Radiation damage at softer X-rays

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Outline

- Basis of X-ray induced Radiation Damage
- Radiation damage – physics, chemistry and biological aspects
- How to prevent it
- Energy dependent radiation damage effects

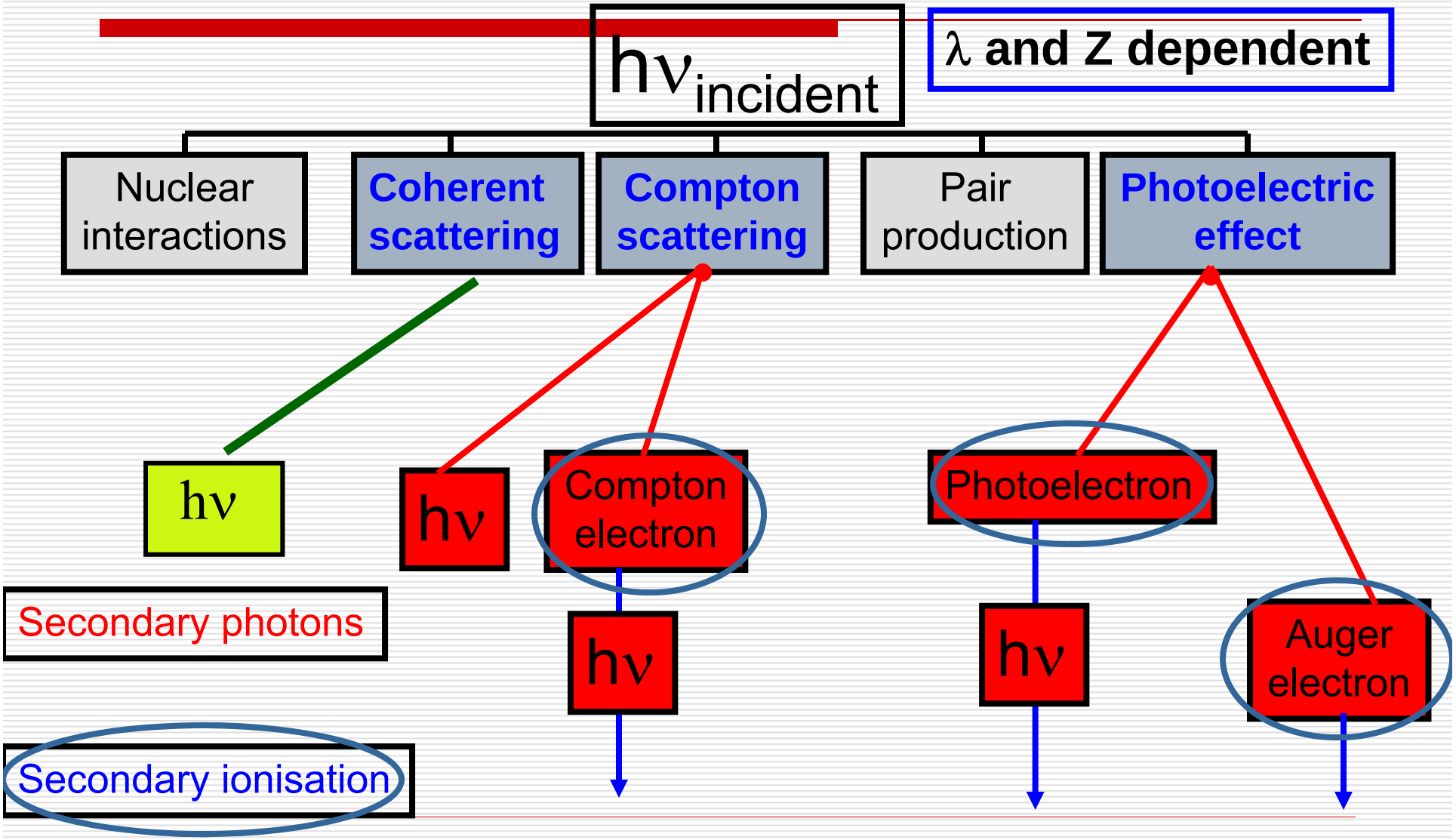
History

- First reported study on radiation damage at room temperature on myoglobin crystals by Blake and Phillips was in 1962
  - Blake C, Phillips DC: Effects of X-irradiaton on single crystals of myoglobin. In *Proceedings of the* ***Symposium on the Biological Effects of Ionising radiation at the Molecular Level***, Vienna 1962: 183–191.
  - Quotes:
    - Damage is proportional to dose and might be structurally specific.
    - Each absorbed 8 keV photon disrupted 70 molecules and disordered another 90.

History

- Data collection at 100 K prolongs the crystal lifetime by a factor of 70, on in-house X-ray sources
- Cryo-cooling becomes a routinely used in 90s
- Advent of third generation synchrotron beamlines in late 1990s: radiation damage to cryocooled crystals increases
- Pertinent problem/fenomenon in modern macromolecular crystallography

Interaction of X-rays with matter

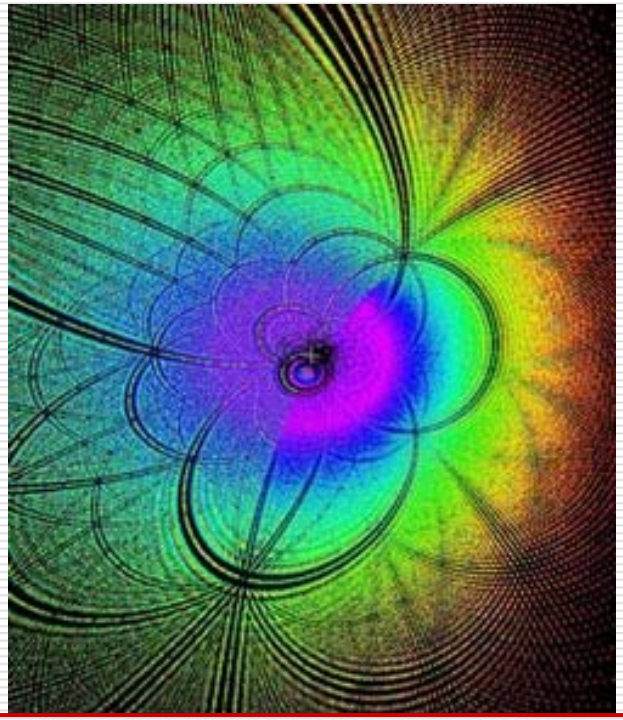


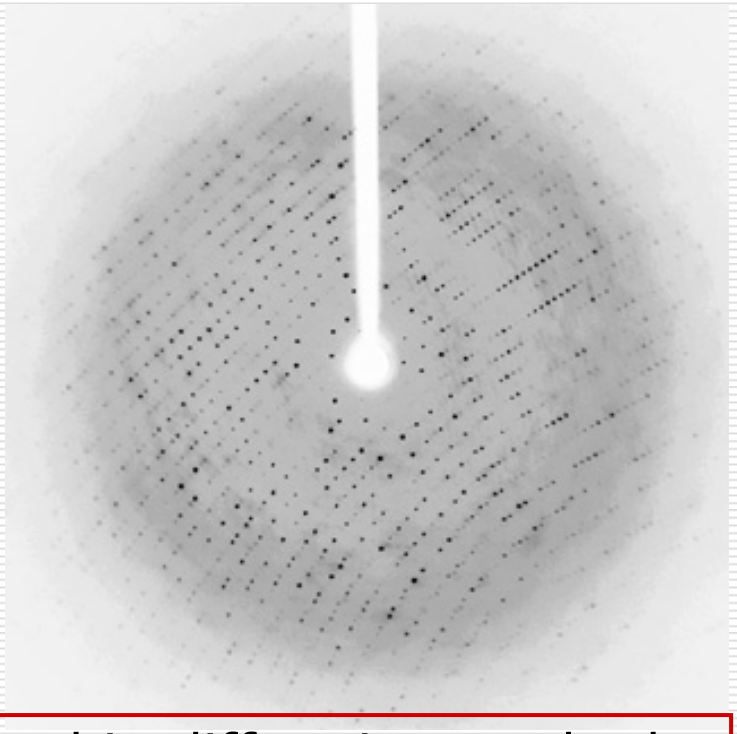
Interaction of X-rays with matter

- Coherent/Elastic/Rayleigh Scattering
- Compton/Inelastic Scattering
- Photoelectric Effect

