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X-ray reduction correlates with soaking accessibility as judged from four non-crystallographically related diiron sites.

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X-ray reduction correlates with soaking accessibility as judged from four non-crystallographically related diiron sites

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Keywords: diffusion, diiron, metalloprotein, ribonucleotide reductase, X-ray reduction

Abbreviations: RNR, ribonucleotide reductase; R2F, class Ib ribonucleotide reductase R2 protein; MES, 2-(4-morpholino)-ethane sulfonic acid

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X-ray reduction of diiron sites in a metalloprotein crystal correlates with their soaking accessibility, indicating that a diffusion component is involved in the reduction process.

Abstract

X-ray crystallography is extensively used to determine the atomic structure of proteins and their cofactors. Though a commonly overlooked problem, it has been shown that structural damage to a redox active metal site may precede loss of diffractivity by more than an order of magnitude in X-ray dose. Therefore the risk of misassigning redox states is great. Adequate treatment and consideration of this issue is of paramount importance in metalloprotein science, from experimental design to interpretation of the data and results. Some metal sites appear to be much more amenable to reduction than others, but the underlying processes are poorly understood. Here, we have analyzed the four non-crystallographically related diiron sites in a crystal of the ribonucleotide reductase R2F protein from *Corynebacterium ammoniagenes*. We conclude that the amount of X-ray reduction a metal site suffers correlates with its soaking accessibility. This direct observation supports the hypothesis that a diffusion component is involved in the X-ray reduction process.

Introduction

In order to obtain information about their three-dimensional structure at near-atomic resolution, crystals of biological macromolecules are exposed to high energy X-rays. These X-rays also cause radiation damage in the crystalline molecules, thereby limiting the amount of usable data that can be obtained.^{1,2} Radiation damage can prevent structure solution or hamper correct

interpretation of the obtained structure. The latter is a problem in particular in the case of metalloproteins because X-rays can reduce metal sites in proteins.³⁻¹³ Photoreduction may occur at X-ray doses orders of magnitude lower than are required to have obvious effects on the diffraction pattern, and often long before a complete dataset has been collected.^{7, 11, 14} This can make it difficult to unambiguously assign redox states to obtained structures and to acquire the molecular structure of oxidized states. The phenomenon has, however, also been elegantly taken advantage of to obtain information on the enzymatic mechanism of metalloproteins,^{6, 7, 12} and in some cases more serendipitously used to obtain the structure of a particular, previously not observed redox state.⁴ Some metal sites are more amenable to X-ray reduction than others, but the molecular determinants and underlying chemical processes are poorly understood.² Since crystals of different proteins react differently to X-ray exposure, and the chemical properties of the buffer and the particular protein also differ, it is complicated to dissect which factors influence the amount of reduction suffered.

The diiron sites in ribonucleotide reductase (RNR) R2 proteins are well characterized both structurally and spectroscopically and display very distinct and clearly identifiable structural differences between the reduced and oxidized states. While determining the structure of the oxidized diiron form of the class Ib RNR R2F subunit from *Corynebacterium ammoniagenes* we observed different amounts of reduction in the four non-crystallographically related diiron sites in the asymmetric unit of the crystal.⁸ For studies of the mechanism of X-ray reduction this is an advantageous situation, as the metal sites are chemically identical, but the monomers have slightly different environments in the crystal packing.

In the same study we also presented the structures of the reduced ($\text{Fe}^{\text{II}}\text{-Fe}^{\text{II}}$) diiron site as well as the dimanganese-bound protein. Although the protein is active with a diiron cofactor, the dimanganese site was recently shown to in fact most likely be the physiological cofactor of class

Ib R2F proteins.^{15, 16} Unlike the diiron site, however, the dimanganese site is not oxidized by molecular oxygen, but requires an additional subunit to deliver a reduced form of oxygen to the site.¹⁵⁻¹⁷ The dimanganese-bound structure therefore represents the reduced ($\text{Mn}^{\text{II}}\text{-Mn}^{\text{II}}$) state of the protein. The two latter structures were obtained by soaking crystals of metal-depleted protein with the respective metal ions, while the protein as produced in *E. coli* under oxidizing conditions contains an oxidized ($\text{Fe}^{\text{III}}\text{-Fe}^{\text{III}}$) diiron site.⁸ Here, we perform further analysis and modelling of the data for these three structures and show that the amount of reduction a site suffers correlates with its soaking accessibility.

