X-ray radiation damage to crystalline proteins

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Protein X-ray crystallography

Synchrotron radiation is a powerful tool, but price must be paid.
Radiation damage to proteins

Disulfide breakage

Decarboxylation
• Radiation damage
  - global indicators
  - specific structural damage
  - primary or secondary damage?
  - influence on specific damage of: pKa, solvent access., chemical environment

• Actives sites are most radiation sensitive - biological information altered
  - bacteriorhodopsin, malate dehydr, cholinesterase, DNA photolyase, IrisFP
  - redox proteins (e.g. metalloproteins)
  - online spectroscopy complements crystallography

• Practical issues
  - is there a dose-rate effect?
  - critical dose? how to calculate it?
  - how to minimize radiation damage?
  - wavelength-dependence of radiation damage?
  - radiation damage and MAD
  - how to use it?
  - T-dependence of raddam and beamheating
  - raddam in SAXS experiments

• Radiation-induced changes and temperature-controlled cryo-crystallography to study macromolecular function
  - T-dependent disulfide-radical lifetime
  - trapping metastable species on the P450cam reaction pathway
  - radiolysis of substrate analogue of acetylcholinesterase
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Global indicators of radiation damage


**Resolution (Å)**

**Mosaicity (degrees)**

**Wilson B-factor (Å²)**

**Relative unit cell volume (%)**
Relative B-factor increase per residue type

Most affected by radiation damage:
- Cysteine residues
- Acidic residues (Glu, Asp)

Specific structural damage?
Data collection series

• crystals of acetylcholinesterase (AChE)

• ESRF undulator beam line ID14 - EH4

• 9 complete data sets (A - I) at 100 K

• dose: $10^7$ Gy/data set
  
  (for comparison:  natural dose for humans: 0.002 Gy/year
  lethal dose for humans: 5 Gy )

Carboxyl groups lose definition (ACHE, Glu306)

Decarboxylation?
Disulfide bond C254 - C265 breaks in AChE
Disulfide bond C402 - C521 does not break...
Disulfide bond C402 - C521 does not break ... but elongates
New rotamer position for C94 in HEWL

Grey: $2F_o-F_c$, 1.5 $\sigma$

Green: $F_o-F_c$, 3.5 $\sigma$

Ravelli & McSweeney (2000) Structure 8, 315
Damage to a Tyr and a Met in myrosinase

Glu409

Tyr330

Met393

Specific damage: primary or secondary event?

Primary damage

Solvent

Protein

Secondary damage
Primary events
at 12.7 keV ($\lambda=0.98$ Å)

Murray et al. (2005) J. Synchrotron Rad. 12, 268

- 98% of incident photons don’t interact at all
- 2% interact:
  - Elastic (Thomson) scattering (diffraction): 8%
  - Compton scattering: 8%
  - Photoelectric effect: 84%

  each photoelectron produces 500 ionization events

Ravelli et al. (2005) J. Synchrotron Rad. 12, 276
Specific damage is mostly secondary event

... because:

- only a few photons are absorbed per unit cell and per data set
- differential sensitivity for chemically identical groups (see disulfides)
- specific damage is temperature-dependent
Radiolysis of water

\[
\begin{align*}
\text{H}_2\text{O} & \xrightarrow{\text{Ionizing radiation}} \text{H}_2\text{O}^{+\cdot} + e^- \quad \text{(ionization)} \\
\text{H}_2\text{O} & \xrightarrow{\text{Ionizing radiation}} \text{H}_2\text{O}^* \quad \text{(electronic ionization)} \\
\text{H}_2\text{O}^{+\cdot} + \text{H}_2\text{O} & \rightarrow \text{H}_3\text{O}^+ + \cdot\text{OH} \\
e^- + n\text{H}_2\text{O} & \rightarrow e^-_{\text{aq}} \\
\text{H}_2\text{O}^* & \rightarrow \text{H}^\cdot + \cdot\text{OH} \\
e^-_{\text{aq}} + \text{H}^+ & \rightarrow \text{H}^\cdot
\end{align*}
\]