Coherent/Elastic/Thompson Scattering

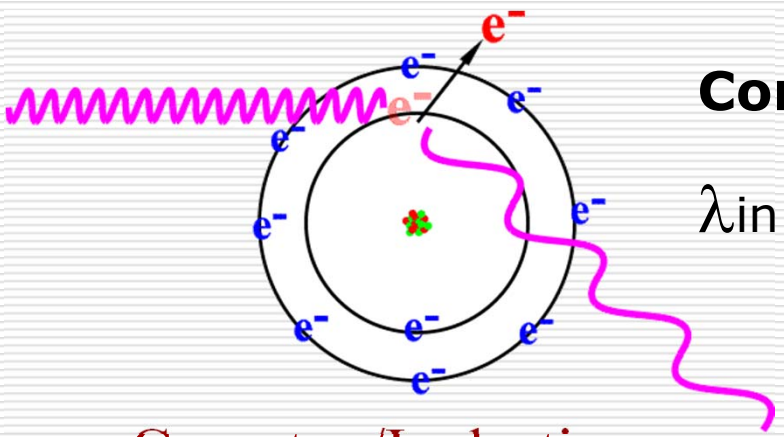
- $\lambda_{in} = \lambda_{out}$
- Rayleigh Scattering by particles much smaller than the wavelength of the light
- Coherent/elastic scattering arises from the interaction of the X-rays with the atom





…this is the interaction exploited in diffraction methods…

Compton/Incoherent/ Inelastic Scattering



Compton/Inelastic Scattering

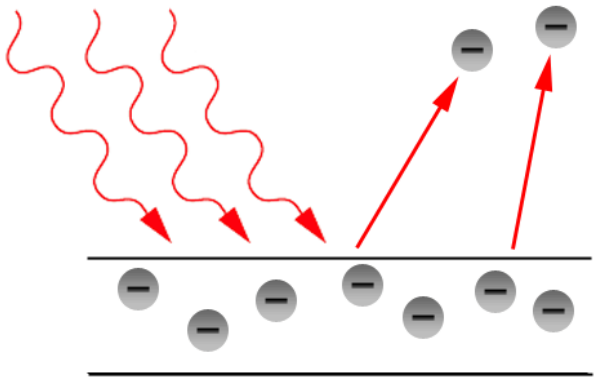
Compton shift:

$\lambda_{in} < \lambda_{out}$

Incoming photon is scattered incoherently on electrons, passing a small amount of energy to the electron (which is ejected).

Atom becomes ionised.

Photoelectric effect



Photoelectric effect

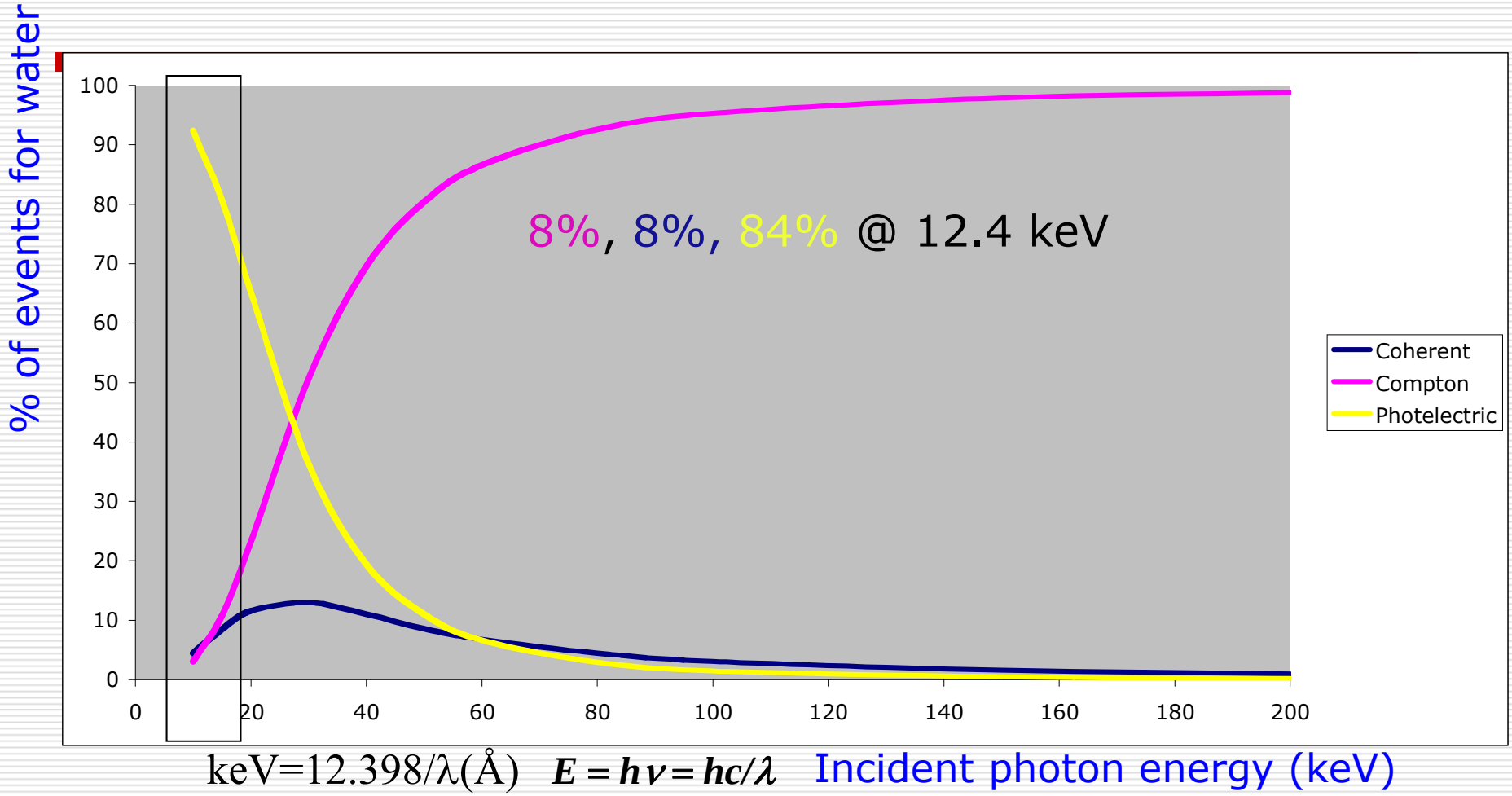
Emission of electrons from matter upon absorption of electromagnetic radiation (such as UV or X-rays).

