

# Abstracts

## SCAVENGERS IN MACROMOLECULAR CRYSTALLOGRAPHY: DO THEY HELP?

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Free radical scavengers and radioprotectants have long been suggested as a possible means of reducing the rate of radiation damage suffered by protein crystals. Early room temperature (RT) experiments seemed to show that styrene and PEG might have a positive effect on the dose tolerance of crystals, but the idea was not systematically pursued. We have previously reported that 0.5M-1 M ascorbate incorporated by cocrystallisation was effective in quenching the disulphide breakage in lysozyme (HEWL) crystals during 100 K data collection [1]. The screening of a large number of potential radioprotectants was then undertaken with an on-line microspectrophotometer using cystine and cysteine to model protein disulphide bonds and thiol groups respectively and observe any quenching of the disulphide radical anion peak. Evidence for the potential of ascorbate as a radioprotectant was strengthened, and 1,4 benzoquinone, 2,2,6,6-tetramethyl-4-piperidone (TEMP) and reduced dithiothreitol also showed promise [2].

Work at EMBL Hamburg has identified Nicotinic acid and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) as effective scavengers [3], although the validity of this work has recently been challenged [4].

In recent work to search for RT radiation damage mitigation strategies, three of these putative radioprotectants were tested for their ability to prolong HEWL crystal lifetime at 293 K. The results indicate that ascorbate and 1,4-benzoquinone are effective radioprotectants, whereas studies on TEMP were inconclusive. Ascorbate, which scavenges OH<sup>\*</sup> radicals ( $k_{OH} = 8 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ ) and electrons with a lower rate constant ( $k_{e-(aq)} = 3.0 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ ), doubled the crystal dose tolerance, whereas 1,4-benzoquinone, which also scavenges both OH<sup>\*</sup> radicals ( $k_{OH} = 1.2 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ ) and electrons ( $k_{e-(aq)} = 1.2 \times 10^{10} \text{ M}^{-1}\text{s}^{-1}$ ), offered a 9 fold increase at the dose-rates used, judged by decay in total intensity [5].

Most interestingly, the universally previously observed exponential form of the RT diffraction intensity decay was modified by the addition of scavengers to become linear as is observed at 100 K without scavengers present. The underlying radiation damage mechanisms were elucidated by these results, which enabled postulates to be made on the radical species causing the majority of the damage at 100 K [5].

The RT results with Ascorbate and 1,4 benzoquinone led us to seek a radioprotectant which only scavenges electrons, with the aim of separating the effects of electron and OH radical damage. New results from data collections at 100K using the electron scavenger, sodium nitrate, soaked into HEWL crystals will be discussed [6].

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[3] Kauffmann, B., Weiss, M. S., Lamzin, V. S. & Schmidt, A. (2006). *Structure* **14**, 1099-1105

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[6] de la Mora, E, Carmichael, I & Garman, EF. In preparation.

## **TO SCAVENGE OR NOT TO SCAVENGE: THAT IS THE QUESTION**

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Analysis of a series of diffraction data sets measured from four native as well as four nicotinic acid-soaked crystals of trypsin at 100 K shows a high variability in radiation-sensitivity among individual crystals for both nicotinic acid-soaked and native crystals. The level of radiation-sensitivity and the extent of its variability is statistically indistinguishable between the two conditions. This suggests that this potential scavenger does not have any statistically significant effect on the amount of radiation damage incurred in the crystals on X-ray irradiation. This is in contrast to previous results [1] where only one crystal specimen was used for each condition (native and nicotinic acid-soaked).

[1] Kauffmann et al. (2006), *Structure*, 14, 1099–1105

## **A NOVEL METHOD FOR ATTENUATING X-RAY DAMAGE TO PROTEIN CRYSTALS**

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The advent of third-generation synchrotron beamlines has stimulated renewed interest in discovering new ways to attenuate radiation damage to cryocooled macromolecular protein crystals. Accumulated damage decreases the signal-to-noise ratio of collected diffraction images, undermines traditional phasing experiments, and propagates structural changes throughout the proteins within the crystal lattice. We have developed a new method to introduce free-radical scavengers into protein crystals that increases the effective dose-dependent lifetime of several different types of protein crystals on two synchrotron beamlines. Preliminary results will be presented.

## SPATIAL DEPENDENCE AND MITIGATION OF MX RADIATION DAMAGE BY FOCUSING

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Recently, strategies to reduce primary radiation damage have been proposed which depend on focusing x-rays to dimension smaller than the penetration depth of excited photoelectrons (PE's). For a line focus as used here the penetration depth is the maximum distance from the irradiated region along the x-ray polarization direction that the PE's penetrate. Reported here are measurements to determine the penetration depth and magnitude of PE damage excited by 18.6keV photons in a lysozyme crystal. It is found that the x-ray dose has a significant contribution from the crystal's 9 w% solvent NaCl atoms. The 15.8 keV PE's of the Cl atoms and their accompanying 2.8 keV localized dose from the decay of the resulting excited atoms more than doubles the dose deposited in the focused region because of a much greater cross section and higher energy of the excited atom, degrading the mitigation of radiation damage. Eliminating heavier atoms from the solvent will significantly improve the mitigation of damage by focusing. The experimental results showed the penetration depth of ~17 keV PE's is 1.36+/- 0.2  $\mu\text{m}$ , well below previous theory estimates. Such a small penetration depth raises challenging technical issues to mitigate damage by focusing because the optimum requirements are gaussian line focused beams with sigma of 0.15  $\mu\text{m}$  and distance between lines of 1.8  $\mu\text{m}$  to reduced damage by a factor of 2.

