Photoreduction of Superoxide Reductase (SOR) Induced by X-rays

Virgile Adam[ab], Fernando Molina-Heredia[c], Vincent Nivière[c] & Dominique Bourgeois[ab]

[a]*LCCP, UMR 9015, IBS, 41 avenue Jules Horowitz, 38027 Grenoble Cedex 1, France*
[b]*ESRF, 6 rue Jules Horowitz, BP 220, 38043 Grenoble Cedex, France*
[c] *CBCRB, DRDC-CEA, 17 avenue des Martyrs, 38054 Grenoble, Cedex 9, France*

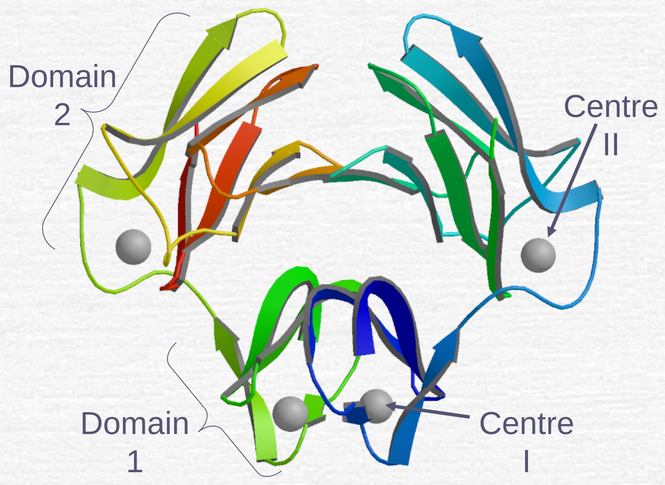

Fig. 1 Structure of superoxide reductase

1) What is superoxide reductase?

Superoxide reductase (SOR) is a metallic-enzyme eliminating the superoxide anion as shown in the following equation:

$$O_2^{-\cdot} + 2H^+ + SOR(Fe^{2+}) \rightarrow H_2O_2 + SOR(Fe^{3+})$$

As it is found in sulphure-reducing and microaerophilic bacteria, the advantage of SOR compared to superoxide dismutase (SOD) is that there is no oxygen release in the cell. However, the reaction catalyzed by SOR is formally the same as the second half reaction catalyzed by SOD.

Desulfoarculus baarsii SOR has two iron centers with the following redox potentials: +4 mV for centre I, whose physiological role remains unclear, and +240 mV for centre II, where $O_2^{-\cdot}$ binds. The enzyme is naturally found in a hemi-reduced form, with centre I oxydized and centre II reduced. Center II is insensitive to di-oxygen, but can be oxidized in solution with powerful oxidizers like hexachloroiridate, hexacyanoferrate and of course, the substrate, superoxide.

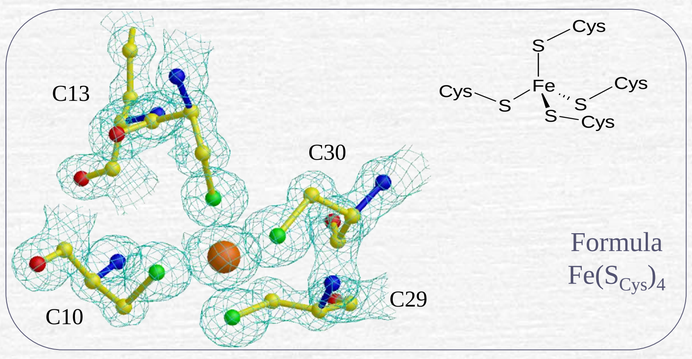

Fig.2 Detail of the SOR center I

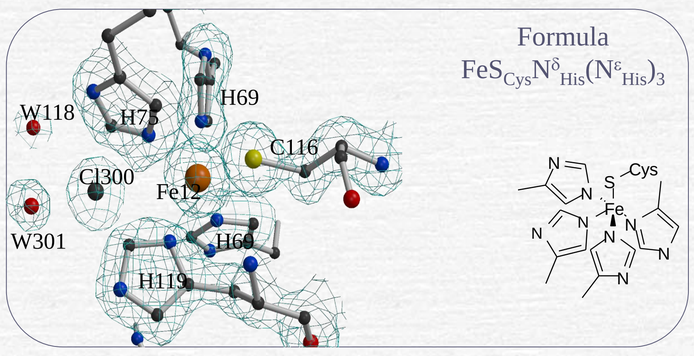

Fig.3 Detail of the SOR active site (center II) and of a stabilized chloride atom close to the iron

