Physical and chemical considerations of damage induced in protein crystals by synchrotron radiation: a radiation chemical perspective†

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Radiation-induced degradation of protein or DNA samples by synchrotron radiation is an inherent problem in X-ray crystallography, especially at the 'brighter' light sources. This short review gives a radiation chemical perspective on some of the physical and chemical processes that need to be considered in understanding potential pathways leading to the gradual degradation of the samples. Under the conditions used for X-ray crystallography at a temperature of <100 K in the presence of cryoprotectant agents, the majority of radiation damage of the protein samples arises from direct ionization of the amino acid residues and their associated water molecules. Some of the chemical processes that may occur at these protein centres, such as bond scission, are discussed. Several approaches are discussed that may reduce radiation damage, using agents known from radiation chemistry to minimize radical-induced degradation of the sample.

Keywords: radiation damage; chemical degradation; dose effects; scavengers; dose rate.

1. Introduction

Radiation-induced degradation of protein or DNA samples by synchrotron radiation is an inherent problem in X-ray crystallography (Gonzalez & Nave, 1994; Garman, 1999; Weik *et al.*, 2000). Radiation damage to protein crystals is expected to be random, occurring at all the amino acid residues. Damage that causes atoms to move through a structural change or a loss of a functional group will lead to gradual degradation of the resolution of the diffraction pattern. This degradation of the protein leads to a reduction of the lifetime of the crystal samples. Recently, evidence has been presented that the damage may be localized at specific sites within proteins (Burmeister, 2000; Ravelli & McSweeney, 2000; Weik *et al.*, 2000). The aim of this review is to present a radiation chemical perspective on some of the physical and chemical processes that need to be considered in understanding potential pathways leading to the gradual degradation of the protein crystals. In consideration of radiation-induced damage to crystals, it has been assumed that the aim is to reduce the influence of the damage on the diffraction patterns, leading to more detailed structural information on proteins. Several approaches are being used or being considered to reduce radiation-induced damage. These approaches include cryocrystallography (Glaeser *et al.*, 2000; Garman, 1999; Kuzay *et al.*, 2001), the addition of agents that intercept diffusible radicals produced in the vitreous support material (radical scavengers), understanding dose-rate effects, and the use of redox agents that may maintain the oxidation state of the protein. It was previously predicted that an X-ray dose of 2×10^7 Gy, delivered by 8 keV photons to small crystals at liquid-nitrogen temperatures, would generally result in destruction of the diffraction pattern of protein crystals (Henderson, 1990). This estimation of dose was based on the observation that an equivalent dose delivered by 100 keV electrons causes significant radiation damage during electron microscopy of proteins.

2. Damage induction in a protein crystal

A 100 keV electron, typical of an electron diffraction experiment, has a mean depth of penetration of ~100–150 μm, depending on the density of the crystal (ICRU, 1984). In comparison, the relative flux of 12 keV (1 Å) photons typically used in synchrotron diffraction experiments is only reduced by ~10% for a penetration depth of 300 μm in a crystal, although the attenuation does depend upon the density of the sample. Deposition of energy in protein crystals by 8–12 keV (1.5–1.0 Å) synchrotron photons causes ionization and other excitation processes. In the ionization process, an electron-loss centre is formed together with a secondary electron of several keV energy, typically ejected from an inner electron shell of an atom of the absorbing material. The energy of the ejected electrons depends on the energy of the incoming photon. Secondary electrons, with mean track lengths of a few micrometres (from 12 keV photons), will induce further excitation and ionization events within the atoms of the crystal, resulting in the formation of a number of electrons, many of which can then ionize other atoms within the crystal. Generally the energy of the photons used in crystallographic studies is significantly above that of the resonance bands of the *K*-shell electrons of the atoms in the molecules, so that the distribution of energy deposition events, to a first approximation, will be proportional to the relative electron densities of the different components of the crystal. The majority of the ionization events induced by a 12 keV photon result from the electron emitted with ~12 keV energy, which will produce ~500 ionizations, assuming 25 eV is required per ionization event. Initially, two radicals are produced, *i.e.* electron-loss centres and electron-gain centres. These events may occur directly in the protein, including its primary shell of hydration (direct events), or in the support material (indirect events), as outlined schematically in Fig. 1. The primary events, through direct ionization of the protein within the crystal, cannot be prevented, whereas the indirect effects may be