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[2] John A. Cowan & Nave, C. (2008). *J. Synchrotron Rad.* **15**, 458–462.

[3] Stern, E. A., *et al.*, *Acta Cryst.* (2009). **D65**, 366–374

## **PRINCIPLES OF RADIATION DAMAGE IN COHERENT X-RAY DIFFRACTION IMAGING**

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In this presentation we will briefly review the history and principles of Coherent x-ray diffraction microscopy (CXDM) and report some of the progress at the Advanced Light Source (ALS) in 3D imaging experiments in which radiation-hard objects of about 2 microns width have been imaged at about 10 nm resolution. We also report progress in the more difficult task of applying CXDM to imaging frozen-hydrated biological samples. The motivation for developing a new imaging method must always be that it will enable important and otherwise impractical new investigations to be carried out. In the case of CXDM the new challenge that is already beginning to be met is to image objects in the one to about twenty micron size range in three dimensions at a resolving power of around  $1000 \times 1000 \times 1000$  voxels. Such objects are too big for 3D imaging at good resolution in the transmission electron microscope while their interesting features are too small to be imaged in the light microscope. Nevertheless, success in this type of imaging is expected to have a decisive impact on the growing activities of nano-science and nano-technology.

Of course, any plan to image biological materials with ionizing radiation must confront the question of radiation damage. We have approached this in two ways. On the one hand we have begun a program of experiments on model samples based on yeast cells and we will report successful imaging of both freeze dried and frozen hydrated samples. We also show how the onset of damage-induced blurring of the images of these samples might be observable experimentally. We note that these samples have proved considerably more difficult to deal with than materials-science samples and we discuss some of the difficulties we have encountered.

On the other hand we have also done calculations to try to establish where the fundamental limits lie. The calculations are based on the so-called "Dose fractionation theorem" which was introduced in 1976 by Hegerl and Hoppe. We explain our use of the theorem to estimate the ultimate damage-limited resolution of the CXDM technique. We make a comparison and reconciliation of our calculations with those of Shen et al which are based on different assumptions. We conclude from this study that Rose-criterion image quality can be obtained for samples consisting of natural protein against a background of vitrified water down to a resolution of around 10 nm. We discuss ways in which one might try to overcome this limit by contrast enhancement and/or the use of samples with reduced information content such as fibers.

## **PUSHING AGAINST DAMAGE LIMITS IN X-RAY MICROSCOPY**

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Radiation damage sets an ultimate limit on achievable resolution in microscopy with ionizing radiation (such as X rays and electrons). We describe methods for evaluating image quality in minimum-dose experiments, and use that measure to compare the damage-resolution coupling of several x-ray imaging methods<sup>1</sup>. We next describe the status of x-ray diffraction microscopy as a dose-efficient method for biological imaging, and highlight recent results with room temperature and frozen hydrated specimens<sup>2</sup>. Finally, we describe efforts to develop next-generation cryo microscopes, and show initial investigations of damage limits in full-field cryo x-ray microscopy.

[1] X. Huang *et al.*, *Optics Express* **17**, 13541 (2009)

[2] X. Huang *et al.*, *Physical Review Letters* **103**, 198101 (2009)

## SINGLE MOLECULE IMAGING BY XFEL: GETTING AND PROCESSING SIGNALS FROM DECAYING MOLECULES

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New intense X-ray free-electron laser light sources offer a new possibility in imaging single biological macromolecules. If realized, it may permit to remove a current bottleneck of crystallization in the X-ray crystallographic structure determination. Problems to be solved before the realization of this possibility originate mainly from the extreme weakness of the scattered light from a single molecule. Intense X-ray light used to overcome this problem, however, causes to destroy the target in a single shot of pulse of X-ray light. Structure determination must be done for a sample which decays during the exposure to the pulse of light used for obtaining necessary information. Another problem due to the extreme weakness is the quantum noise. Algorithms for structure determination must be developed to process the experimental data immersed in the quantum noise. We are carrying out two types of works aiming to solve these problems. First is simulation of the decay process of biological macromolecules due to exposure to the intense X-ray light. Second is development of algorithms for structure determination from experimental diffraction intensity data covered by the strong quantum noise.

To simulate the decay process, we treat a biological macromolecule at the moment as a spherical cluster of solid state density carbon atoms. Intensity of incident X-ray flux is assumed to be in the range of  $10^{18}$  to  $10^{22}$  photons/pulse/mm<sup>2</sup>. Photon energy is assumed to be in the range of 10 to 20 keV, which corresponds to the wave length of 0.12 to 0.06 nm. As elementary processes we consider the following. (1) Ionization due to photon absorption. (2) Compton scattering. (3) Auger process. (4) Electron impact ionization process, where an unbound electron produced by various processes collides with an atom/ion to produce another new unbound electron. (5) Field ionization process, which takes place when the cluster becomes highly charged after emitting many electrons and a very strong electric field is produced near the surface of the cluster. An atom/ion in such a strong field can no longer keep its upper shell electrons and emit them by a tunnelling process. Among these processes, Auger process, one of the secondary processes, becomes important in the time range of 10 fs. To minimize the effect of radiation damage to data of X-ray elastic scattering to be used for structure determination, it is highly desirable that pulse of incident beam be shorter than 10 fs. Results of simulations carried out for various sets of parameters indicate that for high incident fluxes, field ionization process becomes very pronounced.

