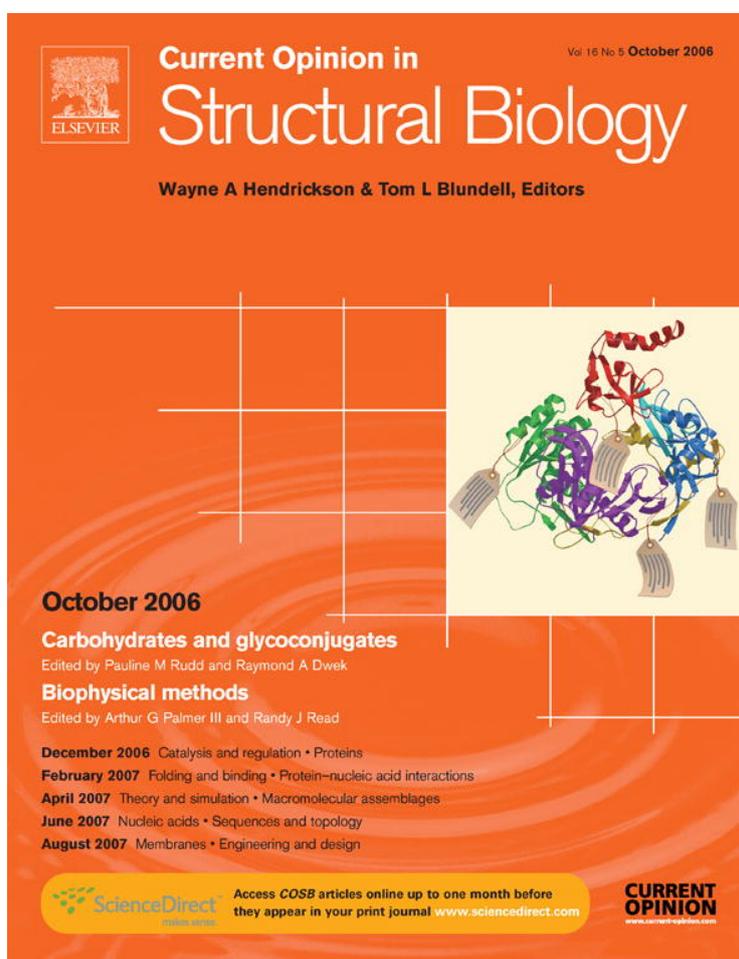


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Radiation damage in macromolecular cryocrystallography

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X-ray radiation damage to cryocooled (~100 K) macromolecular crystals has emerged as a general problem, especially since the advent of third generation synchrotron undulator sources. Interest in understanding the physical and chemical phenomena behind the observed effects is growing rapidly. The specific structural damage seen in electron density maps has to be accounted for when studying intermediates, and can sometimes be related to biological function. Radiation damage induces non-isomorphism, thus hampering traditional phasing methods. However, specific damage can also be used to obtain phases. With an increased knowledge of expected crystal lifetime, beamline characteristics and types of damage, macromolecular crystallographers might soon be able to account for radiation damage in data collection, processing and phasing.

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Introduction

Since the earliest days of macromolecular crystallography (MX), radiation damage has been a major concern because it limits the information that could be obtained from a single crystal. The first reported MX study on radiation damage was carried out in 1962 at room temperature on myoglobin crystals by Blake and Phillips [1]. They concluded that the damage was proportional to dose and might be structurally specific. They calculated that each absorbed 8 keV photon disrupted ~70 molecules and disordered another 90. With the development and widespread use of cryocrystallographic techniques for monochromatic MX, the problem appeared to vanish because data collection at ~100 K usually prolongs the crystal lifetime by a factor of ~70 [2], normally long enough for a complete dataset to be collected from a single crystal. With the advent of third generation

