	Experiment title: Structural Studies of Catalytic Turover by a Decaheme Nitrite Reductase Induced by Photoreduction in a Synchrotron X-ray Beam Followed by Online Monitoring of Spectral Changes	**Experiment number**: MX586
Beamline: 14.2	**Date of experiment**: 5th November 2006 from: 08:30 to: 08:00	**Date of report**: 2/2/2007
Shifts: 3	**Local contact(s)**: John MCGEEHAN	*Received at ESRF:*

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Report:

ABSTRACT Beamline ID 14.2 equipped with an online microspectrophotometer was used to investigate the rate of photoreduction of a crystalline decaheme cytochrome-*c* nitrite reductase in the ESRF X-ray beam at 100K. The rate of photoreduction was rapid and essentially complete by 100 sec total X-ray exposure. Crystals presoaked with nitrite showed heme reoxidation during exposure consistent with reduction of the substrate. However, the nature of the catalytic product is unclear. These results have implications for the assignment of redox state to currently available structures of redox proteins solved using synchrotron radiation.

INTRODUCTION Cytochrome *c* nitrite reductase (NrfA) is a complex decaheme homodimer which catalyses the respiratory six electron reduction of nitrite to ammonium and is central to anaerobic energy conservation in many pathogenic bacteria (1). It can also catalyze the five and two electron reductions of nitric oxide and hydroxylamine, respectively, to produce ammonium ions. As part of our studies of the enzymatic mechanism of nitrite reduction, we sought beamtime at the ESRF to study catalytic turover by the *E.coli* nitrite reductase enzyme induced by photoreduction in the synchrotron X-ray beam. Changes in the oxidation state of the heme redox centres were to be followed by monitoring of spectral changes using an online microspectrophotometer.

METHODS Our initial objective was to carry out X-ray exposures of crystals of native, oxidised NrfA with concomitant monitoring of heme oxidation state by measurement of absorbance at 410 and/or 550 nm. This information was to be used to determine strategies for collection of diffraction data to obtain structures of the enzyme in the fully heme-oxidised and heme-photoreduced states.
Firstly, a crystal of oxidised, native enzyme at 100K was subjected to increasing cumulative time X-ray exposures. Absorbance spectra were measured after each exposure. A separate crystal was exposed for 100 sec, the shutter was then closed and absorbance monitored in order to detect reoxidation. In addition, the crystal was then brought to ambient temperature by blocking the cryocooler gas stream for two periods of six seconds. Reoxidation of the protein by this procedure was qualitatively assessed by re-measurement of the

absorbance spectrum. These experiments were then repeated using a crystal of the native enzyme soaked in a solution containing 100 mM sodium nitrite. All experiments were carried out in duplicate.

RESULTS AND DISCUSSION

Figure 1A shows the results of increasing cumulative X-ray exposure on the crystal absorption spectrum for the native protein at 100 K in the range 450-600 nm. The photoreduction of the heme centres in the protein is essentially complete by 100 sec total exposure time. Inspection of these absorbance spectra suggested that the progress of photoreduction could be most usefully carried out by following the difference absorbance between 550 nm and 583 nm. This difference absorbance is shown as a function of time for a crystal of the native enzyme in Figure 2. In this case, the crystal was exposed continuously for 100 sec and the X-ray shutter was then closed. Monitoring of difference absorbance continued for a further 900 sec. No decay in the signal is detected after closure of the X-ray shutter suggesting that no reoxidation is occurring in the cryoprotected and cryocooled crystal. If this crystal is then exposed to air at ambient temperature (approx 295 K), some reoxidation of the heme centres is observed (see Figure 1B) but the crystal does not return to the fully oxidised state. The rate of photoreduction of the crystalline oxidised enzyme in the beam was significantly more rapid than we had anticipated, giving rise to significant spectral changes within the first few seconds of exposure. This precluded any attempt to collect viable diffraction data from fully-oxidised enzyme.

When these experiments were repeated with a crystal presoaked in 100 mM sodium nitrite, the substrate for the enzyme, before cooling to 100K, similar results were obtained (Figures 1C,D). In this case, however, a slower rate of photoreduction and a drop in difference absorbance after closure of the X-ray shutter (Figure 2) suggests that some transformation of the substrate was occurring in the crystal both during and after exposure. The signal drops to approximately 60% of its value when the X-ray shutter was closed suggesting that full reoxidation of the enzyme through reduction of its substrate is not occurring.

CONCLUSION

The rate of photoreduction of this multiheme cytochrome in the crystalline state is rapid and the protein is effectively fully reduced by 100 sec cumulative exposure of X-rays. Reoxidation does not occur in the dark at 100K. Significant photoreduction of the sample is to be expected at an early stage of data collection during a normal MX experiment. This observation has implications for much of the structural data currently available ascribed to the oxidised form of redox proteins. Crystals of the enzyme presoaked with nitrite displayed evidence of reoxidation of hemes during and after exposure consistent with continuous reduction of the substrate. However, even with an excess (100 mM) of substrate present, the enzyme does not return to a fully oxidised state and so the nature of the catalytic product remains unclear.

