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Radiation damage in protein crystals examined under various conditions by different methods

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Investigation of radiation damage in protein crystals has progressed in several directions over the past couple of years. There have been improvements in the basic procedures such as calibration of the incident X-ray intensity and calculation of the dose likely to be deposited in a crystal of known size and composition with this intensity. There has been increased emphasis on using additional techniques such as optical, Raman or X-ray spectroscopy to complement X-ray diffraction. Apparent discrepancies between the results of different techniques can be explained by the fact that they are sensitive to different length scales or to changes in the electronic state rather than to movement of atoms. Investigations have been carried out at room temperature as well as cryo-temperatures and, in both cases, with the introduction of potential scavenger molecules. These and other studies are leading to an overall description of the changes which can occur when a protein crystal is irradiated with X-rays at both cryo- and room temperatures. Results from crystallographic and spectroscopic radiation-damage experiments can be reconciled with other studies in the field of radiation physics and chemistry.

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There have been significant advances in the understanding of radiation damage in protein crystallography over the past few years, with damage to protein crystals at room temperature as well as cryo-temperature being investigated. A variety of techniques to complement X-ray diffraction have been used, and studies with added scavengers to potentially mitigate radiation damage have been carried out. Those scientists involved in these studies are familiar with the detailed mechanisms and consequences of radiation damage. However, the majority of crystallographers require easily understood guidelines to carry out a successful structure determination in the presence of potentially severe radiation damage, together with advice regarding the identification of any structural artefacts resulting from this damage. An article in this issue (Holton, 2009) is written to fulfil this need. It is also a useful review of radiation damage studies and includes several interesting suggestions which would benefit from further detailed investigation. It therefore merits reading by both non-experts and experts alike.

The most relevant metric against which to measure damage is the absorbed dose (energy per unit mass) deposited in the sample. This depends on the properties of the incident beam and the energy deposited by this beam in the specimen, which in turn is determined by the absorption coefficient of the sample, *i.e.* its constituent atoms. Apparently simple terms such as the incident beam 'flux' or 'intensity' are not consistently defined across scientific disciplines. The potential confusion can only be avoided by careful definition and consistent use of the various terms, as emphasized in the article by Holton (2009). The accumulated dose received by a sample depends on the fluence (photons mm^{-2}) received by the relevant portion of it during the X-ray exposure. The fluence will depend on the flux density (photons s^{-1} mm^{-2}) and the exposure time. The flux density is likewise determined from the beam size and flux (photons s^{-1}). Unfortunately, the flux and beam sizes documented for various protein crystallography beamlines throughout the world are not as reliable as they need to be. Tabulated values can be out of date, calculated rather than measured, or simply not available. A reliable and convenient means of measuring the flux using pin diodes is given in this issue by Owen, Holton *et al.* (2009). Although the pin diodes interrupt the beam, they can be used to measure the beam before and after X-ray data collection and/or to calibrate the ionization chambers often used during the X-ray exposures. For this to be an effective strategy, values from the ionization chambers should be routinely recorded in the diffraction image headers, so they can then easily be converted into flux. The beam size itself is also not simple to define unless a top hat profile, combined with accurately measured apertures, is available. A Gaussian beam (for

example) will deposit energy non-uniformly into the crystal, causing for instance differential cell expansion and varying degrees of specific structural damage through the sample. This phenomenon exacerbates the problems caused by radiation damage since different parts of the crystal are being affected by varying amounts. Finally, an estimate of the dose deposited by the incident beam in the crystal is required. The latest developments of the program *RADDOSE* (Murray *et al.*, 2004) to calculate this conveniently for macromolecular crystallography are described in this issue by Paithankar *et al.* (2009).