synchrotron undulator beamlines in the late 1990s, observations of radiation damage to cryocooled crystals became increasingly widespread and it is now posing a problem on most modern MX synchrotron beamlines. The obvious symptoms of radiation damage are fivefold: (i) decrease of diffraction intensity and resolution, (ii) increase in Wilson and individual atomic B-values (Figure 1a and b), (iii) increase in the unit cell volume, (iv) colour changes in the irradiated volume of the crystal, and (v) site-specific damage. The latter occurs in a well-defined order, starting with the breakage of disulphide bonds, followed by decarboxylation of aspartates, glutamates and the C-terminus, and then loss of the hydroxyl group from tyrosines [3–5]. Non-isomorphism within a data series is induced, and can obscure the dispersive signal necessary for success in multiple-wavelength anomalous dispersion (MAD) phasing, and the anomalous signal during a single-wavelength anomalous dispersion (SAD) experiment. Active sites and metal centres appear to be particularly susceptible [6–8,9*] and, thus, detailed biological interpretations can be misleading if no control experiments are carried out to account for radiation damage artefacts.

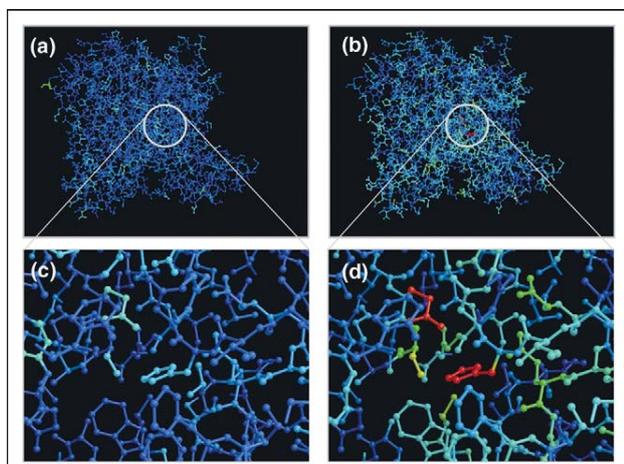
Over the past few years, there has been a revival of interest in the effects of radiation damage in MX. Here, we review a number of systematic studies on different aspects of radiation damage that are underway or have been reported recently. These investigations are increasing our understanding of the physics and chemistry behind the observed effects, and might help to predict the course of the damage. Software that accounts for the effects of damage is actively being developed.

The physics behind radiation damage

The basic physics behind radiation damage at the energies used for MX (5–17 keV) involves around 2% of the primary X-ray beam that interacts with the organic sample via three possible processes: (i) elastic scattering (which contributes to the desired diffraction pattern), (ii) inelastic scattering, and (iii) photo-electric effect, in proportions of 8%, 8% and 84% at 12.4 keV, respectively. Each photoelectron can result in the production of up to 500 secondary lower energy electrons, which then cause further damage.

It was initially thought that crystal heating by the beam might be responsible for radiation damage at cryotemperatures. However, detailed and sophisticated convective-heat transfer modelling studies [10*,11**] show that the external temperature rise for the flux density currently used on the more powerful beamlines (4×10^{14} photons $s^{-1} mm^{-2}$ of 13 keV) is not likely to exceed 15 K.

Figure 1



Acetylcholinesterase (AChE) and its active site coloured according to the relative increase in B-value between an initial and later dataset collected at (a, c) 100 K and at (b, d) 155 K. The colour code goes from blue (no increase), through green and yellow to red (large increase). At the solvent glass transition (155 K in trigonal crystals of AChE) the active site is the most radiation-sensitive part in the entire enzyme, as seen by the large B-value increase of the catalytic histine (red), the catalytic serine (yellow) and a nearby glutamic acid (red) [27*].

These models are being evaluated using an infrared camera [12*], and indicate that crystal heating is not a major problem unless much larger crystal absorption coefficients [13] or flux densities are used. Therefore, it seems unlikely that better cooling protocols (which use, for example, an open-flow helium gas stream) will mitigate damage solely because of reduced crystal heating [10*,11**]. Various studies have investigated other possible benefits of using helium at <100 K, but there is as yet no compelling evidence that helium cooling gives significant improvement in crystal lifetime. Helium is sometimes preferred over nitrogen because of the increased cooling rate [11**] and reduced X-ray background.