A two-step algorithm is developed for reconstructing three-dimensional diffraction intensity of a globular biological macromolecule from many experimentally measured quantum-noise limited two-dimensional (2D) patterns on Ewald sphere, each for unknown orientation. First is a classification of 2D patterns into groups according to similarity of direction of incident X-ray with respect to the molecule and an averaging within each group to reduce the noise. Second is a detection of common intersecting circles between the signal-enhanced 2D patterns to identify their mutual location in the three-dimensional space. The developed algorithm enables to detect signal for classification in such a noisy experimental photon-count data as low as  $\sim 0.1$  photons per an effective pixel. Wave-number of a pixel giving such a limiting photon-count defines the attainable structural resolution. From this fact, resolution attainable by this new technology can be derived for structure-known molecules by calculating their structure factors. To estimate attainable resolution even for structure-unknown molecules, a concept of standard model protein is developed. The model protein is characterized by only two parameters, molecular volume and length, and has an explicit wave-number dependence of the structure factor which is an approximation no worse than a factor 5 to those of real molecules with the same two parameter values. Resolution expected for this model protein is obtained explicitly as functions of intensity of incident X-ray and the size of the molecule. This relation can be used for designing machine and target parameters for the new technology to come.

## **DAMAGE REDUCTION USING FEMTOSECOND XRD - MEASUREMENTS FROM PROTEIN NANOXTALS.**

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Single-shot transmission diffraction patterns have been obtained from protein nanocrystals at 2 kV using the LCLS over a range of pulse durations from about 3 to 200 fs (see [1] for a review of the method). These extend to a wavelength, and detector-size-limited, resolution of about 8 Angstroms. Remarkably, we find little evidence for loss of resolution at 200 fs. Damage mechanisms will be discussed [2]. Membrane protein nanocrystals were prepared in the Fromme lab at ASU and flown across the LCLS beam in a liquid buffer jet in a continuous stream [3], generating 30, 2K x 1K diffraction patterns per second for several days. Strategies for analysing this 20 Tbyte data mountain will be addressed, and methods of phasing these "stills" or "partials" discussed. These preliminary results suggest an entirely new approach to the problem of damage reduction, in which the traditional nexus between dose and damage is finally broken for sufficiently brief pulses.

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[3] J. Phys. D Appl Phys. 41, 195505 (2008) and Shapiro et al J. Synch. Res. 15, 593 (2008).

## **IONIZING RADIATION INDUCED CHEMICAL DYNAMICS STUDIED WITH ULTRAFAST X-RAY LASER RADIATION**

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Early commissioning of the Linac Coherent Light Source (LCLS) has demonstrated x-ray laser radiation at 8 keV with sub-10 femtosecond pulse durations and mJ pulse energies. This unprecedented peak intensity presents a unique opportunity to impulsively initiate the radiative transformation of molecular systems and investigate the fundamental chemical events that generate x-ray beam damage. The presentation will provide an overview of the properties of the LCLS and the experimental facilities that could be utilized to study radiation chemistry in real time.

## RADIATION DAMAGE AND PRACTICAL ASPECTS OF DATA COLLECTION

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Software program BEST (1) for optimal planning of X-ray diffraction measurements from protein crystals is based on modeling the statistical results of data collection. A few initial images are required to make predictions. To describe the radiation damage, two-parametric model is used which accounts for both the average intensity decay and radiation-induced non-isomorphism (2). BEST implementations of radiation damage-driven data collection optimization are being successfully used in everyday practice at the synchrotron beamlines over the last three years; BEST is implemented in a number of automated collection and online data analysis systems like EDNA (3). In most applications, BEST is used to optimize "native" data collection to achieve the highest possible resolution. The optimization is based on the "global" damage model only, but allows for the dose constraints to be applied whenever the data on the radiation sensitivity of a particular "specific" site is available. Optimization is particularly useful for difficult, e.g. very anisotropically diffracting crystals. Choosing an optimal conditions for high resolution data collection often compromises the data quality (both the signal-to-noise and completeness) in the low resolution shells. We introduced a dedicated algorithm for planning separate low resolution path using a small percentage of the total exposure dose required for high resolution data collection, irrespective of the detector overloads. This improves the overall data quality significantly. Yet another specialized optimization strategy, aiming in minimal noise in the Bijvoet differences, is used for collecting highly accurate SAD data. The software is being actively used in crystal screening procedures, where it helps to provide an objective integrated scores for the sample quality - like achievable resolution or signal-to-noise. Newly coming applications involve scanning diffraction experiments, where similar approaches are used for choosing the best spot on a larger heterogeneous samples. Furthermore, in many applications, though still with some involvement of human intelligence, the software was extremely helpful in designing an optimal strategy for collecting a data set using small beams and the multiple crystal centerings. We will present current developments aiming in generalization and automation of such procedures, as well as in the optimized multi-crystal data collection.

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[2] Bourenkov, G. P., Bogomolov, A. & Popov, A. N. (2006). *Fourth International Workshop on X-ray Damage to Biological Crystalline Samples, March 7-8th, Spring-8, Japan*

[3] Incardona, M.F., Bourenkov, G.P., Levik, K., Popov, A.N. & Svensson, O. (2009). *J. Synchrotron. Rad* **16**

## EXPERIMENTAL PROCEDURE FOR CHARACTERIZATION OF MACROMOLECULAR CRYSTAL RADIATION DAMAGE

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Radiation damage limits the information that can be obtained from a single crystal. It induces specific chemical modifications in the protein and so mislead the biological interpretations inferred from an X-ray structure. Effects of radiation damage must be taken into account when designing an optimal data collection strategy.

The most pronounced effect of radiation damage for macromolecular crystals is a resolution-dependent intensity decay. The decrease of average intensity as a function of absorbed dose can be characterized mainly by a linear increase in the Debye-Waller factor. In general, one could expect approximately the same rate of decay for all protein crystals. However, there is a common opinion that some samples are more sensitive to radiation than others. In practice, apparent deviations in radiation-sensitivity often do not arise from a specific feature of a crystal structure but rather from a mismatched beam size, mis-calibration or other technical problems. If such an example is to occur, it could be resolved by applying reliable procedure which can recalibrate the radiation damage model in a preliminary experiment involving a sacrificial sample or a part of the sample.