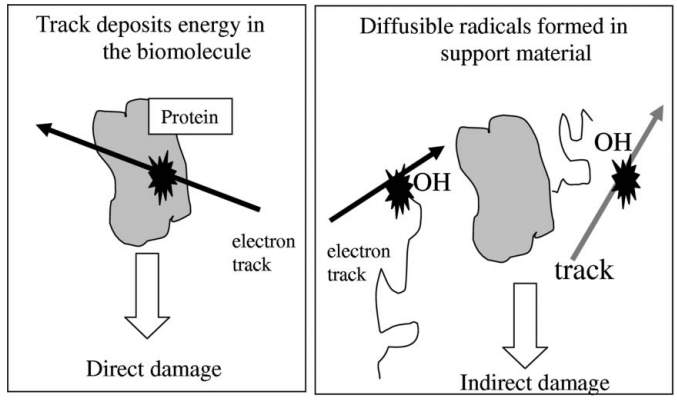

Figure 1
Direct damage and indirect damage caused by ionizing radiation. The indirect effects are minimized by the addition of scavengers, which intercept the diffusible radicals produced in the vitrified cryobuffer support.

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minimized through the addition of scavenger molecules, which intercept the diffusible radicals before they reach the protein. These interceptor molecules are termed radical scavengers.

In the presence or absence of radical scavengers, the electron-gain and electron-loss centres produced by the direct effects may recombine, leading to an excited state, which may or may not cause damage. These recombination processes are in competition with charge separation through migration. Migration of these centres may occur by tunnelling (essentially non-temperature dependent) or hopping (temperature dependent) to given sites in the protein, where the radical sites become localized.

Any species resulting from reactions between radicals and added scavenger molecules within the vitrified cryobuffer may be reactive and may subsequently diffuse. Depending on the temperature, they may be involved in interactions with the protein, causing damage, generally at the interface between the protein and the support material. Thus, a balance needs to be established between scavenging hydroxyl radicals and the production of reactive scavenger products. A high concentration of scavenger is required to prevent reaction of hydroxyl radicals produced by radiation near the protein. For example, a 1 *M* concentration of radical scavenger that reacts at a diffusion-controlled rate with a hydroxyl radical in water at ambient temperatures limits the mean diffusion distance of the hydroxyl radical to ∼1 nm (Roots & Okada, 1975). Lowering the temperature of the crystal during the irradiation will have a significant influence on the level of damage induced, as described below.

3. Effects of temperature and radical scavengers

For at least three decades, the chemistry of the direct effects of radiation on DNA has been investigated by carrying out irradiations with X-rays at low temperature, typically that of liquid nitrogen or helium (Becker & Sevilla, 1993). Irradiation at low temperature minimizes the diffusion of the radicals, namely hydroxyl radicals, electrons and H atoms, induced in the surrounding support material, because any radicals produced in the support material will be essentially immobilized; thus, the direct effects of radiation will predominate. Additionally, if high concentrations of radical scavengers are present in the support material, many radicals produced in the support material will be intercepted prior to their interaction with the protein. Many cryoprotectant agents are efficient hydroxyl radical and H-atom scavengers (*e.g.* glycerol, ethylene glycol, PEG, glucose and other sugars) and since the experiments are undertaken around liquid-nitrogen temperatures it is expected that the major pathway to damage induction in the protein molecules occurs through direct effects. Only radicals produced in the vicinity of the protein would be expected to react with the protein. Caution should be exercised in the use of radical scavengers, since the resulting scavenger radicals may react with protein residues, although at a level generally orders of magnitude lower than that of the primary radicals. The addition of scavengers such as acetone, which is an electron scavenger and therefore may reduce electron-initiated reactions, may bring some gains, although it is anticipated that at low temperature the gain would be limited as the direct effects of radiation should still dominate.

