Resonance Raman Spectroscopy for In-Situ Monitoring of Radiation Damage

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Abstract. Radiation induced damage of metal centres in proteins is a severe problem in X-ray structure determination. Photoreduction can lead to erroneous structural implications, and in the worst cases cause structure solution to fail. Resonance Raman (RR) spectroscopy is well suited *in-situ* monitoring of X-ray induced photoreduction. However the laser excitation needed for RR can itself cause photoreduction of the metal centres. In the present study myoglobin and rubredoxin crystals were used as model systems to assess the feasibility of using RR for this application. It is shown that at least 10-15 RR spectra per crystal can be recorded at low laser power before severe photoreduction occurs. Furthermore it is possible to collect good quality RR spectra from cryocooled protein crystals with exposure times of only a few seconds. Following extended laser illumination photoreduction is observed through the formation and decay of spectral bands as a function of dose. The experimental setup planned for integration into the SLS protein crystallography beamlines is also described. This setup should also prove to be very useful for other experimental techniques at synchrotrons where X-ray photoreduction is a problem e.g. X-ray absorption spectroscopy.

Keywords: Resonance Raman spectroscopy, macromolecular crystallography, radiation damage, photoreduction.
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INTRODUCTION

X-ray induced radiation damage is one of the limiting factors in the structure determination of biological macromolecules at 3rd generation synchrotron sources [1]. While the global effects of radiation damage can be observed directly, for example via reduction of the resolution limit or $I/\sigma(I)$, specific structural damage often only becomes apparent following structure refinement. Alternatively, radiation damage can be monitored by micro resonance Raman (RR) spectroscopy. In this technique the excitation laser line is chosen to be in resonance with an electronic transition of a chromophore in the macromolecule, typically a cofactor or an aromatic amino acid residue. The resonance condition produces a strong enhancement, ca. six orders of magnitude, solely of the Raman bands of the chromophore, providing high sensitivity and selectivity. For metalloproteins, the positions and intensities of the RR bands are particularly sensitive to the redox state, coordination pattern and spin of the metal ion and, therefore, direct markers of radiation damage [2-4].

The most efficient collection of the Raman signal is achieved in back-scattering geometry, i.e. by means of a single microscope objective. Thus, in contrast to UV/VIS spectroscopy which requires two microscope objectives [5], backcattering RR spectroscopy is ideally suited for integration into the spatially restrictive environment at typical macromolecular crystallography diffractometer.

A potential drawback of RR, however, is that high dosis of laser radiation may induce photoreduction and high dose rates may cause thermal damage of the protein crystals.

Here we report a RR study of myoglobin (Mb) and rubredoxin (Rb) single crystals that aims to establish the conditions for the future integration of a Raman set-up into the SLS beam lines.

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EXPERIMENTAL

Protein crystals. Sperm whale Myoglobin (Mb) and Rubredoxin (Rb) crystals were chosen as model systems. Mb is a heme protein containing a Fe^{3+} ion in its stable oxidized state and is among the most studied proteins in RR spectroscopy. The iron is coordinated by four nitrogen atoms from the porphyrin ring and two oxygen atoms from a histidine and a water molecule. Rb is an electron transfer protein containing a single Fe^{3+} ion in a sulphur cluster. Both proteins are known for their sensitivity to photoreduction. Crystals of both compounds were grown according to standard procedures [6,7].

Resonance Raman spectroscopy. RR spectra were measured in back-scattering geometry using a confocal microscope coupled to a single stage spectrograph (Jobin Yvon, XY) equipped with a 1800 l/mm grating and liquid nitrogen cooled back illuminated CCD detector. Elastic scattering was rejected with Notch filters. The laser line was focused onto the surface of the crystal by means of a long working distance objective (20x; N.A. 0.35) resulting in a focal spot size of 10 μm diameter.

To avoid thermal degradation, crystals were cooled to 100 K using an open flow nitrogen cryostat. Mb samples were measured with the 413 nm line of cw Kr^+ laser (Coherent Innova 302) while for Rb the 514 nm line of a cw Ar^+ laser (Coherent Innova 70C) was used.

RESULTS AND DISCUSSION

Upon Soret band excitation, the RR spectra of heme proteins are dominated by the totally symmetric A_{1g} vibrational modes of the porphyrin ring. The high frequency region (ca. 1300-1700 cm^{-1}) displays the so-called marker bands that are particularly diagnostic for specific structural properties of the porphyrin such as the oxidation, spin, and ligation state of the heme iron, while the fingerprint region (ca. 200-500 cm^{-1}) reflects the interactions of the heme with the surrounding protein [4, 8, 9]. For all heme proteins the most intense RR band corresponds to the ν_4 mode which appears at ca. 1360 cm^{-1} for the ferrous form and shifts to ca. 1370 cm^{-1} in the oxidised state and, therefore, constitutes the most sensitive reporter of photoreduction processes.