The program BEST [1,2] has been extended to perform an empirical estimation of radiation damage sensitivity by sacrificing a crystal. From a few initial diffraction images, BEST software defines a *collect* and *burn* strategy plan guaranteeing that the information needed for crystal characterization will be available up to maximum absorbed dose used for the test measurement. Thus, by successive cycles of collecting sub-wedges of data and *burning* the crystal, the average diffraction intensity, along with the B-factors, are determined and displayed as function of crystal absorbed dose.

This functionality aim to be completely transparent to the user. Hence, these developments are being implemented in the context of the EDNA framework [3]. The characterisation of the crystal (indexing and integration) followed by the radiation damage sensitivity study described here are performed in an integrated way using some of the plugins available in this platform.

This procedure has shown to be extremely useful for data collection strategy planning when more than one crystal from the same complex are available.

[1] Popov A.N. and Bourenkov G.P., Acta Cryst., 2003, D59, 1145-1153.

[2] Bourenkov G.P. and Popov A.N., Acta Crystal., 2006, D62, 58-64.

[3] Incardona *et al.*, J. Synchrotron Rad., 2009, 16, 872-879



## THE PARTITIONING EFFECT: RADIATION DAMAGE STUDIES OF PROTEIN-DNA COMPLEXES

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A complete understanding of the mechanisms of X-ray radiation-induced damage (XRID) in biological macromolecules presents a significant challenge, but could provide a more rational approach to some radiation-based therapies. Studies combining monochromatic X-ray radiation therapy with the cancer chemotherapy agent cisplatin have demonstrated a super-additive therapeutic effect, but details of the underlying mechanisms remain elusive [1].

To further investigate this effect, we performed macromolecular crystallography (MX) experiments on crystals of DNA and DNA treated with cisplatin. These studies provided initial evidence that crystallised nucleic acids were particularly radiation resistant in comparison to some proteins, even with covalently bound platinum adducts. However, comparative analysis between DNA and protein crystals can be problematic due to the differences in crystallisation conditions. DNA crystals, for example, often form pseudo-continuous double helices and often crystallise in very different buffers compared to many proteins.

It has been previously shown, using a protein-DNA complex, that in physiological solution conditions, the DNA component can indeed tolerate a higher radiation dose compared to the protein as judged by binding ability [2]. More recent studies have identified specific damage sites on the protein component of these complexes [3]. We followed up this work using MX to look at XRID of protein-DNA complexes. Although these studies provide some interesting findings that are consistent with the solution studies, they were performed on cryo-cooled crystals at 100 K. In an attempt to address this, we employed a humidifier device to allow these studies to be performed under more biologically relevant conditions [4]. The humidifier proved to be an excellent tool for maintaining protein-DNA crystals on the synchrotron beamline at room-temperature, and the initial results of these experiments will be discussed.

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## **TECHNICAL DEVELOPMENTS FOR STUDYING RADIATION DAMAGE ON THE 1-Mm SCALE AT GM/CA-CAT**

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Several recent publications have indicated that radiation damage to macromolecular crystals might be reduced by using micron sized X-ray beams. At GM/CA-CAT (APS), we have explored several X-ray optical schemes for developing both micron-sized beams with circular cross section, as well as a line-focused beam with micron width. Using these beams for radiation damage studies and challenging micro-diffraction experiments from macromolecular crystals (e.g. small crystals) requires a high degree of stability and reproducibility in the beam position and goniometry mechanics. Here, we present details of the X-ray optical layouts and beam characteristics, techniques for stabilizing the beam, and the development of goniometry with a ~1-micron sphere-of-confusion and a crystal-positioning stage with nanometer resolution.

## **A MECHANICAL STRAIN MECHANISM FOR GLOBAL DAMAGE**

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The fading of high-angle diffraction spots is a hallmark of radiation damage, but the exact mechanism connecting dose and the degradation of scattering power has remained unclear. A reasonable assumption has been that random bond breakages culminate in an increasing overall B factor, but this cannot be the case. Applying B-factors to undamaged data does not reproduce the scale or resolution dependence of spot fading measurements. In fact, the observed resolution dependence follows the first power of resolution, not the second power (as with a B factor), and this exponential falloff in reciprocal space implies a Lorentzian distribution of atomic displacements in real space. This departure from the Central Limit Theorem can be explained if a single underlying damage mechanism is at work, and point defects in an elastic medium generate a Lorentzian distribution of strain. Such defects must arise when one unit cell changes shape and its neighbors do not, and these so-called "Kanzaki forces" propagate across dozens of unit cells. The resulting distortions cause not just high-order spots to fade but the relative intensities of the spots to deviate significantly from that expected from the electron density of an average unit cell. This latter effect, combined with the expectation that macromolecular crystals already contain a high concentration of defects, implies that defect-induced strain may be a significant source of systematic error in refinement R factors, and a more complete model of lattice strain could be invaluable not just for compensating for radiation damage effects, but obtaining more accurate macromolecular models in general.

## **ENERGY DEPENDENCE OF HEAVY ATOM RADIATION DAMAGE IN PROTEIN CRYSTALS**

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Considering radiation damage to crystals caused by data collection is important when solving structures and critical when determining protein function, which can often depend on very subtle structural characteristics. In this study, we examine how the rate of damage to specific sites in protein crystals frozen at 100K depends on the energy of the incident x-ray beam. Crystals of a nucleic acid binding protein derivative containing both selenium-methionine and cysteine residues, and lysozyme crystals containing methionine residues and disulfide bridges were each subjected to 3-26 MGy of cumulative x-ray exposure by collecting multiple datasets from each crystal at either 14 keV or 9 keV.