Lowering the temperature to around that of liquid helium (∼4 K) may provide some additional protection, although with DNA it does not lead to a significant (<10%) change in the concentration of DNA radicals observed by electron spin resonance (W. Bernhard, private communication). This small change implies that the number of recombination events is similar at liquid-nitrogen and liquid-helium temperatures. Electron migration in DNA will be minimized at these lower temperatures, so that electron tunnelling should be the main pathway by which species are produced at a distance from the original site of ionization (Messer *et al.*, 2000; Debije *et al.*, 1999). The radicals, resulting from the ionization and which escape recombination, will be trapped in the sample. Whether a significant improvement in protein crystallographic data quality could be obtained by cooling the crystal to below 100 K is as yet not known with sufficient certainty. At the lower doses the radicals resulting from the absorption events arising from a single photon will be separated from events initiated by absorption of other photons, until the photon absorption events are generated sufficiently close to one another, *e.g.* at high dose/dose rate (see next section).

4. Dose-related considerations

4.1. Heating effects

The important requirements for delivery of radiation dose to the protein crystal sample are firstly to maximize the signal to noise ratio of the data collected and secondly to minimize local heating. The latter effect will result in degradation of the sample. This can be achieved by increasing the irradiated sample volume and keeping the dose distribution within the irradiated sample as uniform as possible.

4.2. Dose-rate effects

The rate at which the dose is given also needs to be considered. For instance, interactions of the species produced by different radiation tracks will depend on the photon flux and the lifetime of the radicals in the protein, so that new chemistry (recombination reactions) may occur at the higher photon fluxes when the rate of radical formation is similar to or higher than that of radical loss. These recombination events involving inter-track events may or may not lead to protein degradation, reflecting dose-rate effects. A potential method to utilize the dose rate to advantage would be to use a very high photon flux, so that all the images are captured prior to the onset of the chemical stages of degradation (fragmentation of amino acids *etc.*). At low temperatures, this approach may require the capture of all the diffraction patterns on the picosecond to microsecond timescale and irradiation at very high photon fluxes.

4.3. Dose effects

The absorbed dose, D (Gy $\equiv$ J kg^{-1}), to the surface layer of a sample is the product of the mass absorption coefficient of the sample, μ/ρ (cm^2 g^{-1}), the photon energy, E (eV), the number of photons per second, n, and the irradiation duration, t (s), over the target area, A (μm^2), as described by the following equation:

$$D = \frac{(\mu/\rho)nteE}{A} \times 10^{11},$$

where $e = 1.6 \times 10^{-19}$ J eV^{-1} and the value of 10^{11} is a unit-conversion factor.

If $\mu/\rho = 2.6$ cm^2 g^{-1} (a value for an average protein crystal sample containing 50% solvent), the dose received from a beam of 12 keV photons over an 80 × 80 μm irradiated crystal area is 7.8×10^{-8} Gy photon^{-1}. So 2–3 × 10^{11} photons s^{-1} (equivalent to a flux of ∼4 × 10^{13} photons mm^{-2} s^{-1}, typical of beamline ID14-4 at the ESRF) incident on that surface for 10 s will deliver a dose of ∼2×10^5 Gy to the sample. Since the attenuation of 12 keV photons within a protein sample of 300 μm thickness is such that 91% of the photons do not interact, the dose through the sample will be almost uniform. This possibly allows scope for increasing the sample thickness with no increase in local heating, so improving the diffraction pattern signal.