Scheme from Southworth-Davies & Garman (2007) *JSR* 14, 73
Temperature-dependence of radical mobility

\( T < 115 \, \text{K} \):
- \( \text{e}^- \) are mobile in amorph. ice

\( T > 115 \, \text{K} \):
- \( \text{e}^- \) and \( \text{H}^\bullet \) are mobile in amorph. ice

\( T > 130 \, \text{K} \):
- \( \text{e}^-, \text{H}^\bullet \) and \( \text{OH}^\bullet \) are mobile in cryst. Ice

\( T > 110 \, \text{K} \):
- \( \text{e}^-, \text{H}^\bullet \) and \( \text{OH}^\bullet \) are mobile in amorph. Ice


Sevilla, private comm.

Only electrons are mobile at 100 K
Decarboxylation of Glu/Asp

Oxidation of Glu/Asp by $e^-$ hole

$$R - \text{CH}_2 - \text{CO}_2^- \rightarrow R - \text{CH}_2 - \text{CO}_2^\bullet + e^-$$

$$R - (\text{CH}_2) - \text{CO}_2^\bullet \rightarrow R - \text{C}H_2 + \text{CO}_2$$

Ravelli & McSweeney (2000) Structure 8, 315

$\text{CO}_2$ formation
Can we see CO$_2$ in electron density maps?

Human butyrylcholinesterase

$F_o^E - F_o^A$, ± 5σ

Glu276

J.-Ph. Colletier, PhD thesis

T. californica acetylcholinesterase

$F_o^D - F_o^A$, ± 4σ

Trp279

Glu278

Colletier et al. (2008) *PNAS* 105, 11742

Possibly …
Gas formation after RT annealing

After irrad. at 100 K

After irrad. at 100 K + RT annealing

protein X

Mb

CO$_2$ ?  H$_2$ ?
Evidence for $\text{H}_2$ formation during X-irradiation in protein crystals

Gas chromatography of bubbles: 80% of bubbles is $\text{H}_2$

Meents et al. (2010) *PNAS* 107, 1094
Disulfide bond breakage

\[ \text{RSSR} + e^- \rightleftharpoons \text{RSS}^\cdot \text{R} \]

\[ \text{RSS}^\cdot \text{R} + H^+ \rightleftharpoons \text{RSH} + \cdot \text{SR} \]

\[ \text{RSS}^\cdot \text{R} + H^+ \xrightarrow{\text{+ flexibility}} \text{RSH} + \cdot \text{SR} \]

Favaudon et al. (1990) Biochemistry 29, 10978
## Disulfide-radical signatures

<table>
<thead>
<tr>
<th>Entity</th>
<th>S-S distance (calculated)</th>
<th>Absorption max. (in proteins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSSH</td>
<td>2.1 Å</td>
<td>-</td>
</tr>
<tr>
<td>HSSH&lt;sup&gt;-&lt;/sup&gt;</td>
<td>2.8 Å</td>
<td>425 - 440 nm</td>
</tr>
<tr>
<td>HSSH&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;•&lt;/sup&gt;</td>
<td>3.5 Å</td>
<td>400 nm</td>
</tr>
</tbody>
</table>

Favaudon *et al.* (1990) Biochemistry **29**, 10978  
Influence on specific damage of ...
... solvent accessibility of carboxyl groups

No correlation between solvent accessibility and radiation-sensitivity

Fioravanti et al. (2007) J. Synchrotron Rad. 12, 84
... calculated pKa of carboxyl groups

No correlation between pK_a and radiation-sensitivity
(see also Ravelli & McSweeney (2000) Structure 8, 315)

Fioravanti et al. (2007) J. Synchrotron Rad. 12, 84
... chemical environment of carboxyl groups