The integrated electron density surrounding each sulfur and selenium atom was calculated for each data set and the change in electron density was evaluated on the basis of dose at the two energies. The rate of electron density decrease per cubic Å per MGy was determined to be greater at 14 keV than 9 keV for disulfide bridges and methionine residues in lysozyme crystals. Sulfur and selenium atoms observed in the nucleic acid binding protein crystals followed that same trend but did not show statistically significant differences.

## **UNARMED IN THE WILD: MANAGING RADIATION DAMAGE IN REAL LIFE**

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We here submit that, despite the vastly improved understanding of radiation damage, the tools available for managing the phenomenon are unequal to the demands of data collection in real life. We present observations from hundreds of synchrotron datasets collected for the SGC-Oxford since 2005 at both PXII (SLS) and Diamond, during which we have routinely tried to assess crystal lifetimes using sacrificial datasets. The only metrics available, namely the change in scaling B-factor and high resolution limit per frame (estimated only by mosflm), have both proved problematic. Not only are they ambiguous to interpret, but are logistically to obtain as they require data to be processed, which is (in general) utterly non-trivial to do in real time. Moreover, they correlate only approximately with the lifetime of the non-sacrificial crystal, underestimating it for large crystals, while overestimating it for small, weak diffractors when resolution must be maximised. On the physical side, there is no way to understand the critical parameter, namely the intersection of the beam and crystal: crystal shape can only be guessed, and measuring a beam's profile -- dimensions as well as flux density -- apparently remains elusive; and even with both are known, there is no tool to relate their intersection to the observed decay. Nevertheless, we demonstrate that our a posteriori approach has been remarkably effective, and thus should justify a call for more robust tools at the beamline.

## **PROBING RADIATION DAMAGE IN PROTEIN CRYSTALS WITH MICRON-SIZED X-RAY BEAM**

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X-ray induced radiation damage to macromolecular crystals remains a major hindrance in diffraction data collection and interpretation of results in structural biology. Energy deposition by photoelectrons, emitted during irradiation of crystals, is thought to be a primary cause of radiation damage at data collection temperatures (~100 K). Photoelectrons are emitted preferentially along the polarization vector and according to Monte-Carlo simulations [1] at 15keV will be reabsorbed 4 – 4.5 microns from the point of emission. Subsequently, with a small enough beam, photoelectrons will carry energy outside the footprint of the beam resulting in less damage to the sample. Studies of radiation damage as function of beam size indicated that smaller beams indeed reduce damage to the diffracting volume of a protein crystal. Radiation damage as function of dose and of distance from the beam center was also mapped with a 1  $\mu\text{m}$  beam at two energies – 15.1keV and 18.5 keV. Concurrent with the theories, the damage was higher along the polarization vector than in perpendicular direction; Width of the damaged area was 2-3 times larger than the incident beam size; and the damage did not transfer beyond 4 - 5  $\mu\text{m}$  from the incident beam center.

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## ORIGIN OF RADIATION DAMAGE IN BIOLOGICAL SAMPLES AT CRYOGENIC TEMPERATURES

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X-ray crystallography, small angle X-ray scattering, and monochromatic and white beam irradiation experiments have been applied to identify the basic mechanism of radiation damage. Cubic insulin and elastase crystals served as model systems for the crystallographic part of this work. Data were collected for temperatures between 5 K and 160 K. The decay of the recorded Bragg intensities, changes of the unit cell volume and crystal mosaicity, and the increase of the R(free)-value from structure refinements with dose were chosen as radiation damage parameters. Interestingly for all these parameters a local extremum or a parameter jump is observed at 30 K, most prominent for the unit cell volume increase.

Small angle X-ray scattering of cubic insulin crystal was applied to directly follow this radiation induced disordering process. For all temperatures the integrated diffuse scattering signal of cubic insulin crystals increases with dose. At low doses the signal increases moderately in linear fashion with dose. After reaching a certain 'critical dose' the signal starts to grow exponentially, indicating a fast disorder process. This 'critical dose' increases linearly with decreasing temperature demonstrating an ideal gas behaviour, e.g. at 50 K the critical dose is twice as much as at 100 K.

Warming of irradiated protein crystals above their glass transition temperature leads to a gas release. To identify the nature of this gas, we carried out white beam irradiation experiments of different organic compounds. In all the cases hydrogen gas was identified as being the major component of the gaseous radiolysis products. Smaller organic fragments such as methane or ethane were only minor products indicating that direct C-C bond cleavage is not the major cause for radiation damage.

Combining these results lead us to the conclusion that radiation induced cleavage of the C-H bond and the subsequent formation of hydrogen gas is the major cause for radiation in biological samples at cryogenic temperatures. Lowering the data collection temperature from 160 K to 50 K causes a significant reduction of radiation damage as derived from the lower decay of the Bragg intensities and the slower increase of the R(free)-values with dose - or in other words to a better preservation of the structure. At these temperatures hydrogen gas is still mobile inside the sample and can accumulate at grain boundaries or leave the crystal. At 30 K hydrogen gas becomes immobile, as expressed in the faster unit cell and mosaicity increase, and creates unfavourable sample deformations. In the case of crystalline samples these lattice distortions finally result in a faster loss of diffracting power at 30 K.