Frequency of radiation must be above a certain threshold (specific to the type of material)

The X-ray photon is totally absorbed and a lower level core electron is ejected from the atom.

The excited atom or ion might release part of its extra potential energy through a complex cascade, in which the absorbing atom could become multiply ionized; emission of Auger electrons

Contribution of various processes



Each photoelectron can result in the production of up to 500 secondary lower energy electrons, which then cause further damage

Interaction of X-rays with matter

- X-rays are ionizing radiation

- Generation of electrons, ions, secondary ionization events and **free radicals**

Radiation damage

- Result of interaction of matter and electromagnetic radiation in X-ray regime *via*:
  - Photoelectric effect (84%)
  - Compton/inelastic scattering (8%)
- Photoelectron scatters inelastically off surrounding atoms, creating several hundred secondary electrons and positively charged centers
- Secondary electrons are mobile at 100K and directed to high affinity, electron-deficient functional groups causing specific secondary damage

Primary and secondary damage

- Radiation damage initiated by '*primary*' interactions between the molecules in the crystal and the beam (***Photoelectric Effect and Compton Scattering***).

Dose dependent

not uniformly distributed within the sample, deposited in regions called *spurs*

- '*Secondary*' damage: comes from the secondary ionisation events and reactive radicals generated from the polypeptide chain or water molecules by the primary events. The radicals diffuse through the crystal causing a cascade of further damage.

Time and temperature dependent

How fast does damage occur?

AT **ROOM TEMPERATURE**:

Primary damage/event: **femtoseconds** after exposure

Breakage of S-H, O-H, N-H and C-H bonds, formation of reactive species such as solvated electrons, hydroxyl and hydrogen radicals within the spurs: **picosecond** timescale

Secondary damage: breakage of bonds within the macromolecule and generation of other radicals: **microsecond to millisecond**

How fast does damage occur?

AT **100K**:

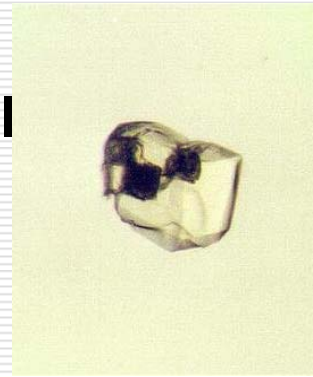
Reactive species are formed, hydroxyl radicals are still mobile. ***Diffusion is limited*** → **secondary damage** is ***slowed down***, but also occurs in frozen crystals on brilliant beamlines at synchrotron sources.

Primary damage is inherent to the use of ionizing radiation and will eventually damage every sample. But **secondary** damage leads to ***specific structural effects***, and should be minimized…

Time

Radiation damage indicators/symptoms

- **Global radiation damage indicators**
  - Loss of **diffraction quality**, and of **high resol** reflections
  - Increase of **Rmeas**
  - Increased sample **mosaicity**
  - Increase of **Wilson B factor**
  - Increase in **unit cell volume**
  - **Colour changes** in the irradiated volume of the crystal
  - **Non-isomorphism** for MAD data sets (the molecule might undergo small rotations and/or translations)
  - Upon warming cryocooled crystals invariably '**bubble**', **releasing trapped gas**: H_2, CO_2



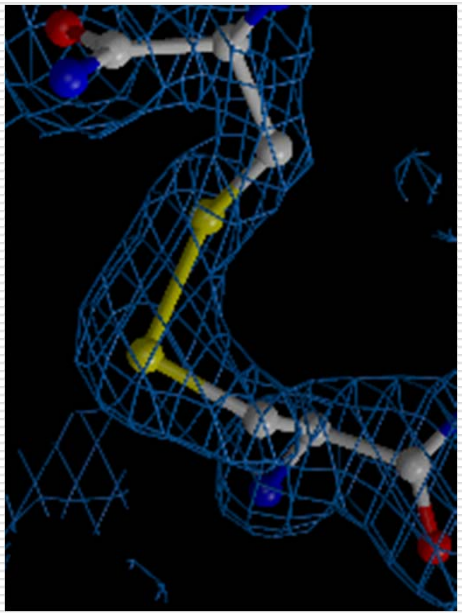
(after Elspeth Garman's website)

Radiation damage indicators

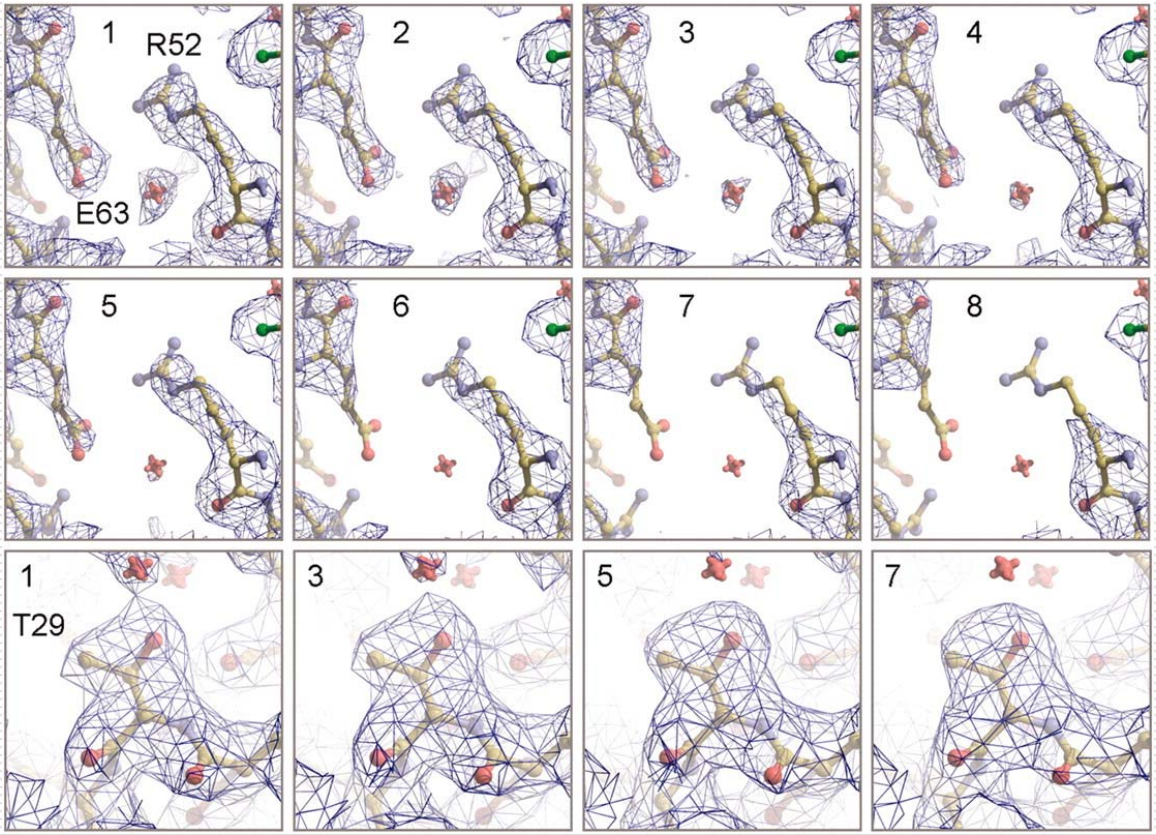
Time

- **Specific structural effects:** secondary lower energy electrons are mobile at cryotemperatures and can migrate to the sites of highest affinity
  - Breakage of disulfide bonds
  - Metal centers reduction
  - Decarboxylation of acidic residues (Asp and Glu)
  - Loss of electron density for hydroxyl groups of tyrosines and methylthio groups of methionines
- Solvent accessibility: is some studies correlation between SSA of residue and RD susceptibility – not conclusive
- Active site residues and metal centers are among the most radiation-sensitive