Low damage to Glu/Asp involved in salt bridges

Higher damage to Glu/Asp if H-bonded to Ser/Tyr/Thr

$F_0^C - F_0^A, \pm 3.5\sigma$

H. marismortui malate dehydrogenase

Fioravanti et al. (2007) J. Synchrotron Rad. 12, 84
... chemical environment of carboxyl groups

*T. thermophilus* lactae dehydrogenase, N. Coquelle, PhD thesis

Wild type

Ternary complex

Proximity of Trp influences radiation damage to Asp

\[ F_o^4 - F_o^1, \pm 0.1 \text{ e}^-/\text{Å}^3 \]
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Active site damage: *HmMaIDH*

Asp168 (deprotonated) in active site most sensitive

DNA photolyase


UV induced DNA damage

Essen (2006) COSB 16, 1

X-ray induced structural changes

FADH cofactor

CPD repair

DNA photolyase/CPD-DNA complex
Active site damage: bacteriorhodopsin

Matsui et al. (2002) J. Mol. Biol. 324, 469

Asp85 (deprotonated) in active site most sensitive
Active site damage: fluorescent protein IrisFP

Adam et al. (2009) JACS 131, 18063

X-ray induced (reversible) structural change of chromophore conformation
Radiation-induced alterations to other enzymatic active sites

Alphey et al. (2003) … photoreduction of the redox disulfide using synchrotron radiation … J Biol Chem. 278, 25919

Roberts et al. (2005) Oxidized and synchrotron cleaved structures of the disulfide redox center … Protein Sci 14, 2414

→ insight into mechanistic redox processes

Dubnovitsky et al. (2005) Strain relief at the active site … induced by radiation damage. Protein Sci. 14, 1489

→ release of active site strain during X-irradiation
Active sites are very radiation-sensitive

doit careful control experiments
before drawing biological conclusions
‘Spectroscopic’ damage to chromophore in BR

Matsui et al. (2002) J. Mol. Biol. 324, 469

- Formation of an inactive orange species
- ‘Spectroscopic’ damage occurs before structural damage
Online spectroscopy
as a complementary tool
to monitor radiation damage:

UV-vis
Raman
XAS

see talk by
Antoine Royant
Online UV-vis microspectrophotometer on ID14-4 (ESRF)

X-ray induced Fe reduction in MetMb crystals at 100 K

ESRF: McGeehan et al. (2009) JSR 16, 163
SLS: Owen et al. (2009) JSR 16, 173

Metal centers are reduced within seconds before full data set is collected

Ostermann, Parak & Weik
X-ray induced reduction of metalloproteins

Schlichting et al. (2000) The catalytic pathway of cytochrome P450cam at atomic resolution. Science 287, 1615
Adam et al. (2004) Structure of superoxide reductase ... upon X-ray-induced photo-reduction. Structure 12, 1729
Baxter et al. (2004) Specific radiation damage illustrates ... structural changes in the photosynthetic RC. JACS 126, 16728
Yano et al. (2005) X-ray damage to the Mn4Ca complex in single crystals of photosystem II ... PNAS 102 12047
Echalier et al. (2006) Activation and catalysis of the di-heme cytochrome c peroxidase ... Structure 14, 107
Pearson et al. (2007) Tracking X-ray-derived redox changes in crystals ... J Synchrotron Radiat 14, 92
Beittlich et al. (2007) Cryoradiolytic reduction of crystalline heme proteins ... J Synchrotron Radiat 14, 11
Kuhnel et al. (2007) Structure and quantum chemical characterization of chloroperoxidase compound 0 ... PNAS. 104, 99
Corbett et al. (2007) Photoreduction of the active site of the metalloprotein putidaredoxin by SR. Acta Crystallogr D63, 951

At ESRF:

rolling access applications for beamtime with online microspec every 6 months
Two other spectroscopic radical signatures often observed with online microspectrophotometry

500 - 600 nm: Hydrated electrons

400 nm: Disulfide radical anion

McGeehan et al. (2009) *J. Synchrotron Rad*; 16, 163
Weik et al. (2002) *J. Synchrotron Rad*. 9, 342

peak: glycerol, PEG
no peak: MPD, glucose
Combining XAS, UV-vis and crystallography (SRS Daresbury, UK)


‘Crystallography with online optical and X-ray absorption spectroscopies demonstrates an ordered mechanism in copper nitrite reductase’
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Important physical quantity in radiation damage context:

**absorbed dose** = absorbed energy / mass

1 Gray (Gy) = 1 J / kg

The higher the absorbed dose, the greater the damage

Don’t mix up with incident photo flux: photons / s
Is there a dose-rate effect (at 100 K)?