These different experiments give us a consistent picture of radiation damage in biological samples as a function of temperature, in particular showing that radiation damage is reduced by a factor of 4 when experiments are carried out at 50 K instead of 100 K.

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## **SLOW COOLING, RADIATION DAMAGE AND ANNEALING IN CRYSTALS OF THERMOLYSIN**

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A recent report showed that protein crystals can be cryogenically cooled to 100 K much slower than is normal practice, yielding lower mosaicities [1]. Here we combine slow-cooling and radiation damage experiments to indirectly characterize mobility in crystals of thermolysin. The crystals could be successfully cooled at 0.1 K/sec from 280 to 100 K, and then rewarmed back to 280 K. The mosaicity increased during cooling until ~200K when it plateaued at a value somewhat lower than typical for flash-cooling. The other crystal parameters ( $1/\sigma$  and cell volume) continued to change below this temperature. Warming the same crystal saw a reversal of this process with some hysteresis. When a crystal was subjected to high doses of radiation at 100 K, there was damage at specific sites in the protein. A subsequent damage experiment at 160 K showed greater overall damage, with less specificity than at 100 K. Additionally, there appeared to be an annealing effect at 160K, resulting in a mosaicity similar to that achieved with slow cooling. Together these results suggest greater mobility in the crystals at 160K than 100K, which can both relieve strain from flash-cooling as well as facilitate transport of damage producing groups.

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## ROOM TEMPERATURE DATA ACQUISITION WITH THE PILATUS 6M PIXEL DETECTOR

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Radiation damage to biological crystals during synchrotron data collection is a major obstacle in macromolecular structure determination. While cryogenic cooling significantly reduces the detrimental effects of ionising radiation, the situation is much less favourable at room temperature. The use of radical and electron scavengers was reported to have a beneficial effect [1]. Moreover, a measurable positive dose rate effect was reported at low dose rates [2] as well as at moderate dose rates [1]. Recently, the high frame rate of 12.5 Hz in combination with continuous data acquisition mode and a dynamic range of 20bit, supported by PILATUS pixel detector has offered the opportunity for data to be collected with unprecedented speed and hence dose rate. Therefore, we investigated the room temperature data acquisition at different dose rates using PILATUS 6M [3], with the objective to minimise secondary and global damage effects in ultrafast data collection. We present the first result for a room temperature data acquisition with a silicon pixel detector, where the data are collected in continuous sample rotation mode, with ms read-out time and no read-out noise. Several successive datasets were collected from a single test crystal mounted with a MiTegen RT system. The dose-rate range tested here is between  $\sim 7200\text{Gys}^{-1}$  and  $700\text{Gys}^{-1}$  with corresponding frame rates between 12.5 to 1.565 Hz. The absorbed dose was calculated by using the program RADDPOSE [4]. The dose required to reduce the diffraction intensity to half ( $D_{1/2}$ ) was obtained for different dose rates for Insulin and Thaumatin crystals. The intensity decays in all cases in an exponential manner and a first analysis indicates a negative dose rate effect. In addition, we observe a minor contribution of the crystal size with respect to beam size.

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## FUNCTION/STRUCTURE RELATIONSHIPS AND RADIATION DAMAGE IN PHOTOSYNTHETIC REACTION CENTER PROTEINS

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Early photosynthetic events involve light-induced electron transfer (ET) between donor-acceptor molecules (cofactors) embedded within highly structured matrices in reaction center (RC) proteins. RCs serve as unique examples of molecular systems in which both the cofactors and the surrounding media are tuned for optimized solar energy conversion.

In the RC of *Rhodobacter sphaeroides* an electron is transferred within 200 ps from the excited singlet state of primary the donor, P, to the secondary acceptor, ubiquinone  $Q_A$ , through the intervening bacteriopheophytin acceptor,  $H_A$ , forming a metastable charge separated state,  $P^+Q_A^-$ . This metastable state can decay either by back recombination or by forward ET from  $Q_A$  to  $Q_B$ . In our research, we seek to understand the factors that control the pathways of ET reactions.

It is well established that the  $Q_A Q_B \rightarrow Q_A Q_B^-$  ET is a conformationally gated reaction. The nature of the gate remains unclear. Previously we demonstrated that the earlier proposed movement of  $Q_B$  from the "proximal" to the "distal" site is not a gate for interquinone ET. Can it be that  $Q_A$  is involved in this conformational gating? Using advanced time-resolved (TR) high-frequency (HF) EPR techniques (in collaboration with G. Kothe, Freiburg University) we found that the geometry of  $Q_A^-$ , observed on a nanosecond time scale after light-induced ET to  $Q_A$ , deviates from the  $Q_A$  geometry determined by X-ray diffraction analysis. This observation suggests that  $Q_A$  possibly plays a more important role in forward ET than previously anticipated. We propose that, at longer times after photo-excitation of the RC, the geometry of  $Q_A^-$  relaxes to that determined by X-ray diffraction. Thus, this light-induced reorientation of  $Q_A^-$  in its binding pocket might be a rate limiting step of interquinone ET reaction. This model logically accounts for the striking "Kleinfeld effect": ET from  $Q_A^-$  to  $Q_B$  proceeds in RCs cooled to cryogenic temperature under illumination but does not proceed in RCs cooled in the dark.

In order to support our model and determine the rationale for the deviation of time-resolved EPR and X-ray structures, a systematic study of the effects of radiation on Fe-removed/Zn-replaced RC crystals using HF (130 GHz) EPR spectroscopy was carried out. It was demonstrated that radiation induces free radical formation and subsequent bond breakage as well as cofactor oxidation state changes that alter the native activity of the protein. We suggest that radiation induced reduction of the quinones can provide an explanation for the multiplicity of quinone binding sites observed in RC crystal structures, and for discrepancies between spectroscopic and crystallographic measurements of quinone site structures [1]. This work was supported by the U.S. Department of Energy, Office of Basic Energy Sciences, Division of Chemical Sciences, Geosciences, and Biosciences, under contract DE-AC02-06CH11357.