Specific damage



Acetylcholinesterase
Weik *et al.* PNAS, 97 (2), 623-628 (2000)



Owen R *et al.*, PNAS, 103 (13), 4912-4917 (2006)

Dose: deposited energy/mass [J/kg; Gy]

- **Depends on**
  - Beam parameters (size, flux and profile)
  - Crystal size
  - Crystal constituents
    - Can be calculated e.g. by **RADDOSE**

$$D \propto \frac{I_0}{\lambda V}[1 - \exp(-\mu_{abs}\, t)].$$

D=dose
λ=wavelength
V=volume of crystal
μ = abs. coefficient
t = path length in beam
Io = incident beam intensity

$\mu = N\sigma$

N - number of atoms per unit V

σ - total absorption cross section

$\sigma = 2\,\lambda\,\Delta\,f''$

Dose limits for protein crystals

- **There is a maximum amount of photons/cell volume that a crystal can handle before the crystalline diffraction is lost.**

- **Dose at which the diffracted intensity of a cryocooled protein crystal *drops to half* (proposed from electron diffraction experiments):**

- **Henderson's limit 2×10^7 Gray (JKg^{-1})**
  - **Henderson, R. (1990) *Proc. R. Soc. London Ser. B* 241, 6–8.**

- **At a 3rd synchrotron generation source this limit can be reached with 200-400 seconds exposure....**

Dose limit for protein crystals: experimental determination

- **Experimental dose evaluation**
  - **E. Garman PNAS (2006), 134912-4917**
- **Dose limit: 4.3×10^7 Gy**
  - **Considering a number of data quality indicators, an intensity reduction to $I_{ln2} = \ln 2 \times I_0$ ($=0.7 \times I_0$)**
- **Dose limit recommended for typical protein crystallography experiments : 3×10^7 Gy**
- **Does it make a difference if a certain dose is delivered over a short or long time interval?**
  - At 100K: no correlation of dose versus dose-rate
  - At RT: faster dose rate, better tolerance: Garman at el. 2011

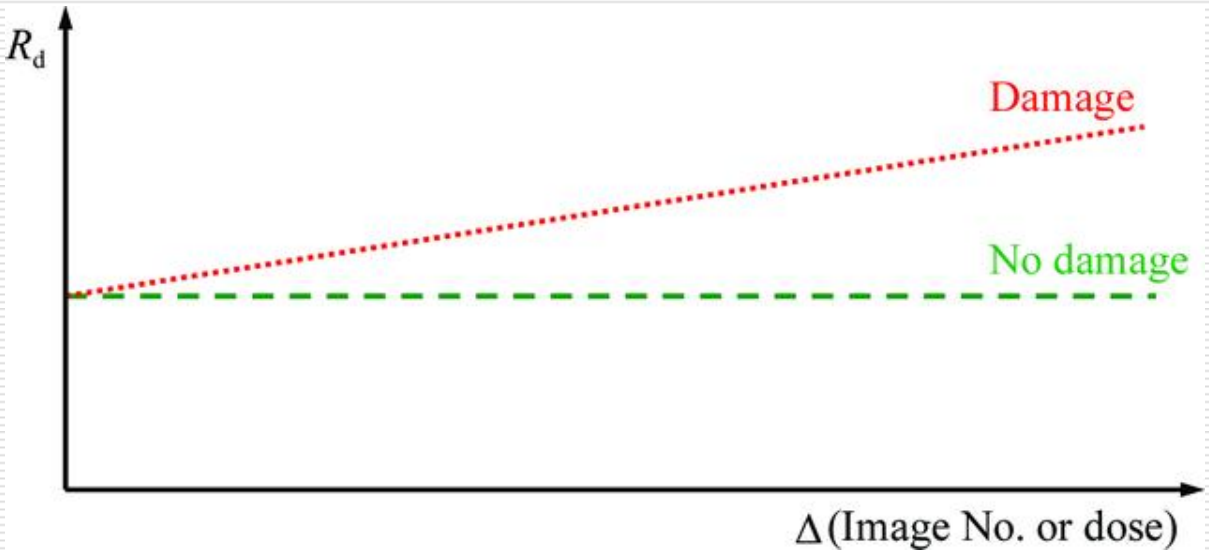
Metrics

- I_D / I_1
  - where I_D is the summed mean intensity (I_{mean}) of a complete data set (or equivalent sections of data) after a dose D and I_1 is the mean intensity of the first data set