If global indicators of radiation damage are considered:

(mosaicity, unit-cell volume increase, $R_{\text{merge}}$ …)

No: Sliz et al. (2003) Structure 11, 13  

Yes: 10x higher dose-rate - 10% shorter crystal life time  
Owen et al. (2006) PNAS 103, 4912

If specific structural damage is considered: Yes, but small

Conclusion: at 100 K dose-rate effect is small compared to absorbed-dose effect

But: significant dose-rate effect at room temperature  
Southworth-Davies et al. (2007) Structure 15, 1531
Which absorbed dose kills your crystal?

Calculated *Henderson* limit
(estimated from EM observations)

Limit reached in:
- 2.5 months on home source
- 24hrs at 2nd generation synchrotron
- 5 min at 3rd gen. synchrotron undulator BL

(Murray et al (2004) JAC 37, 513)

Dose required
to reduce diffracted intensity by half:

\[ D_{1/2} = 2 \times 10^7 \text{ Gy} \]

Experimental *Garman* limit
(determined from X-ray data)

\[ D_{1/2} = 4.3 \times 10^7 \text{ Gy} \]

\[ D_{\ln2} = 3 \times 10^7 \text{ Gy} \] (recommended)


Owen *et al.* (2006) *PNAS* 103, 4912
Important: calculate absorbed dose …

… with RADDPOSE

Input:  
- protein and buffer composition and crystal content  
- X-ray beam energy, dimensions and flux

Output:  
- absorption coefficient  
- exposure time after which a critical dose is absorbed

Paithankar et al. (2005) J. Synchrotron Rad. 16, 152

Get program from:  
Raimond Ravelli (r.b.g.ravelli@lumc.nl)  
Elspeth Garman (elspeth@biop.ox.ac.uk)
Minimize raddam: Composite data sets

Spread of X-ray dose over many crystals

Oxidized redox intermediate in HRP

Reduction by X-ray - generated electrons

Berglund et al. (2002) Nature 417, 463
Minimize raddam: Composite data sets

Spread of X-ray dose over different parts of a long crystal

Important observation: Raddam limited to irradiated area (at 100 K)


Scavenger: **Vitamin C** keeps your crystal fit


1st data set

Crystal without ascorbate

6th data set

Crystal with 1 M ascorbate

Cys76-Cys94 disulfide bond in HEWL

Further effective scavengers:

- **Quinone**, **TEMP**, **reduced DTT** (Southworth-Davies & Garman (2007) JSR **14**, 73)
- **Potassium hexacyanoferrate** (Macedo *et al.* (2009) JSR. **16**, 191)
Is radiation damage wavelength-dependent?

Theoretically: yes (slightly)

Experimentally: no

\[ \lambda = 1 \text{ Å} \quad \text{versus} \quad \lambda = 2 \text{ Å} \]


Same qualitative and quantitative damage …

… if far from any absorption edge

Absorption of SeMet protein crystal

Dose efficiency 34% higher at LER compared to peak wavelength

Murray et al. (2005) J. Synchrotron Rad. 12, 268

Related comment: Back soak heavy-atom soaked crystals
Radiation damage: particularly harmful in MAD

... because:

- high absorption of anomalous scatterers (e.g. Se) used for phasing
- induces non-isomorphism (e.g. unit-cell volume increase)

Radiation damage can swamp anomalous signal: minimize dose

Gonzalez et al. (2005) J. Synchrotron Rad. 12, 285
Ravelli et al. (2005) J. Synchrotron Rad. 12, 276
How to use radiation damage?