Materials and methods

Expression, purification, crystallization and crystal soaking

Expression and purification of R2F from *C. ammoniagenes* have been described previously.¹⁸ Crystallization, crystal soaking with Fe^{II} under reducing conditions and Mn^{II} as well as data collection, processing, structure determination, model building and refinement have also been described.⁸ In short, the protein was crystallized in mother liquor consisting of 30% (w/v) PEG 4000, 200 mM ammonium acetate and 100 mM sodium citrate at pH 6-6.5. To be able to perform metal soaks, crystals of apoprotein or manganese-substituted protein were first soaked for 1 h in mother liquor containing MES buffer instead of citrate because citrate is a metal chelator. Apoprotein crystals were then soaked in MES-buffered mother liquor additionally containing 10 mM $\text{Fe}(\text{NH}_4\text{SO}_4)_2$, 1% (w/v) dithionite, 1 mM phenosafranin and 15% glycerol (for cryoprotection) for one hour to obtain the reduced diiron site. Crystals of manganese-substituted protein were soaked in MES-buffered mother liquor additionally containing 10 mM MnCl_2 and 15% glycerol for 30 minutes before being flash cooled. Diffraction data were collected on a

MAR 345 imaging plate or a CCD area detector (ADSC Q4 CCD) at beamline I711 of the MAXII synchrotron in Lund, Sweden, or ID14-2 at ESRF Grenoble, France. Crystals were kept at 100 K during data collection. Data processing and refinement statistics for the original structures can be found in reference 8 and are also shown in Table 1.

Occupancy refinement

To refine the occupancies of the metal sites in the reduced Fe-soaked structure (PDB ID 1KGO) and the Mn-soaked structure (PDB ID 1KGP), the average B factors for the backbone atoms of the four-helix bundles that harbor the metal site were calculated for each of the four non-crystallographically related subunits. The B factors of the metal ions were then fixed at the corresponding values before individual occupancy refinement of all metal ions with CNS.¹⁹

Assignment of oxidation states

To judge the oxidation state of the four non-crystallographically related diiron sites in crystals prepared under oxidizing conditions (PDB ID 1KGN), a simulated annealing $F_o - F_c$ map was calculated with CNS¹⁹ with the two carboxylate ligands E202 and E168 as well as any non-protein ligands omitted for all subunits. This map was then compared with the oxidized and reduced conformations of the site. Figures were prepared with PyMOL (The PyMOL Molecular Graphics System, Schrödinger, LLC).

Results

This study is based on three crystal structures (Table 1). The first structure was obtained from a crystal of iron-bound *C. ammoniagenes* R2F protein prepared under oxidizing conditions. This

crystal therefore contained the oxidized ($\text{Fe}^{\text{III}}\text{-Fe}^{\text{III}}$) diiron site before exposure to X-rays.

However, the metal sites in the four non-crystallographically related subunits in the asymmetric unit display varying degrees of reduction. For structures two and three, crystals of metal-depleted R2F protein were soaked with ferrous or manganese ions. These structures are used to assess the differential accessibility of the active sites in the four non-crystallographically related subunits by comparing their occupancy with metal ions after soaking.