- **V**
  - Volume of the unit cell

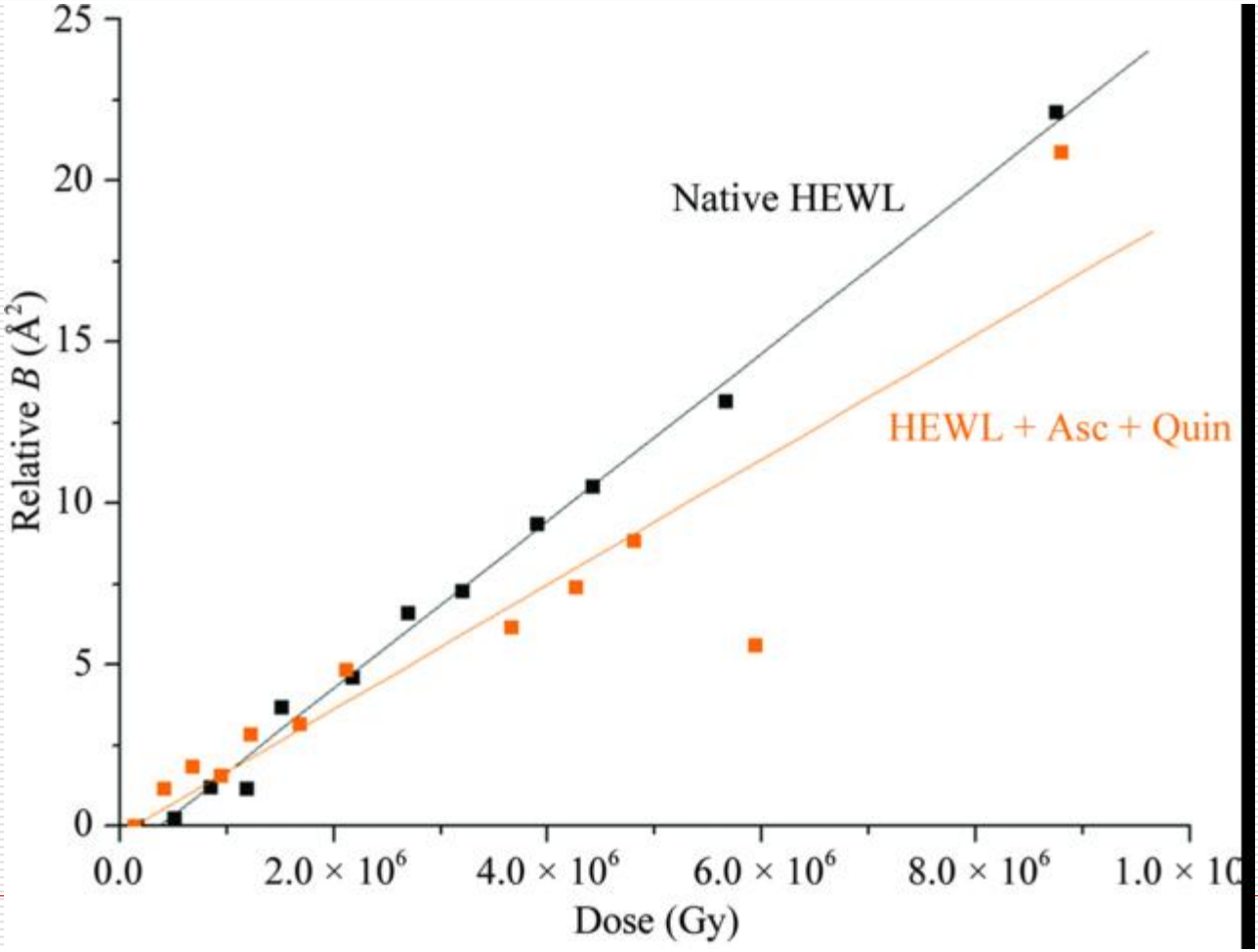
Metrics

- R_d
  - the pairwise *R* factor between identical and symmetry-related reflections occurring on different diffraction images, plotted against the difference in dose, *D* (Diederichs, 2006)



Metrics

- **B factor**



Monitoring on-line

- Formation of the disulfide-anion radical $RSSR^{\cdot -}$: UV-vis microspectrophotometry @ 400 nm
- Solvated electrons UV-vis microspectrophotometry @ 550-600 nm
- Reduction of metal centers UV-vis microspectrophotometry
- Raman

Alleviating Radiation Damage

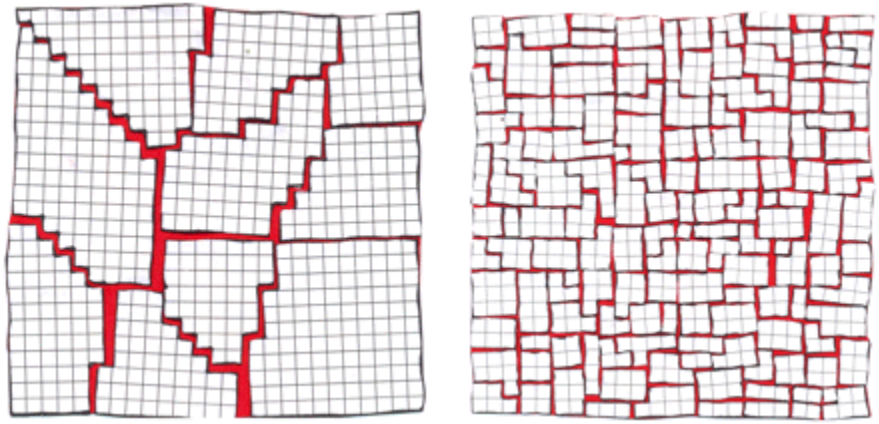
- Addition (soak into crystals) of radio-protectants (electron and radical scavengers)
  - Ascorbate, nicotinic acid, 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), $NaNO_3$,…
  - Not a standard approach
- Cooling X-tals at 100 K during data collection
  - Reduce diffusion of free radicals
    - Act on secondary damage effects
  - Standard since beginning of 90s
  - ***T lower than 100 K***
- Energy of radiation

Cooling crystals to 100 K or lower?

- ***Diffusion is limited*** → **secondary damage** is ***slowed***
- Cooling from RT to 100 K improves dose tolerance by a factor of 70
- Crystal heating by the beam is **NOT** responsible for radiation damage at cryotemperatures.
- Some improvement at 50 K observed with EXAFS/XANES on metals reduction (Grabolle, JBC 2005; Corbett, ActaD 2007)
- At 50 K, specific radiation damage to disulfide bridges is reduced by a factor of 4 compared to 100 K and dose toleration increased by a factor 2.6-3.9 (Meents, PNAS 2009)
- **No** compelling **evidence** that helium cooling gives significant improvement in crystal lifetime (Meents, PNAS 2009)

T lower than 50 K ?

- Damage to the crystal lattice at temperatures of 50 K (left) are higher at 30 K (right)



- 50–160 K, the hydrogen formed inside the sample as a result of x-ray irradiation can diffuse inside the crystal.
  - Accumulates at lattice imperfections → macromosaicity
- At 30K it is trapped
  - → micro-cracs and loss of loss of short range order

Effects of different X-ray energies

- Is there and optimal energy for MX experiments?
- Is there an optimal energy to minimize RD?
  - Experimental and theoretical approaches

Is there and optimal energy for MX experiment?

- ***The Ultimate Wavelength for Protein Crystallography*?** (Polikarpov, ActaD 1997)

- No optimal energy, it depends on the crystal size

  - Aim of experiment design to:
    - Increase integrated diffraction I and decrease absorption

    - Optimize ratio between integrated diffraction I and energy of absorbed X-ray photons (diffraction efficiency)

Is there and optimal energy for MX experiment?

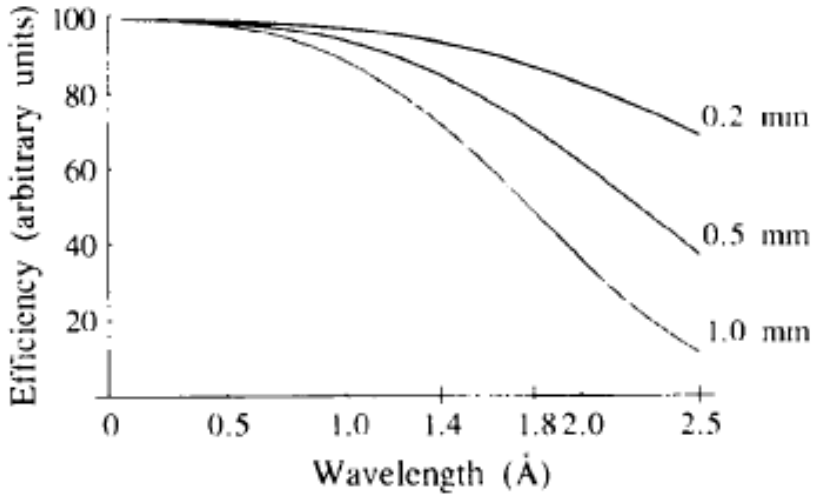


Fig. 1. Wavelength dependence of the diffraction efficiency [equation (1)] normalized to 100% at the short-wavelength limit for three different thicknesses t of the crystal. The maximum optimum wavelengths, 1.4, 1.8 and 2.5 Å, for the crystal sizes, 1, 0.5 and 0.2 mm, respectively, are indicated [see equation (2)].

$$(P/E_{\mathrm{abs}}) \propto [\lambda^3 t \exp(-\mu t)]/[1 - \exp(-\mu t)].$$

Is there and optimal energy for MX experiment?