2) Structure of SOR

The structure of SOR (mutant E47A) (fig.1) shows a globular homodimeric protein. Each monomer is composed of two domains, each holding an iron center.

In center I (fig.2), the iron atom is surrounded by four cysteines in a square-pyramidal conformation, a commonly found coordination pattern.

In center II (fig.3) the iron atom is coordinated by four histidines and a cysteine, a quite unusual coordination pattern. In the reduced state, the sixth coordination, where superoxide is expected to bind, is vacant. The substrate binding pocket is a strong anion attractor and we have noticed the presence of a chloride atom (present the crystallization medium) stabilized at 4Å from the iron.

By optimizing crystallization conditions, we could solved the structure of reduced SOR to 1.15Å (fig.4).

In native oxidized SOR, Glu47 has been found to occupy the sixth coordination to the center II iron. However, the mutant we are using (E47A), which is thought to stabilize a (hydro) peroxo intermediate along the reaction pathway, lacks this glutamate. It is therefore of interest to obtain the structure of this oxidized SOR mutant,

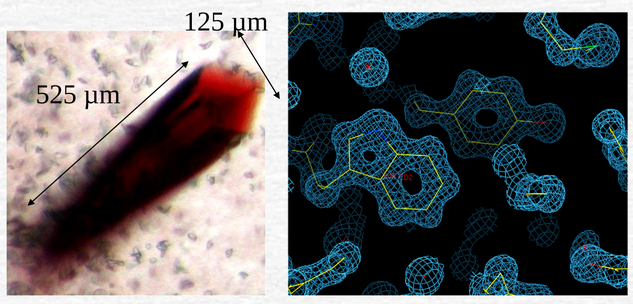

Fig.4 SOR crystal in its hemi-reduced form and the electron density map at 1.15Å

3) Spectrophotometry

In solution, the oxidization state of center II cannot be easily seen because the corresponding absorption band (650 nm) is overwhelmed by the signal from center I (at 370 and 503 nm). To see the 650 nm band from oxidized center II, a difference spectrum must be computed (fig.5). At cryogenic temperatures in the crystal, spectra are slightly different (they are better resolved) but again the 650 nm band may not be directly seen.

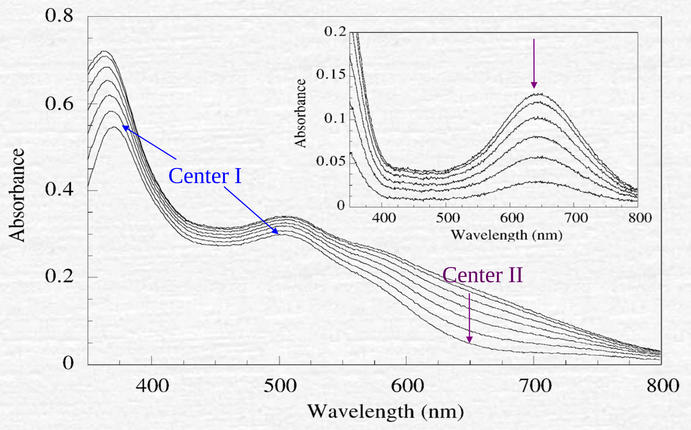

Fig.5 Differential spectra of SOR oxidized by the superoxide anion

4) Structure of the oxidized form

Soaking SOR crystals in solution containing oxidizing agents failed, possibly due to the presence of the chloride ion in the active centre II. However, co-crystallization of SOR with hexacyanoferrateIII also known as ferricyanide ($Fe(CN_6)^{4-}$) was successful. Unexpectedly, the structure clearly shows a ferricyanide bound to center II iron through a bent cyanide group (fig.6). Also unexpectedly, the excess of ferricyanide added to crystallization drops resulted in the loss of 65% of the center I iron atoms. Therefore we find partial occupation in this center for cysteines forming disulphide bridges.