Assignment of oxidation states

There are several structural features that identify the different oxidation states of the dimetal site of R2 proteins, these being rotamer changes of two glutamate ligands, the presence of an oxo or hydroxo bridge in the oxidized metal site, as well as characteristic metal-metal distances.^{4, 5, 8, 20-22}

In the case of the *C. ammoniagenes* R2F protein, the two carboxylate ligands E168 and E202 adopt different rotamers in the oxidized and reduced state. Two terminal water ligands and a metal-bridging ligand additionally characterize the oxidized, di-ferric form of the site, but are absent in the reduced state. In addition, the iron-iron distance is 3.4-3.5 Å in the oxidized site as compared to ~3.9 Å in the reduced site (Fig. 1).⁸ These characteristic structural differences allow robust assignment of oxidation states to observed structures, even in the absence of spectroscopic information. In this particular case, spectroscopic information would indeed be of little help in assigning oxidation states to the four metal sites in the asymmetric unit of the crystal, as it would show a mixture of oxidation states with no possibility to assign states or ratios to specific positions in the asymmetric unit.

An omit electron density map of the ligands is the best way to judge the conformation of the glutamates and the absence or presence of the non-protein ligands. Figure 2 shows a simulated annealing $F_o\text{-}F_c$ electron density map calculated with E168, E202, and the non-protein ligands

omitted for the four different sites A-D in the structure of iron-containing crystals obtained under oxidizing conditions (PDB ID 1KGN). Several atoms in the reduced and oxidized structures occupy nearly the same positions in space. For example, the reduced conformation of E202 overlays with the positions of the bridging ligand and one of the water ligands in the oxidized site. However, when taking the whole site into account, the differences between oxidized and reduced conformations are striking enough to allow confident assignment of the redox state. Sites C and D are mainly oxidized and mainly reduced, respectively. Sites A and B appear to represent mixed states, as judged primarily by the presence of the water molecule coordinated to Fe²⁺, and traces of density for E202 in both its reduced and oxidized conformations. Site A is tentatively assigned as less reduced than site B because of the somewhat clearer density for E202 in the oxidized conformation.

Regarding the iron-iron distance, the iron atoms refine to an intermediate distance depending on the relative occupancy of the two oxidation states. The refined Fe-Fe distance thus reflects the relative populations of reduced and oxidized sites. The refined distance for site C is the shortest with ~ 3.5 Å, whereas in site D the Fe-Fe distance refines to ~ 3.7 Å, and the distances for sites A and B are in between (Table 2). This agrees with the assignment of oxidation states based on the ligand omit electron density. Thus, the amount of reduction of the four metal sites can be assigned as follows: $D > B \approx A > C$.

We have considered other reasons than X-ray reduction for the observed differences in structure, for example that all sites were not oxidized upon crystallization. However, this is improbable since the protein was aerobically prepared in the iron-bound state. Moreover, any pre-crystal effect would imply a selection during crystallization so that specific sites ended up in the same symmetry positions in the crystal lattice, which also seems unlikely in this case.

Assignment of soaking accessibility

We assessed the accessibility of the metal sites by refining the metal occupancies in two crystals of metal-depleted protein soaked with either Mn^{II} or Fe^{II} under reducing conditions. To be able to obtain relative occupancies of the bound metal ions their B factors have to be estimated, as B factor and occupancy are highly correlated. Here, we chose to fix the B factors of the metal ions to the average B factor of the backbone atoms of the four-helix bundle harboring the respective site. It should be noted that this leads to a slight overestimation of the occupancies, and hence occupancies greater than 1 can be obtained. However, the occupancies only changed slightly, and, most importantly, the internal order of occupancies did not change for any of the sites if all metal B factors were fixed at $20 \text{ \AA}^2$ during occupancy refinement, indicating that the assignment of relative occupancies is robust. Table 3 shows the average B factors and refined occupancies of the metal atoms for the two metal-soaked structures. In both cases, site D is most accessible followed by site B, site A and finally site C.