We have investigated the threshold of laser induced damage in Mb crystals as a representative example of a heme protein prone to photoreduction. Figure 1 shows a typical RR spectrum of a Mb crystal measured at low laser power, ca. 200 μW. Despite the relatively long laser exposure (8 minutes), the RR spectrum of the crystal is identical to that of ferric Mb in solution measured in a rotatory cuvette under ideal conditions and no indication of laser-induced damage of the protein crystal is observed.

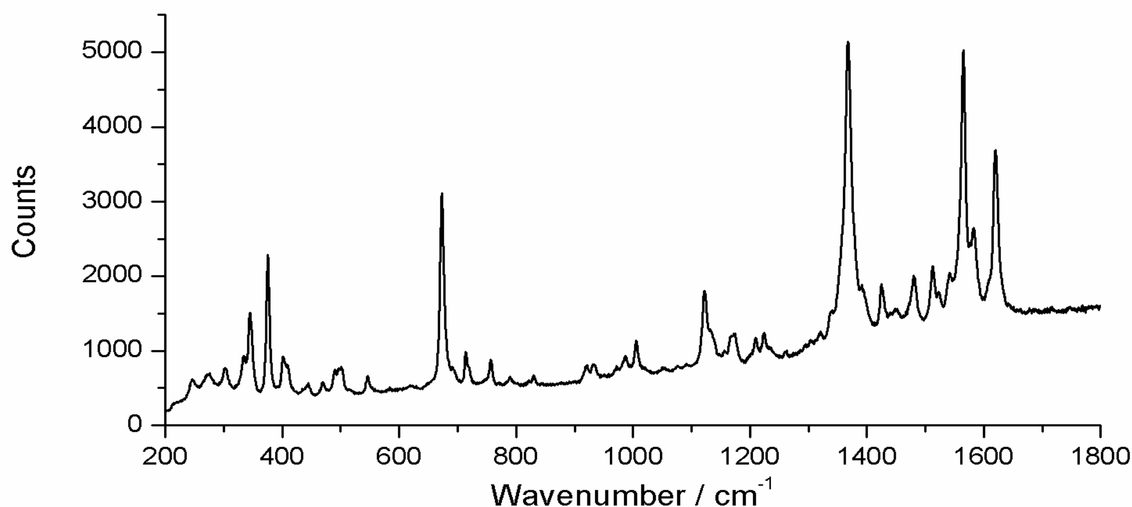

FIGURE 1. Resonance Raman spectrum from a Myoglobin crystal (excitation wavelength 414 nm). The total exposure time was 480s at a laser power of 0.23 mW.

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At substantially higher laser powers we observe the accumulation of photoreduced Mb that is reflected in the RR spectra by the appearance of a shoulder in the ν_4 band at 1357 cm^{-1}, as well as a shoulder in the ν_3 band whose position for ferric Mb is at 1480 cm^{-1} and shifts down to 1475 cm^{-1} in the photoreduced form. This effect is exemplified in Fig. 2A, where spectra recorded at 9 mW with spectral accumulation times of 2 seconds are shown as a function of the exposure time to the laser beam.

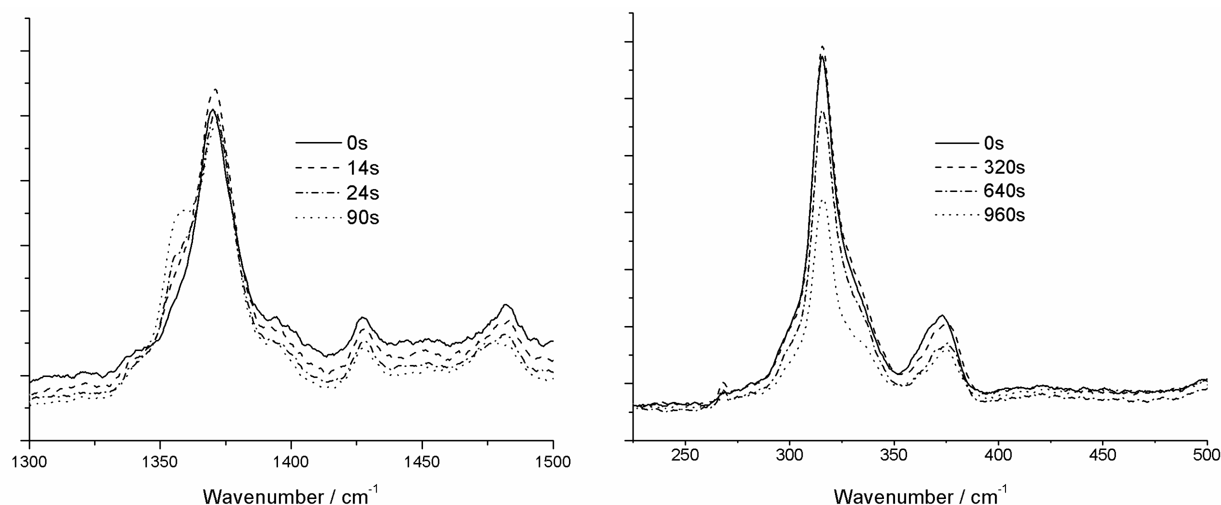

FIGURE 2. Band shifts in Mb (left) and Rb (right) spectra as a function of absorbed dose.