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## CORRELATED SINGLE-CRYSTAL SPECTROSCOPY AND X-RAY CRYSTALLOGRAPHY AT BEAMLINE X26-C OF THE NSLS

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Understanding the complex relationships among atomic structure, electronic structure, and chemistry is critical for obtaining fundamental insights into biological processes that underlie human diseases, and to the realization of new strategies to achieve our future clean energy needs. Inspiration and breakthroughs will come from new tools developed to probe biological processes with several complementary techniques. To achieve these goals, one needs to establish an integrated infrastructure to enable the simultaneous measurement of spectroscopic and high resolution crystallographic data. These capabilities do not currently exist in the United States, but will impact a broad scientific cross section. Therefore, with supplemental ARRA funding, we are accelerating the construction of an integrated facility at beamline X26-C of the National Synchrotron Light Source (NSLS) for single crystal X-ray diffraction and spectroscopic analysis of macromolecules. This includes the simultaneous measurement of at least three types of complementary data -- X-ray diffraction to high resolution, optical absorption spectroscopy, and Raman spectroscopy -- from the same sample under the identical experimental conditions.

We routinely collect correlated X-ray diffraction and single-crystal electronic absorption spectroscopy. The spectroscopic data is obtained within the readout time of the X-ray detector from a ~25µm diameter region of the crystal that intersects the X-ray beam. We are also commissioning a non-resonance and resonance Raman spectroscopy instrument at the beamline. This includes two excitation lasers (785nm and 532nm) and we plan to add more lasers in the near term (e.g. 633nm, 442/ 405nm). The Raman microprobes are designed for coaxial, backscattering mode. They excite the same ~25µm diameter region of the crystal that intersects the X-ray beam and the electronic absorption spectroscopy. We are integrating the controls for these three techniques into the beamline operations software. We also plan to add instrumentation to support steady-state and time-resolved fluorescence spectroscopy from single crystals. A complementary off-line laser spectroscopy laboratory immediately adjacent to the beamline is also under construction.

Our diffraction and spectroscopy facility is available full-time to the general user community at the NSLS. It is also, by its very nature, highly interdisciplinary. Results from the facility provide unique insights into the biochemistry of macromolecules relevant to energy metabolism, metal transport and homeostasis, photosynthetic and light sensing systems, the fluorescent protein superfamily, nitric oxide synthesis and signaling, redox-state<sup>1,2</sup> and stress-response systems, and managing the toxicity of reactive oxygen<sup>3</sup> and nitrogen species.

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## **EXPLOITING X-RAY INDUCED LUMINESCENCE OF MACROMOLECULAR CRYSTALS AS A METRIC FOR RADIATION DAMAGE**

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Radiation damage is a limiting factor in the collection of X-ray diffraction data at synchrotron sources and an important step of any X-ray experiment is determination of the point at which data become compromised and data collection, or processing, should be halted. Traditionally this point can either be defined by comparison of the dose absorbed by the crystal to established dose limits, or analysis of diffraction images collected. The use of complementary methods, such as UV-Vis absorption spectroscopy, allows the tracking of radiation damage on much shorter timescales and have proved incredibly useful in, for example, tracking the reduction of metal centres. Complementary methods do suffer from limitations however: spectral changes are difficult to follow during the X-ray experiment and visible spectroscopies are generally only applicable to chromophore-containing enzymes.

The luminescence of protein crystals during irradiation is a little-known side effect of the X-ray experiment and is a phenomenon common to almost all enzymes. During, and for a short time subsequent to, X-ray exposure crystals emit visible light in a process known as X-ray Excited Optical Luminescence (XEOL). The nature and dose dependent behaviour of XEOL from several enzymes and common cryoprotectants has been analysed and compared to diffraction based metrics.

## **RADIATION DAMAGE EFFECTS AT FOUR SPECIMEN TEMPERATURES FROM 4 K TO 100 K**

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Radiation damage is the primary factor that limits resolution in electron cryo-microscopy (cryo-EM) of frozen-hydrated biological samples. Negative effects of radiation damage are attenuated by cooling specimens to cryogenic temperatures using liquid nitrogen or liquid helium. We have examined the relationship between specimen temperature and radiation damage across a broad spectrum of resolution by analyzing images of frozen-hydrated catalase crystal at four specimen temperatures: 4 K, 25 K, 42 K, and 100 K. For each temperature, "exposure series" were collected consisting of consecutive images of the same area of sample, each with  $10 \text{ e}^-/\text{\AA}^2$  exposure per image. Radiation damage effects were evaluated by examining the correlation between cumulative exposure and normalized amplitudes or IQ values of Bragg peaks across a broad range of resolution (4.0-173.5 Å). Results indicate that for sub-nanometer resolution, liquid nitrogen specimen temperature (100 K) provides the most consistent high-quality data while yielding statistically equivalent protection from radiation damage compared to the three lower temperatures. At lower resolution, suitable for tomography, intermediate temperatures (25 or 42 K) may provide a modest improvement in cryo-protection without introducing deleterious effects evident at 4 K.