Does it make a difference if a certain dose is delivered over a short or long time interval? Blake and Philips [1] postulated that damage in protein crystals depends solely on the dose, irrespective of the dose rate. Sliz *et al.* [14] found no evidence for a dose versus dose-rate effect when inspecting data reduction statistics using dose rates up to 10^{15} photons s^{-1} mm^{-2} . Another study described similar observations when monitoring global data quality indicators, but concluded that there could be a second-order dose-rate effect on the basis of an analysis of electron density difference maps [15]. A small dose-rate-dependent lifetime decrease was observed by Owen *et al.* [16*].

Any systematic radiation-damage study relies on an accurate determination of the dose, expressed in energy deposited per mass (Gray [Gy] = Joules/kg). The dose

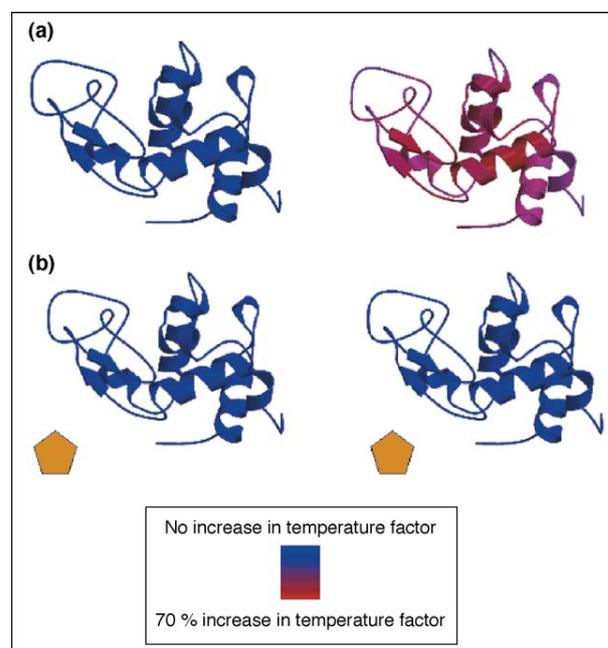
can be calculated from knowledge of the beam parameters (size, flux and profile) and crystal size and constituents, for example using RADDOSE [17]. Henderson [18] predicted that protein crystals would lose half of their diffracting power at 77 K at a dose of 2×10^7 Gy. This has recently been experimentally investigated, resulting in a dose value of $4.3 (\pm 0.3) \times 10^7$ Gy and a proposed limit of 3×10^7 Gy, which corresponds to the dose above which the crystal loses 30% of its initial diffracted intensity between 50 and 2.4 Å [16*]. The damage seems to be independent of the X-ray wavelength [19*]. Most dose calculations assume that the initial photoelectron deposits all its energy within the crystal, which is not true for highly energetic X-ray beams and microcrystals; this might result in 'reduced damage' [20*].

The unit-cell volume of cryocooled crystals normally increases with dose [3,4,13,21,22]. The cause of this phenomenon is not yet fully understood. Upon warming after irradiation, cryocooled crystals invariably 'bubble', releasing trapped gas, the identity of which is unknown. Interestingly, a room temperature study on microcrystals of lysozyme shows a unit-cell volume 'collapse' [23].