Discussion

Studies of location-dependent X-ray reduction in protein crystals represent particular challenges because spectroscopy cannot be readily used to corroborate the structural data. In these cases, the crystallographic data must be used to the fullest extent and as much information as possible extracted, given the resolution and other factors. Here we have assessed X-ray reduction and accessibility to metal soaking, using two independent measures for each parameter, for four crystallographically independent metal sites. Although the absolute numbers are approximate, the trends are clear and consistent in all cases.

The presented data support a correlation between solvent accessibility and X-ray reduction of metal sites in proteins. The more accessible a site is, the more easily it is reduced by X-radiation. This suggests that the reduction process has a diffusion component. At temperatures below 110 K, the only mobile species are solvated electrons.^{1, 23, 24} These originate mostly from water undergoing radiolysis and will diffuse to the closest electron-affinic site, i.e. in this case the metal ions. The observed correlation between solvent accessibility and reduction sensitivity might therefore be due to the higher concentration of solvent at the more accessible sites.

Our observations are in agreement with previous studies proposing higher solvent accessibility to be the reason for the higher radiation sensitivity of certain sites as compared to others of the same chemical composition.²⁵⁻²⁷ It should be noted that there are counter-examples and other propositions to explain the observed differential sensitivity.^{2, 28-30} However, as specific radiation damage proceeds via different mechanisms at chemically different sites, there might also be more than one mechanism at play to explain the differential radiation sensitivity of chemically identical sites.

A large number of R2 and R2-like proteins from different organisms have been crystallized to date.^{5, 8, 20-22, 31-35} Interestingly, although their structures and especially active site architectures are very similar, they nonetheless show remarkably different sensitivity towards X-ray reduction. For example, a large number of datasets have been collected on crystals of the *E. coli* R2 protein in its oxidized state.²⁰⁻²² Despite a number of these datasets being collected without any attempt made to reduce X-ray dosage, no signs of photoreduction of the active site at cryo temperatures have been reported. On the other hand, *C. ammoniagenes* and *S. typhimurium* R2F are easily reduced in the X-ray beam.^{5, 8} Since the aim of these studies was not to investigate radiation damage, the total X-ray dose the crystals were exposed to were not recorded, and a systematic analysis of these data is hence not possible. It has been suggested that the presence of glycerol in

the mother liquor facilitates photoreduction because it scavenges electron holes.^{5, 36, 37} It is noteworthy, however, that while *S. typhimurium* R2F crystals only suffered photoreduction in the presence, but not in the absence of glycerol,⁵ *E. coli* R2 crystals are not reduced in the presence of glycerol unless they are allowed to thaw after intense irradiation.^{4, 22} In a systematic analysis, no significant effect of the glycerol concentration on the photoreduction rate in myoglobin crystals was observed, although reduction proceeded slightly more slowly in the absence of glycerol.¹¹ The mother liquor composition is very likely to have an influence on the reduction rate.^{11, 24, 30} However, the data presented here indicate that the chemical composition of the mother liquor is not the only determinant, but that site accessibility might also play a role. These results support the hypothesis that a diffusion component is involved in X-ray mediated metal reduction. This should be considered when designing schemes to reduce radiation damage. The complete picture of X-ray reduction of metal sites is clearly still being painted. Given the large number of crystallized R2 and R2-like proteins, they appear well suited as model systems to study the mechanisms of radiation damage. An in depth understanding of this phenomenon would greatly augment metalloprotein structure-function studies using several methods.

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Tables

Table 1. Data processing and refinement statistics for the included structures.