This loss of center I iron atoms, is fortunate as it resulted in the possibility to observe the 650 nm absorption band of center II much more easily (see paragraph 3). We could thus verify that in the crystals of the complex, before X-ray data collection, the center II iron is oxidized.

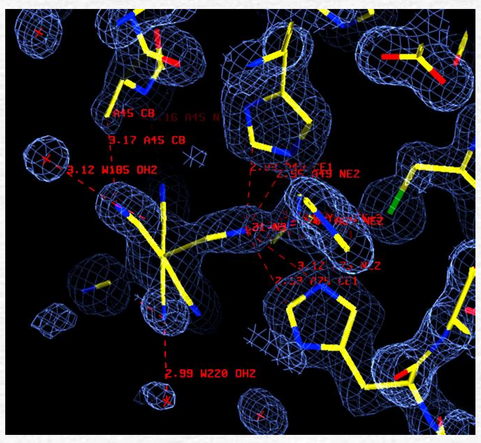

Fig.6 Structure of the complexed and oxidized superoxide reductase at 1.5Å

5) Radiation damage

Data collection on the SOR-$FeCN_6$ crystals (grey-green), resulted in clear radiation damage induced by X-rays, as assessed by visual inspection (fig.7). Further study of this radiation damage by following its time-dependence on the high brilliance beamline ID14-4 revealed that rapid photoreduction of both centers I and II occurred during data collection.

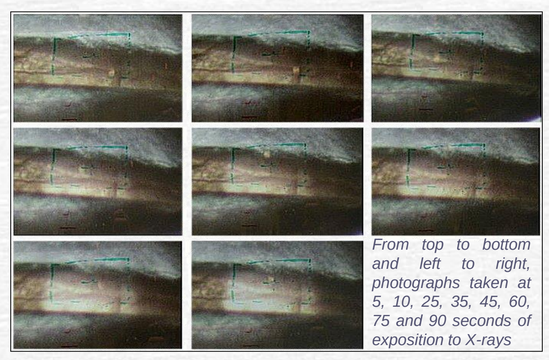

Fig.7 Development of radiation damage on a needle-shaped crystal of complexed SOR

6) Composite datasets

A real-time absorbance measurement in the crystal during X-ray exposure (fig. 8) showed a very fast reduction of the 650 nm band (t1/2 = 10 seconds). In order to obtain a "low-dose" oxidised structure and a "high-dose" reduced structure, we collected several partial data sets with an attenuated beam on the same long needle-shaped crystal, and recombined them to form two "composite" data sets (fig.9). The "low-dose" dataset corresponded to 6 seconds of exposition under the attenuated beam and the "high-dose" dataset corresponded to 80 seconds of exposition under the non-attenuated beam (fig.10).

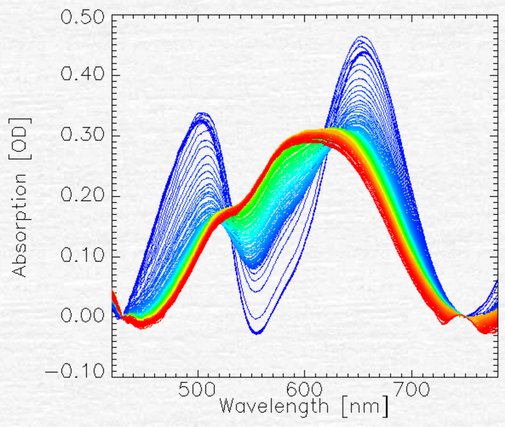

Fig.8 Experimental decrease (spectra from blue to red) of the 503nm and 650nm peak, with concomitant increase of a non-specific 600 nm band.

7) Fourier difference maps

The difference maps between the oxidized and reduced structures of the complex with $FeCN_6$ (solved at 1.7Å) are consistent with X-ray induced reduction of the molecule. As an internal control, a clear reduction of two disulphide bridges formed by the cysteines of center I can be observed (fig.11a). Modelization of these cysteines turned out to be much easier in these structures than in a dose-dependant (standard) data set where mixed conformations exist.