Radiation chemistry

The secondary lower energy electrons are mobile at cryotemperatures and can migrate to the sites of highest

Figure 2



Atomic B-value increase in the structure of hen egg white lysozyme following X-ray irradiation of (a) native crystals and (b) those co-crystallized with ascorbate [21]. (a) The left panel shows the values for the initial dataset from a series, and the right panel for a later dataset. The crystals with ascorbate (b) show no observable increase, whereas native crystals exhibit a rise of around 70%.

electron affinity, such as metal centres [9[•]] and disulfide bonds [4], long before the crystalline diffraction is lost. Disulfide bonds show different susceptibility within a given protein, emphasizing the role of secondary processes and local environment. Decarboxylation of acidic residues (Glu and Asp) has been explained as the result of oxidation [3–5]. Thus far, no clear evidence of other oxidative processes has been found.

Radical scavengers might reduce the damage rate. For example, sodium ascorbate has been shown to be effective in protecting lysozyme [21] (Figure 2), and several studies extending this work are now underway.

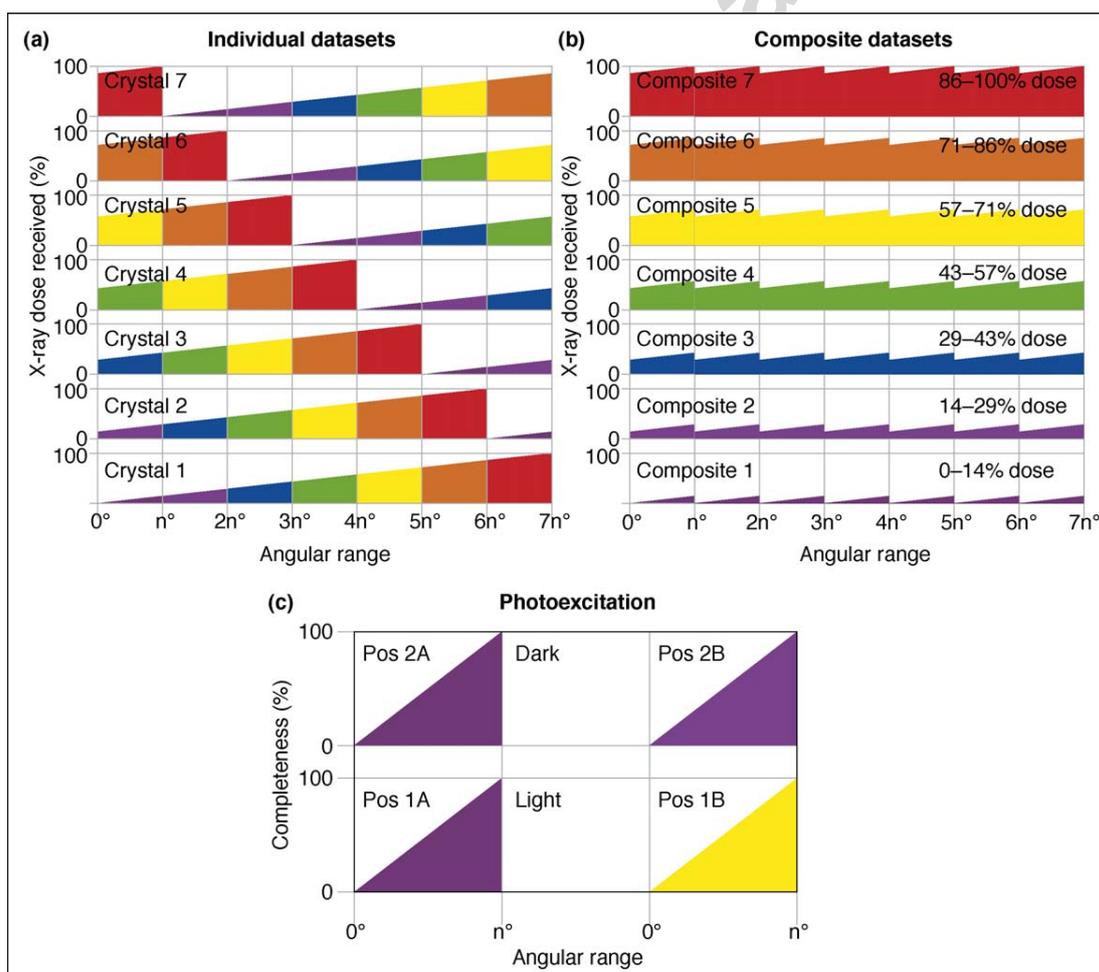
The exact chemistry of the rupture of C–I [22,24] and S–Hg [25[•]] bonds is, to our knowledge, still unknown.