Data statistics	Fe-containing (1KGN)	Fe ^{II} -soaked (1KGO)	Mn ^{II} -soaked (1KGP)
beamline	I711/MAXII	ID14-2/ESRF	ID14-2/ESRF
wavelength (Å)	1.030	0.933	0.933
spacegroup	P2 ₁	P2 ₁	P2 ₁
unit cell parameters (Å)			
<i>a</i>	50.81	49.95	50.67
<i>b</i>	90.71	91.15	91.20
<i>c</i>	136.81	137.27	137.27
unique angle:β (°)	91.24	91.27	91.45
resolution (Å)	30-1.85	25-2.25	25-2.0
(outer shell)	(1.88-1.85)	(2.29-2.25)	(2.03-2.00)
no. of observations	193418	135221	149319
unique reflections	101333	55206	77244
R _{merge} ^a (outer shell)	0.048 (0.260)	0.079 (0.294)	0.071 (0.209)
Completeness (%) (outer shell)	95.9 (94.7)	94.7 (96.4)	91.1 (85.4)
I/σ(I) (outer shell)	11.8 (2.9)	10.7 (3.0)	15.0 (5.4)
Refinement			
R _{work} ^b (%)	15.8	16.3	17.7
R _{free} ^c (%)	21.3	23.7	23.6
RMS dev. bonds (Å)	0.014	0.010	0.009
RMS dev. angles (°)	1.44	1.29	1.21
Ramachandran plot ^d , % of residues			
Most favored	97.2	96.4	97.0
Allowed	2.8	3.6	3.0
Generously allowed	0.0	0.0	0.0
Disallowed	0.0	0.0	0.0

^a $R_{\text{merge}} = \sum_j \sum_h |I_{hj} - \langle I_h \rangle| / \sum_j \sum_h I_{hj}$ where I_{hj} is the j th observation of reflection h .

^b $R_{\text{work}} = \sum ||F_{\text{obs}} - F_{\text{calc}}|| / \sum |F_{\text{obs}}|$, where F_{obs} and F_{calc} are the observed and calculated structure factor amplitudes, respectively. ^c R_{free} is equivalent to R_{work} for a 5% subset of reflections not used in

the refinement. ^d Ramachandran plots were generated with Procheck³⁸.

Table 2. Refined iron-iron distances in the four non-crystallographically related subunits of the Fe-containing *C. ammoniagenes* R2F protein (PDB ID 1KGN).

subunit	Fe-Fe distance (Å)
A	3.5(6)
B	3.5(7)
C	3.4(7)
D	3.6(6)

Table 3. Average B factors for the backbone atoms in the respective four-helix bundles and the refined metal occupancy for the different metal sites in the four non-crystallographically related subunits of *C. ammoniagenes* R2F. As B factor and occupancy are highly correlated, the B factors of the metal ions were fixed to the average B factor of the backbone atoms of the four-helix bundle harboring the respective site before occupancy refinement. It should be noted that this leads to a slight overestimation of the occupancies, and hence occupancies greater than 1 can be obtained (see text).

Subunit	Average B ($\text{\AA}^2$) 4-helix backbone Fe ^{II} -soaked (1KGO)	Average B ($\text{\AA}^2$) 4-helix backbone Mn- soaked (1KGP)	Refined occupancy Fe ^{II} -soaked (1KGO) Fe1/Fe2	Refined occupancy Mn-soaked (1KGP) Mn1/Mn2
A	20.5	18.4	0.52/0.56	0.37/0.39
B	24.6	20.4	0.71/0.71	0.51/0.58
C	18.8	19.4	0.40/0.47	0.29/0.27
D	20.2	22.2	1.09/1.16	1.06/1.17

Figures

Fig. 1 Structure of the diiron sites of *C. ammoniagenes* R2F in the reduced (top, PDB ID 1KGO) and oxidized state (bottom, PDB ID 1KGN, site C). Note especially the non-protein ligands in the oxidized site and the rotamer changes of E168 and E202.

Fig. 2 Simulated annealing F_0-F_c map of the metal sites of the different monomers A-D in the four non-crystallographically related subunits of the Fe-containing *C. ammoniagenes* R2F protein (PDB ID 1KGN) contoured at 5σ . The side chains for E168 and E202 as well as the non-protein ligands were omitted from the map calculation. Maps are compared to the reduced (blue) and oxidized (red) structure. Sites C and D are most easily interpreted as mainly oxidized and mainly reduced, respectively. Sites A and B show mixed structures.