- 0.9 Å shall be is sufficiently short to minimize radiation damage
- 1.3-1.6 Å for smaller crystals
- Soft X-rays (2.5 Å) combined with microcrystals (20 μm) – factor 8.8 gain in diff. beam E

Optimal wavelength (in the absence of RD)

- ***On the Choice of an Optimal Wavelength in Macromolecular Crystallography***. Tepliakov ActaD 1998
- Integrated diffraction intensity depends on:

$$I \propto \lambda^2 t^3 \exp(-\mu t)$$

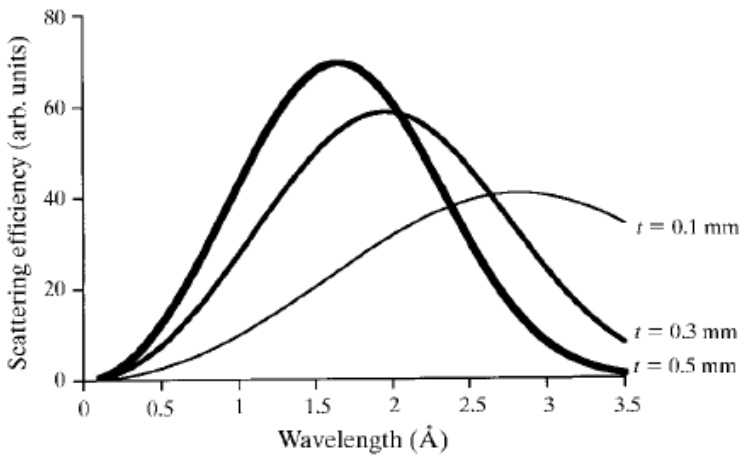


Fig. 1. Scattering efficiency of the protein crystal of thickness t as a function of the wavelength. To be in the same scale, the curves are normalized on the crystal cross section t^2.

Optimal wavelength (in the absence of RD)

- A series of experiments at 0.9 and 1.3 Å, crystal dimensions (0.4x0.4x0.2mm, 0.5x0.5x0.25 mm, 0.2x0.2x0.2 mm)
- Higher quality of the data can be achieved by using the wavelengths of 1.1- 1.3 Å or even 1.5 Å as compared with 0.8 Å - 0.9 Å
- $\lambda > 1.1$ Å particularly advantageous for small X-tals – small absorption

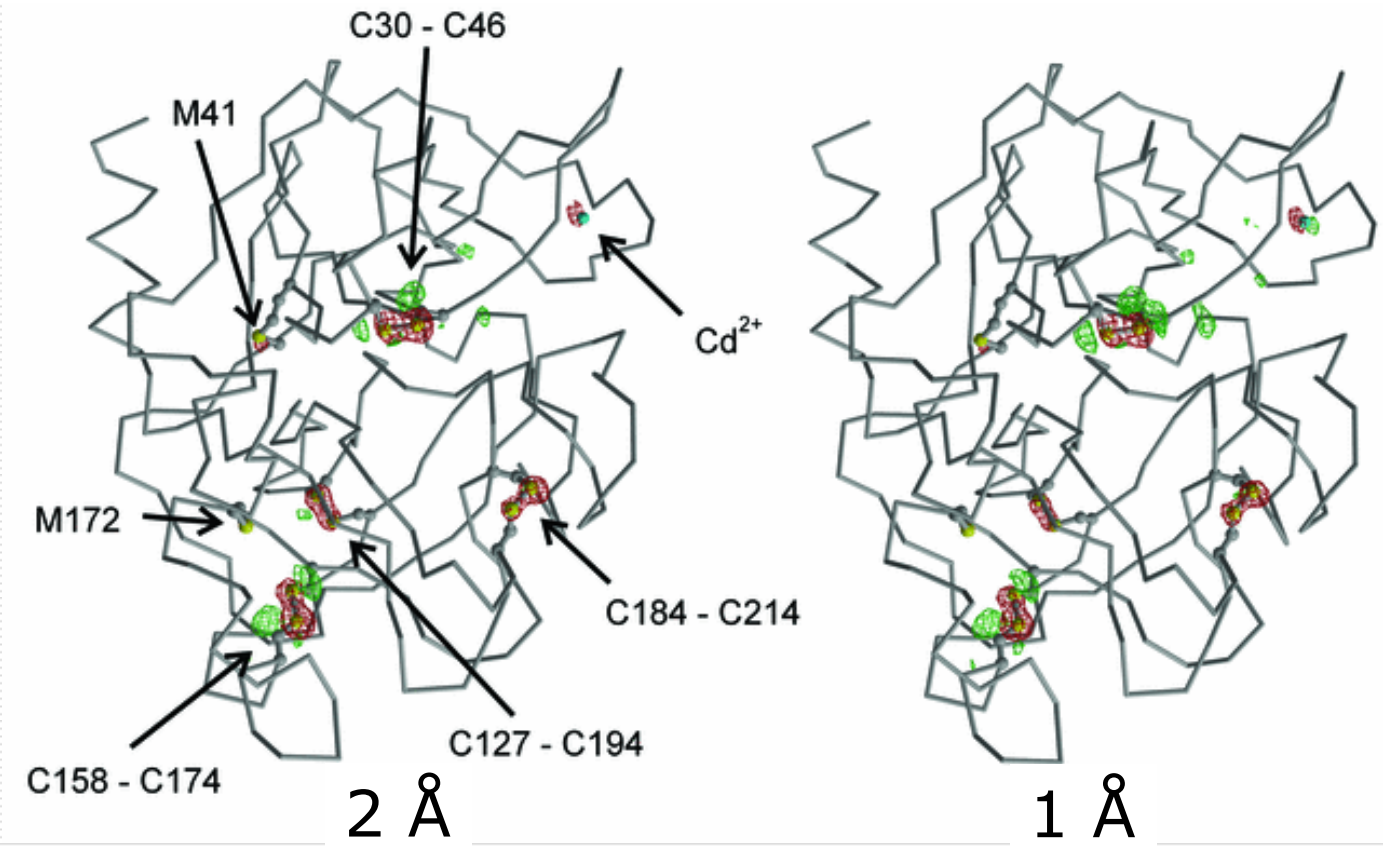
Effect of λ on specific RD effects

- ***On the influence of the incident photon energy on the radiation damage in crystalline biological samples.*** Weiss JSR 2005

- Series of datasets collected at 1.0 and 2.0 Å on Cd derivative of porcine pancreatic elastase (PPE)

- Assessment:
  - Based on difference Fourier syntheses between datasets with increased absorbed dose – neg. el. density shows RD susceptible sites: Cys, Cd, Met

Effect of λ on specific RD effects



Effect of λ on specific RD effects

- **Conclusion**: no significant differences in radiation damage between long and short **λ**

CAVEAT (MSW): this might be different if you are close to the absorption edge!

$$\mu = N\sigma$$

N - number of atoms per unit V

σ - total absorption cross section

$\sigma = 2\,\lambda\,\Delta\,f''$

Effect of λ on global RD effects

- ***Dose dependence of radiation damage for protein crystals studied at various X-ray energies.*** Shimizu, JSR 2007
  - X-ray energies (6.5, 7.1, 8.3, 9.9, 12.4, 16.5, 20.0, 24.8 and 33.0 keV

X-ray energy (keV)	Dose per data set (Gy)
33.0	8.2×10^5
24.8	1.5×10^6
20.0	1.1×10^6
16.5	5.7×10^5
12.4	1.3×10^6
9.9	9.1×10^5
8.3	1.7×10^6
7.1	2.2×10^6
6.5	2.5×10^6

Effect of λ on global RD effects

- Using global indicators (R_{merge}, B-factors, I/σ(I)):