REFERENCES

(1) Richardson,D, Reyes,F, Pitts,K, Hemmings,AM, Seward,H, Thomson,A, Dobbin,P & Sawers,G (2003) Bacterial iron (III) respiration *J.Inorg.Biochem.*, 96, 67-67.

FIGURES

Figure1 (A) Absorbance spectra of an oxidised NrfA crystal before X-ray exposure (thick black line), after 10 sec exposure (grey line), 100 sec (dashed line), 200 sec (thin black line). **(B)** Absorbance spectra of reduced crystal (thin black line), after two successive 6 sec annealings (dashed line). The oxidised spectrum is also shown (thick black line). **(C)** Absorbance spectra of a NrfA crystal soaked in 100 mM sodium nitrite before X-ray exposure (thick black line), after 10 sec exposure (grey line), 100 sec (dashed line), 200 sec (thin black line). **(D)** Absorbance spectra of reduced crystal soaked in 100 mM sodium nitrite (thin black line), after two 6 sec second annealings. Oxidised spectrum is also shown (thick black line).

Figure 2. Change in absorbance on reduction of oxidised NrfA crystals with a 100 sec X-ray pulse. Magenta line - native enzyme crystal, green line - native enzyme soaked with 100 mM sodium nitrite. In each case, the X-ray shutter was opened for 100 sec. Monitoring continued for a further 900 sec with the shutter closed.

FIGURE 1

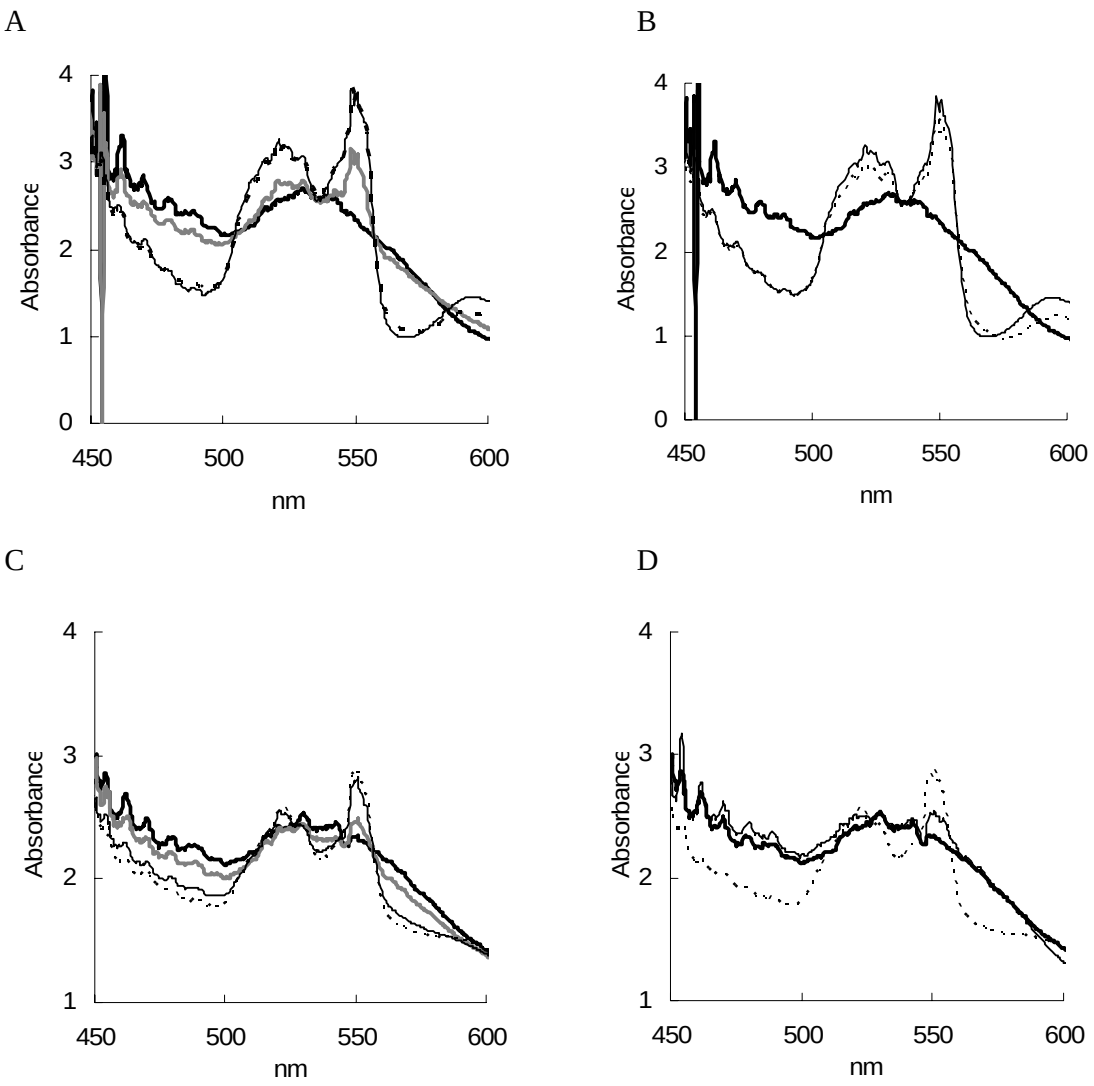

FIGURE 2