To estimate the extent of damage to amino acid and protein structure we can convert absorbed dose to eV deposited per dalton (Da) using the conversion equation

$$D\ (\mathrm{eV/Da}) = 1.04 \times 10^{-8} D\ (\mathrm{Gy}).$$

If we assume that on average an amino acid has a molecular weight of 120 Da, that the protein consists of 400 amino acids and that on average a deposited energy of 25 eV results in an ionization event, then for a total dose of 10^6 Gy one ionization will be induced per 20 amino acid residues and thus 20 ionized amino acid residues per protein molecule. The probability that an ionization leads to permanent damage to the protein is as yet unknown, but if this probability is significant we would expect this level of ionization to lead to major conformational changes, with a resulting loss of information from the diffraction patterns. It should be noted that this calculation does not take into account any sample heating induced by the beam.

5. Chemical events – bond scission

The majority of the chemical studies on radiation-induced degradation of proteins have focused on irradiation of the proteins in aqueous solution at ambient temperature (Houvee-Levin & Sicard-Roselli, 2001). Under these conditions, water radicals (*i.e.* indirect effects) induce the majority of the damage. Only a few studies have investigated radicals produced during protein irradiation at low temperatures between 77 and 195 K, where the majority of the damage will arise from direct effects or involve radicals produced in the vicinity of the protein molecules (*e.g.* Sevilla *et al.*, 1979; Jones *et al.*, 1987; Filali-Mouhim *et al.*, 1997; Audette *et al.*, 2000; Houvee-Levin & Sicard-Roselli, 2001).

In general, reactions of water radicals (indirect effects) are varied. For instance, the interaction of hydroxyl radicals with aliphatic residues is very inefficient compared with the high reactivity of hydroxyl radicals with thiol and aromatic residues in proteins. In fact, the ease of oxidation of residues by hydroxyl radicals follows the order tryptophan > tyrosine > thiols > disulfides $\simeq$ aliphatic residues. Ionization of water molecules in the first hydration shell of the protein to give $H_2O^{+\bullet}$ may either lead to the oxidation of specific amino acid residues or the $H_2O^{+\bullet}$ may be removed by an ion–molecule reaction with a neighbouring water molecule. The probability of oxidation of amino acid residues by $H_2O^{+\bullet}$ is expected to mirror the ease of oxidation seen with the hydroxyl radical. In DNA, the water radical cation oxidizes the π system in preference to oxidation of the aliphatic sugar phosphate backbone (Yokoya *et al.*, unpublished data; O'Neill, 2001). At low temperatures (77–195 K) deprotonation of electron-loss centres induced in amino acid residues by direct effects or reactions with $H_2O^{+\bullet}$ may lead to persistent damage. The major radical produced at 77 K on irradiation of proteins in the presence of scavengers that remove diffusible electrons (Jones *et al.*, 1987) is shown in reaction (1),

$$\begin{array}{c} -\mathrm{CH}R-\mathrm{C(O)NH}-\mathrm{CH}R'- \\ \downarrow -\mathrm{e}^- \\ -\mathrm{CH}R-\mathrm{C(O)N}^{\bullet}-\mathrm{CH}R'- \quad + \quad \mathrm{H}^+. \end{array} \tag{1}$$

One-electron oxidation of carboxylic groups by electron-loss centres produced in the protein by direct effects of radiation at 77–100 K (Sevilla *et al.*, 1979; Ravelli & McSweeney, 2000) may lead to decarboxylation [reaction (2)],

$$\begin{array}{c} -\mathrm{CH}R-\mathrm{CO_2^-} \\ \downarrow -\mathrm{e}^- \\ -{}^{\bullet}\mathrm{CH}R \quad + \quad \mathrm{CO_2}. \end{array} \tag{2}$$