In center II (fig.11b), a slight but significant coordination expansion is observed. All atoms around the iron moved slightly away. The the bridging cyanide is found less bent in the high-dose structure as compared to the low-dose structure, as a result of the back motion of bound ferrocyanide. All these results are consistent with a decrease of the iron charge by on electron, thereby reducing the electrostatic attraction of the coordinating ligands.

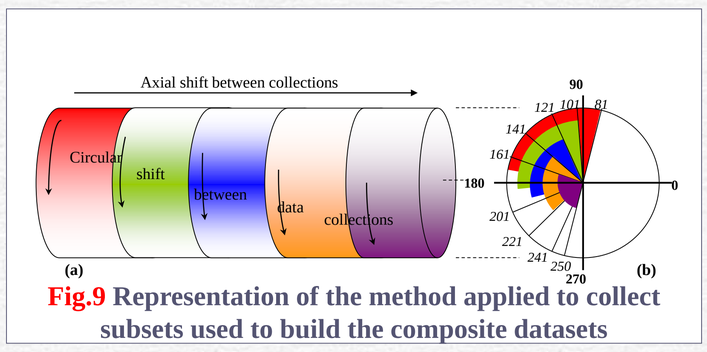

Fig.9 Representation of the method applied to collect subsets used to build the composite datasets

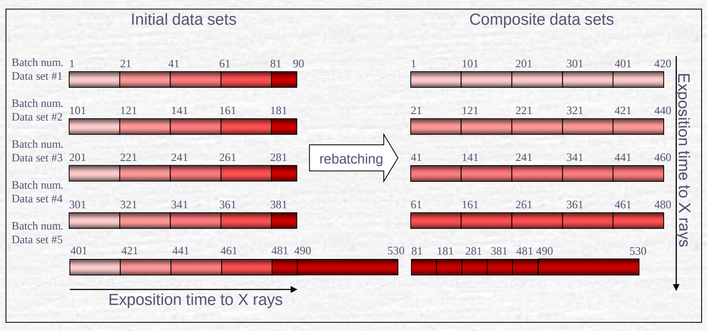

Fig.10 Rebatching of the partial data sets in order to build composite datasets independent of time exposition to X-rays

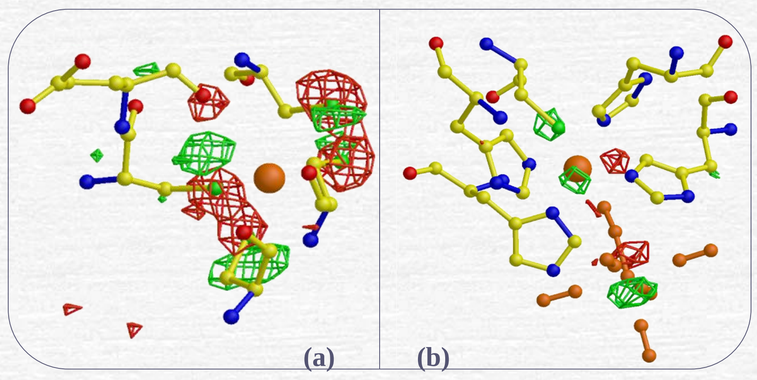

Fig.11 Difference maps of the reduced-oxidized structures for center I (a) and center II (b)

8) Conclusion

The described SOR crystal structure in complex with hexacyanoferrate is the first protein structure solved with this compound.

Our results show that it is possible to follow in real time by microspectrophotometry the reduction of a metalloenzyme by a synchrotron X-ray beam. The technique of composite datasets allows to observe the associated subtle coordination expansion with unprecedented accuracy in a protein.

Preliminary biochemical studies show that ferrocyanide could be a partial inhibitor of the enzyme or a scavenger of superoxide, and may thus have a significant physiological relevance.

Références

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Acknowledgements

Grenoble
ESRF
IBS
Institut de Biologie Structurale (Grenoble)