RIP : Radiation damage-induced phasing
Ravelli et al. (2003) Structure 11, 217

Low-dose data set A → X-ray burn → Low-dose data set B
(1 $10^7$ Gy)

Radiation-induced structural changes used then for phasing (similar to SIR)

See also UV-RIP : UV-induced damage used for phasing
Radiation damage and crystallographic software


see talk by
Sasha Popov
Temperature dependence of radiation damage

$T < 100 \text{ K}$
(He - cooling - 40 K)

- Specific and non-specific protein damage:
  - small/no dependence
  - (max 2 - 4-fold reduction)

- Reduction of metal sites:
  - large dependence
  - (30-fold reduction)

$T > 100 \text{ K}$
(N$_2$ - cooling)

- large dependence

Meents et al. (2010) PNAS 107, 1094

Weik et al. (2006) Protein Sci. 10, 1953
Borek et al. (2007) JSR 14, 24
Colletier et al. (2008) PNAS 105, 11742
Crystal heating by X-ray beam?

Around 5 K at 100 K on 3rd generation synchrotron undulator BL

Experimentally: Snell et al. (2007) JSR 14, 109

Theoretically: Mhaisekar et al. (2005) JSR 12, 318
Radiation damage in SAXS experiments

Kuwamoto et al. (2004) J. Synchrotron Rad. 11, 462

Radius of gyration increases

Aggregation occurs

400 Gy: critical dose
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Metastable species on the P450cam reaction pathway


Cytochrome P450cam catalyses hydroxylation of camphor: $2e^-$ redox reaction
Metastable species on the P450cam reaction pathway


Electron generation by X-irradiation (1.5 Å) for 3h

Thawing: increasing protein and substrate flexibility, backbone flip
Radiolysis of substrate analogue in acetylcholinesterase

- hydrolyses acetylcholine into choline and acetate in CNS
- involved in Alzheimer disease
- very rapid enzyme: turnover 20 000 s$^{-1}$

Colletier et al. (2006) *EMBO J.* 25, 2746
Consecutive data sets at 100 and at 150 K:
(ID14-4, ESRF)

100 K: A, B, C, D
cumul. dose: $0.92 \times 10^7$ Gy

150 K: A, B, C
cumul. dose: $0.95 \times 10^7$ Gy

$= \frac{1}{3}$ Garman limit ($3 \times 10^7$ Gy)
Owen et al. (2006) PNAS 103, 4912
choline reorientation
Backdoor movement

\[ F_{0}^{100 \text{D}} - F_{0}^{100 \text{A}} (+, - 4\sigma) \]

\[ F_{0}^{150 \text{C}} - F_{0}^{150 \text{A}} (+, - 4\sigma) \]

Colletier, Bourgeois, Sanson, Fournier, Sussman, Silman & Weik (2008) PNAS, 105, 11742
Radiation damage control - native AChE at 150 K

\[ F_{0}^{150 \text{ C}} - F_{0}^{150 \text{ A}} (+, - 4\sigma) \]
Backdoor movement

\[ F_{o, 100 \text{D}} - F_{o, 100 \text{A}} (\, +, \, - 4\sigma) \]

\[ F_{o, 150 \text{C}} - F_{o, 150 \text{A}} (\, +, \, - 4\sigma) \]

Summary

- Synchrotron radiation produces specific damage even at 100 K
  - decarboxylation of Glu/Asp
  - disulfide bond breakage or elongation
  - damage to Tyr, Met …

- Specific damage is mostly secondary effect

- Active sites are particularly radiation-sensitive, metalloproteins get reduced

- Great tool: combination of X-ray crystallography and online spectroscopy

- Practical issues:
  - (absence of major) dose-rate effect, dose limits, \( \lambda \) (in)dependence, scavengers, raddam and MAD, RIP, T-dependence, raddam in SAXS

- specific radiation damage and T-controlled crystallography to study biology
Merci à …

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