Carbonyl groups (Symons 1995; Garrison, 1987; Sevilla *et al.*, 1979; Filali-Mouhim *et al.*, 1997) and disulfide bridges (Burmeister, 2000; Ravelli & McSweeney, 2000) are two major sites for localization (trapping) of the electron, produced either by direct ionization of the protein or indirectly by ionization of water at 77–195 K. The electron-gain sites at the carbonyl group may undergo the following fragmentation reactions (Sevilla *et al.*, 1979; Filali-Mouhim *et al.*, 1997):

$$\begin{array}{c} -\mathrm{CH}R-\mathrm{C(O)NH}-\mathrm{CH}R'- \quad + \quad \mathrm{e}^- \\ \downarrow \qquad (3) \\ -\mathrm{CH}R-{}^{\bullet}\mathrm{C(O^-)NH}-\mathrm{CH}R'- \\ \downarrow \qquad (4) \\ -\mathrm{CH}R-\mathrm{C(O)NH_2} \quad + \quad {}^{\bullet}\mathrm{CH}R'-. \end{array}$$

These latter processes may lead to degradation of specific amino acid residues. Degradation of the samples may lead to conformation changes, the timescale of which with respect to the photon fluence, *i.e.* time of irradiation, may need to be considered.

Other sites of charge trapping are aromatic residues (*e.g.* tryptophan, tyrosine) and metal redox centres. The use of electron scavengers, *e.g.* acetone, in conjunction with the cryoprotectant agents may be worth pursuing to assess whether electron damage can be minimized by involving interception of electrons produced in the vicinity of the protein molecules. Minimization of interactions of diffusible electrons with DNA is seen at 77 K if electron scavengers are present in the support material (Becker & Sevilla, 1993).

The use of agents known to minimize radical-induced degradation in radiation chemistry, namely chemical repair agents, is a possible avenue to explore. For instance, electron donors (commonly known as anti-oxidants or reductants) may react with the electron-loss centres by donation of an electron. Thiols are also known to be radioprotective by electron donation to electron-loss centres, radiolytically induced in DNA at 77 K (for a review, see Becker & Sevilla 1993). This approach would require the use of high concentrations of electron donors; furthermore, the effects of lowering the temperature from 293 to 77 K are not known. In aqueous solution at ambient temperature, the use of electron donors such as ascorbic acid is known to 'repair' tryptophan radicals by transferring the radical site to the electron donor, the mechanism being based upon their redox potentials (Wardman *et al.*, 1989):

$$\begin{array}{c} \mathrm{Tryptophan}^{\bullet+} \quad + \quad (\mathrm{reductant})^- \\ \downarrow \qquad (5) \\ \mathrm{Tryptophan} \quad + \quad (\mathrm{reductant})^{\bullet}. \end{array}$$

Similarly, electron acceptors (oxidants) may 'repair' electron-gain centres by accepting the electron prior to fragmentation of the amino acid radical as shown in reaction (4):

$$\begin{array}{c} -\mathrm{CH}R^{\bullet}\mathrm{C(O^-)NHCH_2}R' \quad + \quad \mathrm{oxidant} \\ \downarrow \qquad (6) \\ -\mathrm{CH}R\mathrm{C(O)NHCH_2}R' \quad + \quad (\mathrm{oxidant})^-. \end{array}$$

6. Summary

It is predicted that at low temperatures the majority of the radiation-induced damage in proteins arises from their direct ionization. In this discussion it has been assumed that collection of X-ray diffraction data is generally undertaken at low temperatures. The following approaches, based on chemical considerations, may bring some benefit in reducing degradation of the resolution of diffraction patterns with increasing radiation dose.

(i) Addition of very high concentrations of agents that may 'shuttle' electrons into electron-loss centres or away from electron-gain centres in the protein. These approaches have been successfully applied with DNA irradiated at 77 K.

(ii) Addition of high concentrations of electron scavengers to complement the hydroxyl radical scavenging of the generally used cryoprotectant support material.

(iii) The use of very high photon fluxes so that the diffraction patterns are collected prior to any chemical degradation that affects the diffraction profiles. This approach may require the development of ultrafast detection methods and the delivery of doses of $\gg 10^7$ Gy within $\ll 10^{-9}$ s.

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