Similar experiments were performed for Rb crystals, as a representative example of proteins containing iron-sulphur clusters. As in the case of Mb, RR spectra do not undergo time dependent changes when measured at laser powers below 500 µW and display the typical features of fully oxidised Rb in its native state (data not shown).

At higher laser powers laser-dose dependent accumulation of photoreduced Rb becomes evident as a drop of the RR signal due to the lower extinction coefficient of the reduced form at the excitation line (Fig. 2B). For a laser power of 7 mW photoreduction of Rb starts to become evident only after irradiation times of ca. 600 seconds while RR spectra of good quality can be recorded in less than 20 seconds.

CONCLUSIONS AND FUTURE PERSPECTIVES

The test experiments show that RR is well suited to monitor the X-ray induced photo-reduction of metal centres in protein crystals. Complete RR spectra of good signal-to-noise ratio can be obtained in few seconds using sufficiently low laser powers that do not induce sample damage even after exposure times of several minutes.

Based on these results, we have started the installation of a micro RR setup at the SLS protein crystallography beamlines that is schematically shown in Fig. 3. The light source is a Kr^+ or Ar^+ laser, which is focused on the sample by the use of a microscope objective. Backscattered light is collected by the same objective and delivered to the Raman spectrometer. The objective used has a 10 x magnification and a numerical aperture of 0.28. The working distance is 33 mm. 1 mm holes were drilled in all lenses at the PSI workshop using water-cooled diamond hollow drills. Elastically scattered light is rejected by means of a notch filter placed in front of the spectrograph entrance slit. The spectrograph is an Andor Shamrock 303 equipped with an electron multiplying CCD and 3600 l/mm grating that provides a resolution of ca. 3 cm^{-1} at 413 nm excitation. The use of the on-axis geometry, with the X-ray beam passing through the drill hole in the middle of the objective, ensures that the focused laser spot probes the same sample volume which is exposed to the X-ray beam.

Installation of the RR device at the beamline will be finished by the end of July and the first experiments are scheduled for September 2006.

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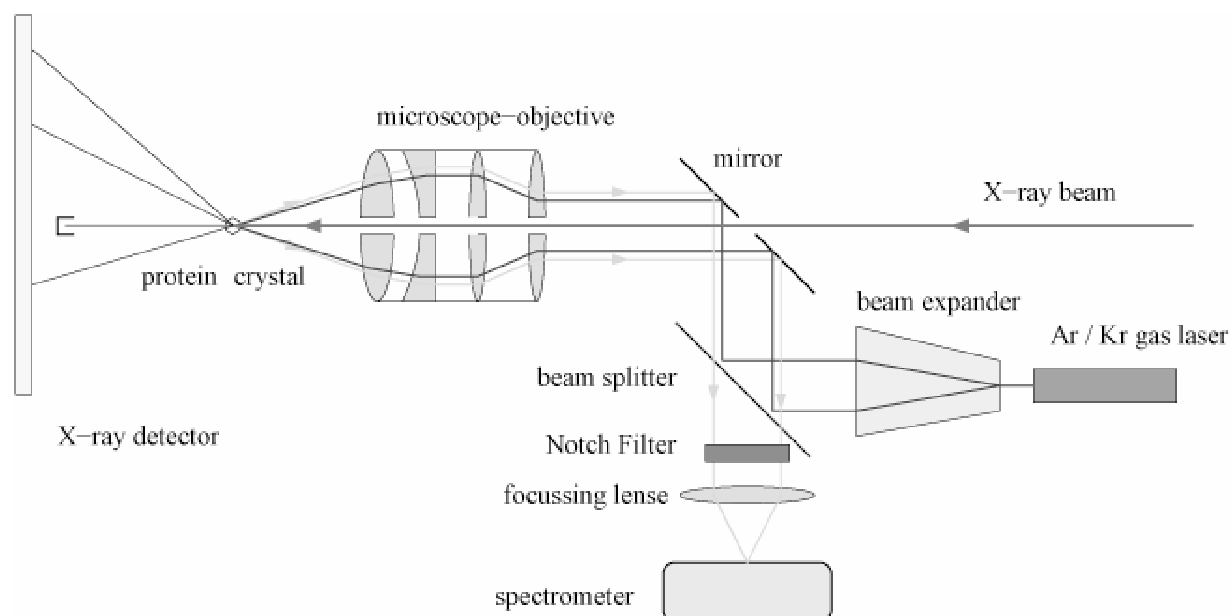

FIGURE 3. Experimental setup of a Raman microscope planned for in-situ monitoring of X-ray induced radiation damage at the SLS protein crystallography beamlines.

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