- **Conclusion**: RD NOT depended on photon E, but only on absorbed dose

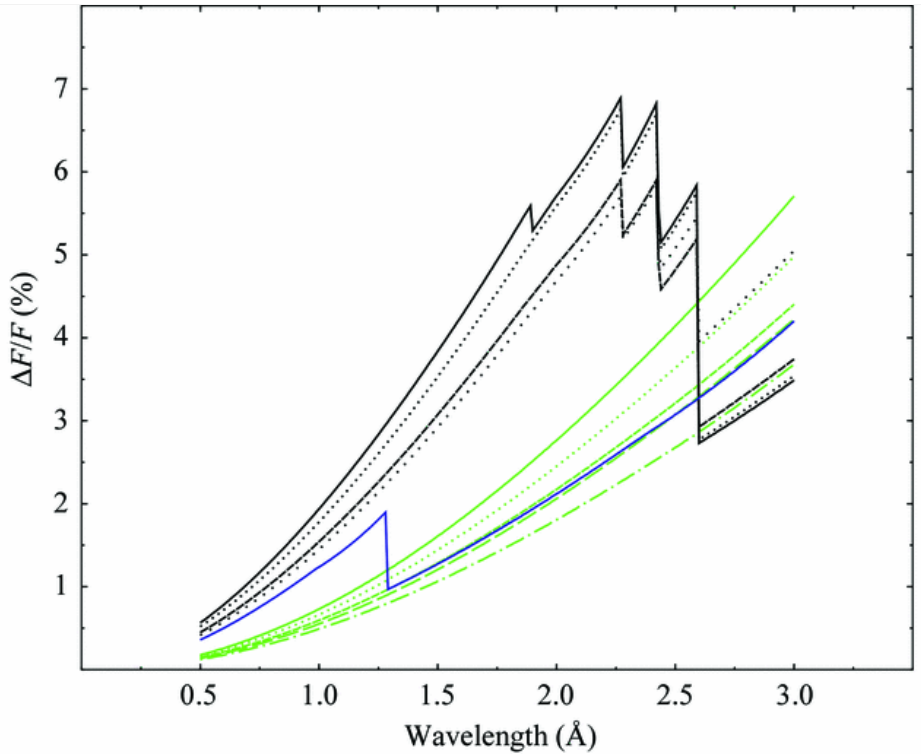
Optimal wavelength for best anomalous signal-to-noise ratio

- ***On the routine use of soft X-rays in macromolecular Crystallography P-II***. Mueller-Dieckmann, ActaD 2005
- 10 different systems (protein, DNA)
- Range 0.80 and 2.65 Å
- Monitor: magnitude of the anomalous signal-to-noise ratio
- Assessment: quotient $R_{anom}/R_{r.i.m.}$

Optimal wavelength for best anomalous signal-to-noise ratio

- Estimated anomalous diffraction ratio $\Delta F/F$ as a function of the wavelength:

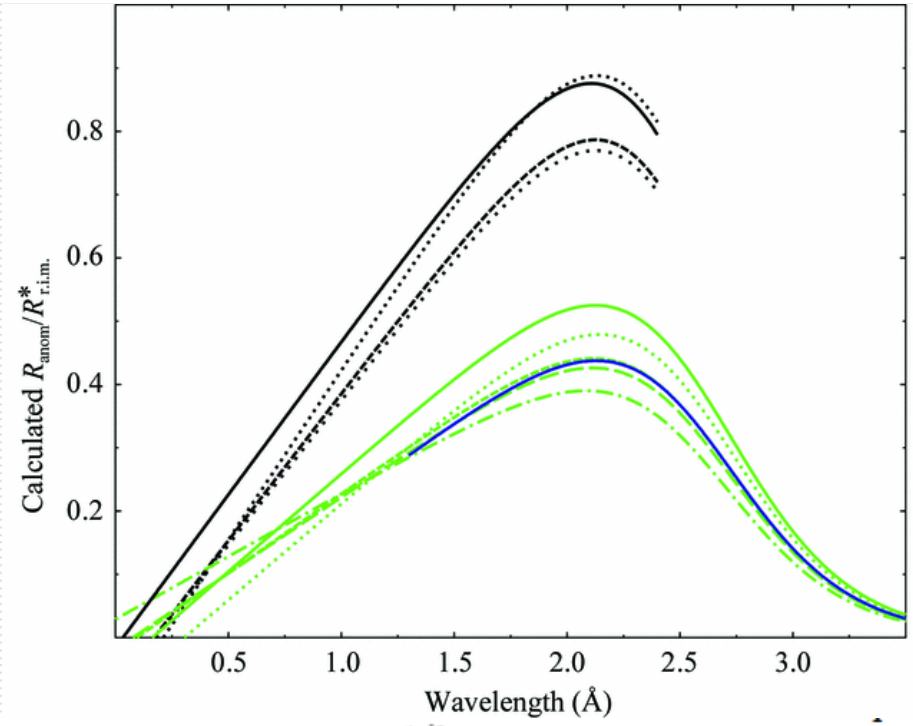
$$< \frac{\Delta F}{F} > \approx \frac{1}{\sqrt{2}} \frac{\sqrt{N_A}\, 2\, f''_{\lambda_{i,\max}}}{< | F_T | >}$$



ΔF/F used in experiments

Optimal wavelength for best anomalous signal-to-noise ratio

- Assessment: quotient $R_{anom}/R_{r.i.m.}$



$$R_{r.i.m.} = 100 \sum_{hkl} [N/(N-1)]^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$$

$$R_{anom} = 100 \sum_{hkl} |I(hkl) - I(\overline{hkl})| / \sum_{hkl} \langle I(hkl) \rangle$$

Optimal wavelength for best anomalous signal-to-noise ratio

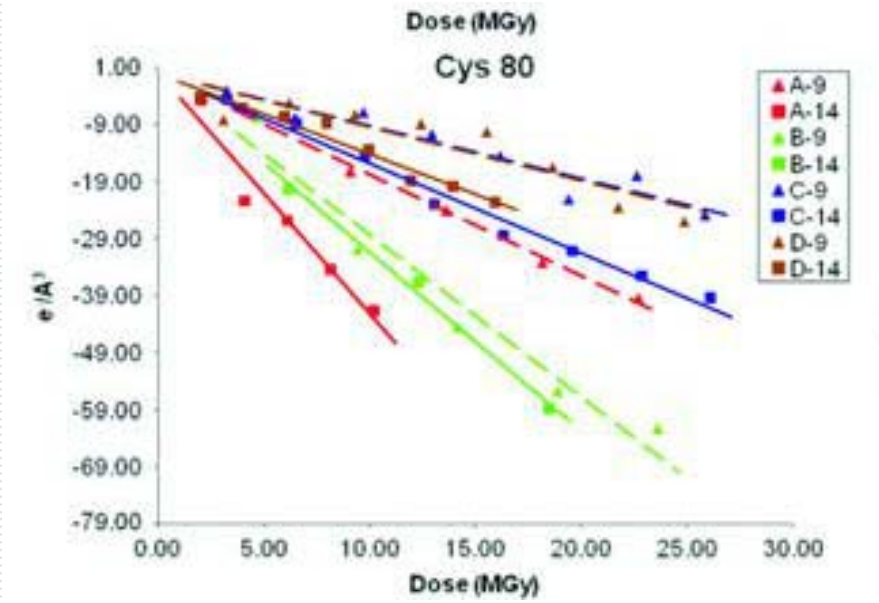
- Almost independent of the nature of the anomalously scattering substructure and provided that no elemental X-ray absorption edge is nearby:

- **Conclusion:** Optimal wavelength is **2.1 Å**

Effect of λ on rate of specific RD effects

- *Energy dependence of site-specific radiation damage in protein crystals.* Homer, JSR, 2011
- Lysozyme, 9 keV or 14 keV, 3-26 MGy of cumulative X-ray dose
- Assessment: electron density surrounding S

Effect of λ on rate of specific RD effects



- **Conclusion:** Rate of electron density decrease per cubic Å per MGy was determined to be **greater at 14 keV than at 9 keV** for cysteine sulfurs involved in S-S bridges, Met much less affected

What to do with λ?