Spectroscopic methods are increasingly being applied to the study of radiation damage in protein crystals, including combinations of X-ray diffraction, X-ray spectroscopy and optical spectroscopy (*e.g.* Hough *et al.*, 2008). In this issue, papers by McGeehan *et al.* (2009) and Owen, Pearson *et al.* (2009) describe experimental arrangements which enable routine measurements of this kind, together with (resonance) Raman spectroscopy. EXAFS can be used to measure metal ligand distances to an accuracy of 0.02 Å and is therefore sensitive to small movements of atoms surrounding a metal atom. X-ray spectroscopy in the XANES region is a sensitive measure of the oxidation state of the absorbing atom (*e.g.* a metal atom) and it is found that metal atoms can be reduced by very low absorbed doses of X-rays [*e.g.* approximately 3 MGy to reduce 50% of MnII centres in photosystem II (Yano *et al.*, 2005)] presumably because of their high electron affinity. Fast changes also occur in the redox state at other centres, and these changes can be monitored by UV or visible spectroscopy. IR and Raman spectroscopy are sensitive to bond breakage and formation, ligand binding and conformational change. Finally, X-ray diffraction is only sensitive to larger movements of atoms or groups of atoms and is a technique which monitors the structure averaged over both time and many unit cells. Apparent discrepancies when monitoring radiation damage with different techniques can be explained by the fact that the changes are being observed on different length scales or due to alterations in the electronic state. Many of the movements originating from radiation damage and in principle observable by X-ray diffraction are largely suppressed at cryo-temperatures. This can lead to the false conclusion that the relevant atoms are free of changes in oxidation state. The same consideration provides the reason for the success of cryo-methods in 'suppressing' radiation damage in protein crystals.

The course of radiation damage in protein crystallography will be different for the various temperatures (*e.g.* room temperature, 100 K, 20 K) which are routinely employed, and will depend on the presence or absence of scavenger molecules. A description of the various processes could include the following events.

The primary event is the creation of a photoelectron, created when a photon of around 12 keV is absorbed by a light atom. The photoelectron has a path length of a few micrometres and, for small crystals, can escape, thus causing less damage than if all the energy was deposited in the crystal (Nave & Hill, 2005; Cowan & Nave, 2008). While in the crystal, the photoelectron scatters inelastically off surrounding atoms creating several hundred secondary electrons and positively charged centres (O'Neill *et al.*, 2002). These secondary electrons are mobile, even at 100 K (Jones *et al.*, 1987). They will be attracted preferentially to sites of high electron affinity causing, for example, reduction at metal centres and more general changes in oxidation states of atoms, adding to specifically electron deficient functional groups throughout the protein. Many of these changes can be observed by a variety of spectroscopic techniques [*e.g.* UV/Vis spectroscopy (Beitlich *et al.*, 2007; McGeehan *et al.*, 2009), Raman (Carpentier *et al.*, 2007), XAS (Corbett *et al.*, 2007) and EPR (Utschig *et al.*, 2008)]. When trapped in water, the hydrated (or more generally solvated) electrons give a characteristic broad optical absorption spectrum (Ershov & Pikaev, 1968). This spectrum of the hydrated electron builds up during the X-ray exposure but undergoes partial decay (McGeehan *et al.*, 2009) when the beam is switched off as some electrons recombine (*e.g.* with the various positively charged holes).

Fisher & Devlin (1995) have previously investigated the mobility of protons at cryo-temperatures. For both electrons and protons, tunnelling mechanisms are a possible way of surmounting any energy barriers which cannot easily be overcome by thermal vibrations at cryo-temperatures. Tunnelling of protons has been implicated in the mechanism of some enzyme reactions (Masgrau *et al.*, 2006).

In this issue, Meents *et al.* (2009) postulate that changes in bond lengths observed in a peptide are due to hydrogen abstraction from the amino acids. These hydrogen atoms could be abstracted for example by radicals produced nearby such as hydroxyls or other hydrogen atoms, or by dissociative electron capture.

Large movements of other atoms would be suppressed at 100 K because the amorphous solvent present in protein crystals at cryo-temperatures is a glass (*i.e.* has the structure of a liquid but with rigidly bound atoms). However, local flexibility will be present even at 100 K, so small movements could occur. Such movements are a pre-requisite for identifying damage by techniques such as X-ray diffraction and EXAFS (which is sensitive to smaller movements of the atoms than is X-ray diffraction). The rather unpredictable and ill-defined nature of 'flexibility' means that it is difficult to develop a correlation between the environment of a residue (*e.g.* exposed to solvent or buried) and the susceptibility to damage (if defined as observable by X-ray diffraction). This is an area which remains to be explored more thoroughly. Recent results from acetylcholinesterase crystals irradiated at 100 K and 150 K have shown this to be a fertile area for obtaining dynamic information on proteins which can elucidate biological function (Colletier *et al.*, 2008).