## **EFFECT OF X-RAY RADIATION ON FLAVIN COFACTORS**

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Bearing in mind that more than one thousand structures in the Protein Data Bank contain flavin cofactors,<sup>[1]</sup> it is of interest to assure the flavin geometry and electronic state when using these structures for deducing reaction mechanisms and when analyzing the conformational interplay between the cofactor and its protein scaffold. Inspecting flavin structure with QM/MM methods and monitoring of flavin vibrational modes with single-crystal Raman spectroscopy during X-ray data collection provide important information regarding the actual flavin state. Here we present data from the flavoprotein NrdI, comparing high resolution crystal structures, geometry optimized models, and Raman spectra, showing that the flavin geometry indeed is changed by X-ray radiation.<sup>[2]</sup>

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## UV DAMAGE OF SEMET AS EXPERIMENTAL PHASING TECHNIQUE

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It is widely demonstrated that X-ray radiation damage on biological crystalline samples can induce specific structural changes that may be used as a source of phasing information. This method (radiation damage-induced phasing; RIP) can be regarded as a sort of isomorphous replacement where the 'after' dataset has lost a few atoms that are particularly susceptible to radiation damage. However X-rays introduce many small changes to both solvent and macromolecule and may result in prejudice the diffraction and data quality. Recently Nanao and Ravelli [1] showed how UV radiation can induce specific changes in the macromolecule alone, leading to a larger contrast between radiation-susceptible and nonsusceptible sites. This technique has been optimized on the ESRF beamline ID23EH1, using a UV laser source already installed and the experiment can be routinely performed by users. Furthermore recent results on the combination of UV-RIP with anomalous scattering experiments for phasing experiments will be presented. In particular the effect on UV radiation on Selenium labelled protein will be shown together with its use in phasing.

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## CRYSTALLOGRAPHIC AND SINGLE CRYSTAL SPECTRAL ANALYSIS OF THE PEROXIDASE FERRYL INTERMEDIATE

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The ferryl (Fe(IV)O) intermediate is important in many heme enzymes and thus the precise nature of the Fe(IV)-O bond is critical in understanding enzymatic mechanisms. The 1.40 Å crystal structure of cytochrome c peroxidase Compound I has been solved as a function of x-ray dose while monitoring the visible spectrum at BL9-2 at SSRL. Data was collected with an open air Helium cryostat at 65 K, lowest safe temperature before nitrogen solidifies. 96 crystals were mounted by the SAM robot and were screened or collected on locally or remotely from Irvine, CA. 25 crystals were collected, from which the best 19 were used in the experiment. For each crystal, data collections were carried out in 15 separate runs. Run 1 consisted of 5° of data, representing the first 0.035 MGy of x-ray exposure. Then the same 5° of scanning angle were recollected 12 more times giving runs 2 through 13 with increased x-ray dose. In run 14 a full 120° of data were collected in order to fully reduce the crystal followed by run 15 which again repeated the same 5° representing the highest x-ray dose. The same 15-run data collection protocol was adopted for similarly sized crystals and the scanning angles were chosen to optimize the completeness of the data. Each composite data set was assembled by merging 5° of data with identical run numbers from 19 crystals. Making the composite dataset in such manner ensures measuring the same reflections in every separate run and allows their monitoring in time/dose. In addition the fo-fo difference maps are less noisy and more reliable. A total of 15 structures at 1.40 Å resolution were refined providing a picture of the structural changes associated with increasing the x-ray dose.

The Fe-O bond increases linearly from 1.73 Å in the low x-ray dose structure to 1.90 Å in the high dose structure. The low dose structure correlates well with a Fe(IV)=O bond while we postulate that the high dose structure is the cryo-trapped Fe(III)-OH species previously thought to be Fe(IV)-OH.

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## SPECIFIC RADIATION DAMAGE AND ENZYME RADIATION INACTIVATION

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Active sites are often the most radiation sensitive parts in crystalline proteins as has been shown in several cases. In particular, acidic amino acid residues (Glu, Asp) in active sites become frequently decarboxylated.

In this study, we compared the radiation sensitivity of lactate dehydrogenase from *Thermus thermophilus* (TtLDH) in the native form and in ternary complex with a cofactor and a substrate analogue. Several data sets were collected at 100 K at the ESRF on each of the two forms with a cumulated absorbed dose of 10 MGy. Difference Fourier maps indicate that the aspartic acid in the active site (Asp168) is the most radiation-sensitive residue in the entire protein and that cofactor and substrate-analogue binding do not alter its sensitivity.

In the ternary complex, functionally relevant structural changes were observed with respect to the native protein [1] that displace a tryptophan residue (Trp77). Interestingly, the displacement of Trp77 in the ternary complex reduces the radiation-sensitivity of a nearby aspartic acid that is heavily damaged in the native protein. These observations suggest that radiation-induced decarboxylation is facilitated by electron-hole transfer involving a nearby tryptophan residue.

Detailed inspection of the Glu and Asp radiation sensitivities in both protein forms revealed that involvement in salt bridges does not protect acidic residues from being decarboxylated. This is in contrast to our earlier observations in crystalline lactate dehydrogenase from *Haloarcula marismortui* where Glu and Asp involved in salt bridges were radioprotected [2].

Crystallographic studies have been complemented by irradiation experiments of native TtLDH in solution at room temperature using a <sup>60</sup>Co source. The enzyme was completely inactivated after an absorbed dose of 100 Gy. In order to examine if decarboxylation of the catalytic Asp168 caused the inactivation, limited proteolysis of the irradiated enzyme has been performed, followed by MALDI-TOF analysis. Surprisingly, the mass of peptides carrying the active site residues did not change, indicating that the decarboxylation seen in crystallographic experiments at much higher absorbed doses is not the primary factor causing radiation inactivation. Instead, the MALDI-TOF experiments indicated a mass gain in Gly198 and/or Arg199. These two residues are part of a surface loop whose conformational changes are crucial for enzymatic activity.

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