- Optimised anomalous signal-to-noise: 2.1 Å

- As RD does not depend critically on λ, but integrated diffraction intensity does → regime 1.1 – 1.5 Å

What to do with/against RD

- Do not fry crystals
  - Strategy programs, e.g. BEST for determination of the best data-collection strategy

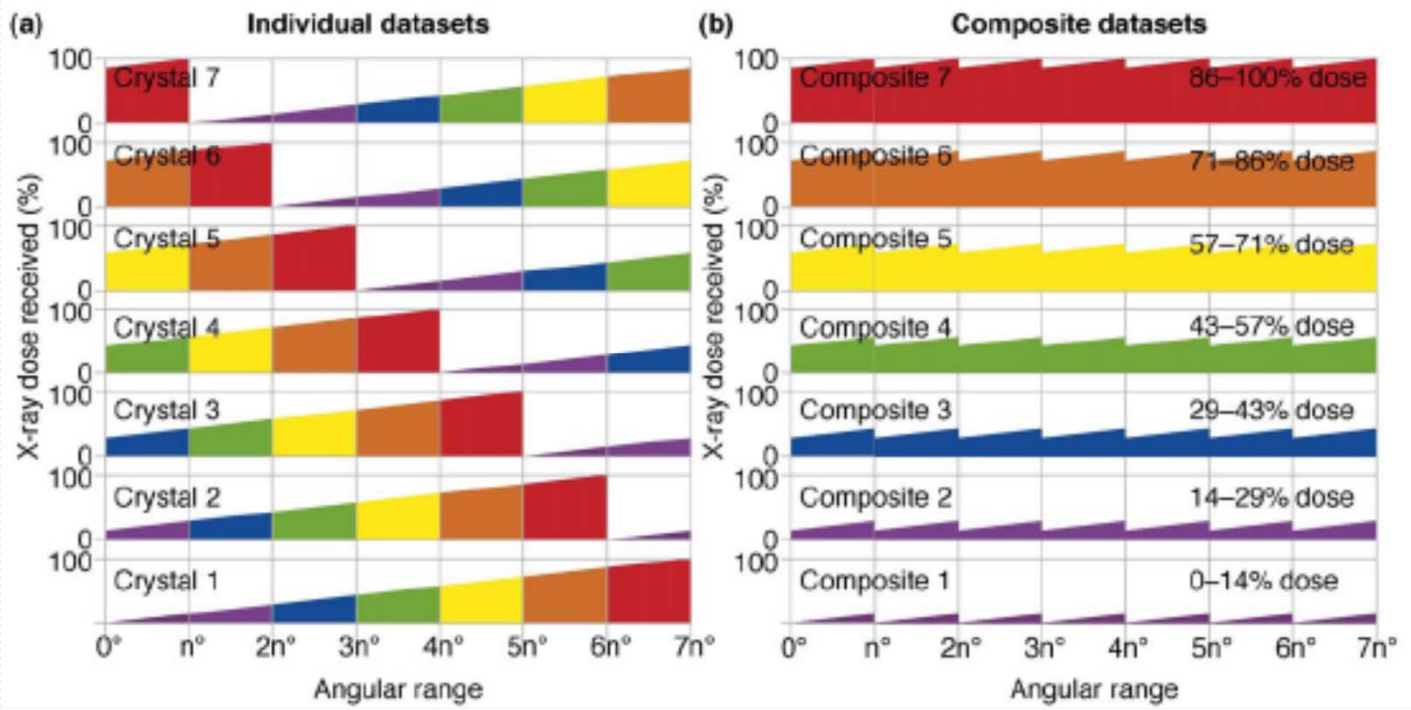
- Use of electron and radical scavengers: "quick soak for a long life of the crystal" (MSW)
  - Ascorbic acid
  - 5,50- dithio-bis-2-nitrobenzoic acid [DTNB]
  - Nicotinic acid….
  - $NaNO_3$
  - …will **NOT prevent metal reduction**

What to do with/against RD

- Liquid helium based cooling at 50K which can drastically reduce the metal reduction rate – XAS exp. And reduced global RD effects
- Correction of intensities of reflections using data by Zero-dose extrapolation (in xscale/XDS)
  - K. Diederichs et al. Acta Cryst. (2003). D59, 903-909
- Composite/multi-crystal data-collection
  - One crystal one shot (at random orientation)

What to do

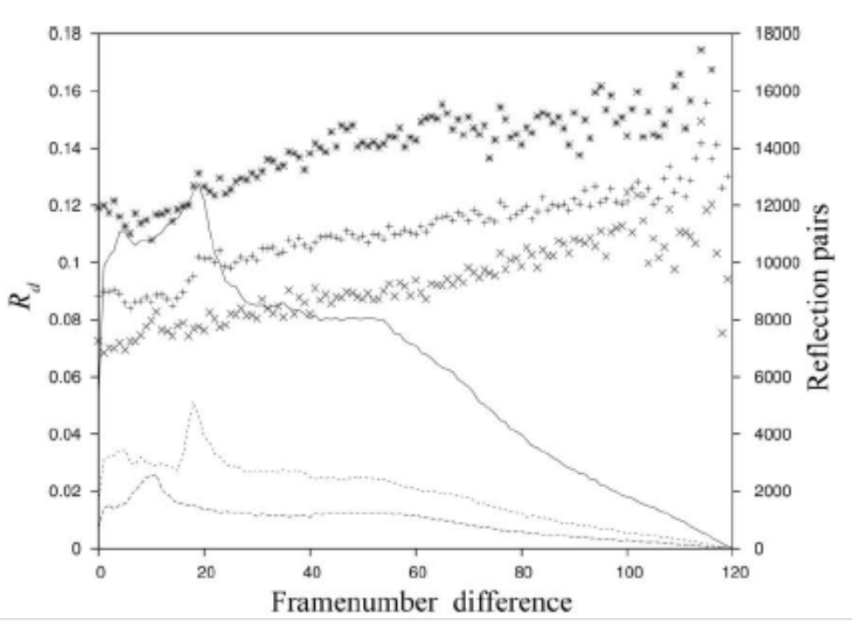
- Composite/multicrystal data-collection
  - Complete datasets are collected from multiple (shots of) crystals starting at different oscillation angles. Composite datasets represent structures that received different X-ray doses.



What to do

- Careful monitoring and analysis of data during and after data-collection
  - Global indicators
  - Decay R-factor
    - Xdsstat

$$R_d = \frac{\sum_{hkl} \sum_{|i-j|=d} |Y_i - Y_j|}{\sum_{hkl} \sum_{|i-j|=d} (Y_i + Y_j)/2}$$



K. Diederichs Acta Cryst. (2006). D62, 96–101

Make it work for you

- RIP on S and Se
- Inform reaction mechanism

“X-ray titrations” of horseradish peroxidase

The catalytic pathway of horseradish peroxidase at high resolution

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Nature 417, 463-468