In some cases the energy barriers could be too high to be surmounted at reduced temperatures (*e.g.* 20 K), thereby demonstrating an apparent decrease in radiation damage at these lower temperatures. There is evidence from both X-ray diffraction and EXAFS (Yano *et al.*, 2005; Grabolle *et al.*, 2006; Corbett *et al.*, 2007; Chinte *et al.*, 2007; Meents *et al.*, 2007) of

less radiation damage between 7 and 40 K compared with 100 K. In principle, a metal atom could be reduced with minimum movement of the surrounding atoms and it might not be expected that additional protection would be conferred at 40 K compared with 100 K. However, significant protection (a factor of 30) has been observed as monitored by XANES measurements (Corbett *et al.*, 2007). The explanation given by Corbett *et al.* (2007) is that 'the electrons generated by X-ray radiolysis are randomly distributed with respect to a metal site and therefore only a small subset would likely be optimized for an athermal reaction'.

The introduction of scavengers for the more mobile species (electrons and protons) could be expected to reduce radiation damage at cryo-temperatures and there is some evidence of this for both specific damage and non-specific damage (Kauffmann *et al.*, 2006; Murray & Garman, 2002; Southworth-Davies & Garman, 2007; Holton, 2007; Borek *et al.*, 2007). As described above, UV/Vis spectroscopy is a sensitive probe of metal oxidation states. In work reported in this issue (Macedo *et al.*, 2009), this has been used to search for scavengers which might slow the rate of reduction in metalloproteins. One candidate was found to be effective, but still did not allow a complete diffraction data set to be collected from the oxidized form of the protein.

The use of scavenger molecules is also attractive at room temperature as they have the potential to interact with many more mobile species than is the case at cryo-temperature. Recent studies at room temperature have yielded some promising results, with significant reductions in the intensity loss in the presence of two scavengers. Interestingly there appears to be a linear decay of the intensity with dose in the presence of scavengers as opposed to the normally observed exponential decay at room temperature. This observation, and its implications for the mechanism of the intensity decay at cryo-temperatures, is discussed in this issue (Barker *et al.*, 2009).

The non-specific damage results from increasing ionization and radical formation at a multitude of sites rather than the identifiable vulnerable sites which are damaged at an early stage. The resultant increase of disorder owing to the movement of many atoms leads to a loss of resolution with increasing dose. This can be referred to as global damage. For the global damage effects, there is much less variation in the susceptibility of protein crystals at 100 K compared with crystals at room temperature. One of the issues still to be resolved is whether the dose required to reduce the intensity of the reflections (*e.g.* to half their initial value) follows a linear relationship with resolution (Howells *et al.*, 2005) or is best described by a linear increase in B factor with dose [as modelled in the program *BEST* (Bourenkov & Popov, 2006)]. This is discussed by Holton (2009). It is noted that the analysis of Howells covered a much greater resolution range than that assumed in the program *BEST* so there could be some sampling issues which explain this apparent discrepancy. Although both models imply that a single number to define the safe dose limit which would apply at all resolutions cannot be specified, the guideline of a 30 MGy dose limit given by Owen *et al.* (2006) is nevertheless useful.

Many of the events described for crystals at cryo-temperatures will also take place at room temperature, but they are normally overwhelmed by the large movements of damaged and reactive species which can occur during and after data collection at room temperature. Damage (if defined as a loss in resolution during a diffraction experiment) under these conditions is much harder to predict and accurate intensity monitoring combined with programs like *RADDOSE* and *BEST* becomes less useful. Following the observation of an inverse dose rate effect at low dose rates of 6–10 Gy s^{-1} (Southworth-Davies *et al.*, 2007), it could be anticipated that there would be an optimum dose rate at room temperature. At very high dose rate, small temperature rises in the uncooled specimen could occur, hastening the diffusion of damaging species. As the diffusion still occurs even if the beam is absent, too long a data collection time with a weaker beam could result in increased damage and a lower recombination rate of mobile and reactive species owing to their lower density. An additional factor, probably to which the inverse dose rate effect is attributable, is the timescale required for recombination of damaged species: a beneficial reaction to set against the adverse ones.

The above description of radiation damage is an attempt by the authors to summarize results obtained by many investigators. Some parts of the description are well established, others much less so.

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