Specific damage could shorten the effective lifetime of a crystal if, for example, crystal contacts are particularly susceptible [26]. Weik [27[•]] has shown how a macromolecule can adapt itself to a radiation-induced altered energy landscape when a crystal is held at the glass transition temperature (Figure 1).

Biological aspects of radiation damage

Radiation damage can be both a benefit and a curse in biological investigations. The X-ray beam induces reduction of metal centres and alters ‘stressed areas’ [5]. Active sites and chromophores are often stressed areas and, therefore, special care needs to be taken to deconvolute radiation damage artefacts from intermediate states. A variety of ingenious data collection strategies have been used to investigate these (Figure 3). The characterization

Figure 3



(a,b) Multicrystal data collection strategy according to [32[•]]. Complete datasets are collected from multiple (shots of) crystals starting at different oscillation angles. Composite datasets represent structures that received different X-ray doses. (c) Photoexcitation study with radiation damage control according to [30]. Two complete datasets were collected twice on different, isomorphous parts of the same crystal. $|F_{\text{pos2B}}| - |F_{\text{pos2A}}|$ gives a control of the radiation damage in the absence of photoexcitation. Thereafter, $|F_{\text{pos1A}}|$ and $|F_{\text{pos1B}}|$ are collected while the crystal is exposed to the excitation light. The $|F_{\text{pos1B}}| - |F_{\text{pos1A}}| + k(|F_{\text{pos2B}}| - |F_{\text{pos2A}}|)$ show the radiation damage corrected light-induced changes.

of the changing states in the crystal during X-rays exposure will benefit from complementary techniques, such as UV/Vis microspectroscopy [28], UV/Vis fluorescence, X-ray spectroscopy [9] and Raman spectroscopy.

A number of studies describe the careful deconvolution of X-ray damage from photoexcitation. These include work on bacteriorhodopsin [29], photoactive yellow protein [30], FAD reduction in DNA apophotolyase [31], photosystem II [9] and the photosynthetic reaction centre [7]. Other studies exploit the reductive power of the X-ray beam to elucidate enzymatic mechanisms. These studies include: the catalytic pathways of horseradish peroxidase [32] and cytochrome P450cam, the mechanism of a new class of nickel-containing superoxide dismutases [8], strain relief at the active site of phosphoserine aminotransferase [33], conformational switching in thioredoxin and trypanothione [34,35], *in situ* repair by the X-ray beam of a DNA lesion in duplex DNA bound to photolyase [36], and the reduction of Fe^{3+} in superoxide reductase [6].

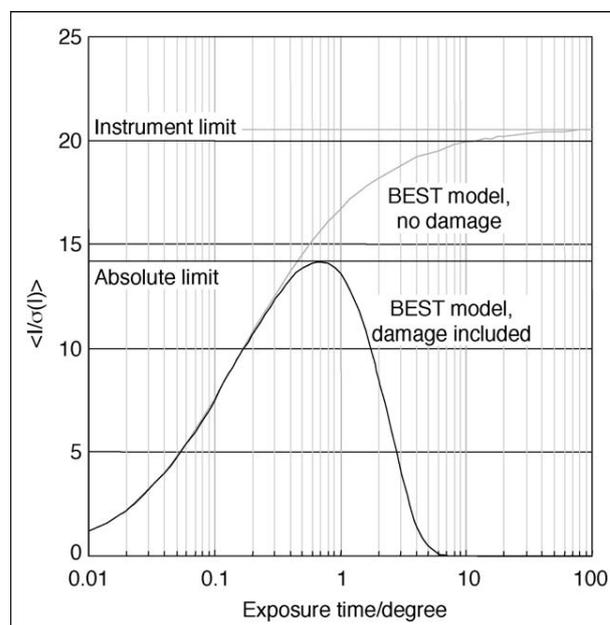
The structure factor as a function of dose

Traditionally, a scale and B-factor are applied to each recorded diffraction image to correct for radiation damage. This is insufficient to account for changes such as unit-cell volume increase, rotation and translation of molecules, and specific structural changes. The structure factor should ideally be treated as a function of dose, $F(\text{dose})$.

