T. Imai, and A. Urushiyama, manuscript in preparation). *S. solfataricus* ARF contains the consensus motif characteristic for the oxygenase-associated Rieske-type ferredoxins, -Cys-Xaa-His-/-Gly-(Xaa)₂-(Val/Ile)-Xaa-Cys-Xaa-(Leu)-His (Mason and Cammack 1992; Link 1999), where the two cysteines and two histidines (Cys-42, Cys-61, His-44, His-64) probably serve as ligands to a Rieske-type [2Fe-2S] cluster. Purified recombinant ARF was concentrated initially by pressure filtration with an Amicon YM-10 membrane, and further by placing the samples under a stream of dry argon gas (Iwasaki et al. 2000). Dithionite reduction was performed anaerobically at room temperature under argon atmosphere. The resultant samples (~0.6–0.8 mM), containing 30% (v/v) glycerol, were frozen in a 24 × 1 × 2 mm polycarbonate cuvet with a Mylar-tape front window for XAS studies.

Recombinant AntDO was overexpressed and purified to homogeneity (Eby et al. 2001). The removal of mononuclear iron from the catalytic site (to avoid complication of Fe K-edge XAS analysis) and the preparation of oxidized and reduced Rieske cluster in AntDO were performed as described previously (Coulter et al. 1999). Briefly, Fe(II) was removed from the catalytic site by dialysis against three changes of 2 L of buffer containing 25 mM MOPS (pH 7.3), 5 mM EDTA over 12 h. To remove exogenous Fe and EDTA, the mononuclear Fe-removed protein (apoAntDO) was dialyzed against two changes of 2 L buffer containing 25 mM MOPS (pH 7.3) over 12 h. ApoAntDO was then passed through a HiTrap 5-mL desalting column (Pharmacia) equilibrated with 25 mM MOPS, (pH 7.3) for complete removal of Fe and EDTA. Loss of Fe(II) from the catalytic site was confirmed by monitoring

absorbance at 340 nm when apoAntDO was added to a spectrophotometric NADH oxidation assay (0.5 mM anthranilate, 0.1 mM NADH, 0.5 μM apoAntDO, 0.18 μM AntC in 50 mM MES, pH 6.3 and 100 mM KCl; Eby et al. 2001). The absence of detectable catalysis of aromatic substrate was also confirmed in the same assay when anthranilate was monitored by HPLC (Eby et al. 2001). The apoAntDO sample was then split into two aliquots. A final concentration of 10% (vol/vol) glycerol was added to one aliquot, placed into XAS cuvetts, and frozen in liquid nitrogen (oxidized apoAntDO). The other aliquot was made anaerobic by first mixing the sample with N₂ gas for 5 min, then reduced by careful titration with an anaerobic sodium dithionite solution (20 mg/mL) in an anaerobic chamber until the red-brown color faded from apoAntDO, a characteristic of reduced Rieske [2Fe-2S]. Glycerol at 10% (vol/vol) final concentration was added to reduced apoAntDO, placed into XAS cuvetts, and frozen in liquid nitrogen. The appearance of reduced Rieske centers was confirmed by presence of the characteristic UV/VIS and EPR spectra (Eby et al. 2001).

XAS data were collected at 10 K at Stanford Synchrotron Radiation Laboratory, beamline 7-3, with the SPEAR storage ring operating at 3.0 GeV. A monochromator containing a Si[220] crystal and a 13-element Ge solid-state X-ray fluorescence detector (provided by the NIH Biotechnology Research Resource) were employed for data collection. No photoreduction was observed in comparisons of the first and last spectra collected for a given sample. The first inflection of the edge of an Fe foil (assumed to be 7111.2 eV) was used for energy calibration. All other data collection parameters were as described previously (Casper et al. 1999). EXAFS analysis was performed with the EXAFSPAK software (www-ssrl.slac.stanford.edu/exafspak.html) according to standard procedures (Scott 1985).

Electronic supplemental material

Plots of curve-fitting results for the EXAFS of oxidized and reduced *Sulfolobus solfataricus* and *Acinetobacter* sp. Rieske-type proteins is available as supplementary information.

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