Quality control factors such as R-factors do not take radiation damage into account. A new 'decay R-factor' has been proposed [37] that can be used to assess the amount of radiation damage within a dataset. Raw data could be corrected for radiation damage during data reduction and scaling, provided that there is enough multiplicity [38–40]. Alternatively, correction can be achieved by refinement of heavy-atom parameters as a function of dose against multiple observations during phasing [41].

The function $|F(\text{dose})|$ pivotally depends on the ratio of crystal to beam size. This function shows a 180° modulo when the beam is much smaller than the rotating crystal, whereas it is independent of the oscillation angle when the uniform beam matches or is larger than the size of the crystal [37,42]. Automated data collection and processing projects such as DNA (www.dna.ac.uk) have started to record the relevant beam and crystal parameters, which could be coupled to, for example, RADDOSE [17]. The program BEST [43] (Figure 4) has recently been extended to take radiation damage into account, during the prediction of the best data collection strategy. This should eventually allow the user to calculate the true maximum obtainable resolution if a full dataset has to be collected from a single crystal.

Figure 4



$\langle 1/\sigma(I) \rangle$ ratio (unmerged data) as a function of exposure time, modelled with BEST [43] for a crystal of bovine trypsin in the resolution shell 1.75–1.70 Å. Two simulations were made for an $180 \times 0.5^\circ$ oscillation dataset, one without and one with taking radiation damage into account. The simulations were based on data taken at ID29, ESRF, and a dose rate of 10^5 Gy/sec.

Radiation damage can be both a threat and an opportunity for phasing [22,24,39,40,41,44,45,46,47]. The use of specific radiation damage to solve macromolecular structures has been named radiation-damage-induced phasing (RIP) [44]. Phasing programs are being adapted to take the particularities of RIP into account [41,47]. Specific damage has been used to identify correct molecular replacement solutions [48] and to orient the ligand vinblastine in a low-resolution map of tubulin [49].

The utility of specific radiation damage for phasing greatly depends on the contrast between general non-isomorphism versus localized changes. Popular phasing methods such as Se-MAD and S-SAD call for a better treatment of both forms of damage. It has been shown recently that UV-radiation damage can be used to enhance this contrast, thus benefiting RIP [46].

Conclusions and future prospects

The past few years have seen a revival of interest in MX radiation damage. This review has focused on publications from the past three years, but even for this limited period it is impossible to give a complete overview. Unfortunately, none of these studies promise to solve the problem of radiation damage; it is still best to improve the initial crystal quality. However, important progress has been made in the understanding of radiation damage,

resulting for example in its use to study catalytic pathways. Software packages have been and are being adapted, allowing users to account for the resolution decay of their crystals during data collection and to exploit the loss of anomalous scatterers for phasing. These trends will benefit from improved crystal and beamline characterization, as well as automation projects in which relevant parameters can be efficiently harvested. The next half-decade could show great advances in the treatment of radiation damage, thus paving the way for even larger challenges, such as the treatment of damage in soft [50**] and hard X-ray diffraction imaging.

Update

The November 2006 edition of the Journal of Synchrotron Radiation will include at least eight papers concerning topics presented at the Fourth International Workshop on X-ray Damage to Biological Crystalline Samples held at SPring-8 in March 2006. A recent paper [51] has reported on the effectiveness of some radical scavengers for MX.

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