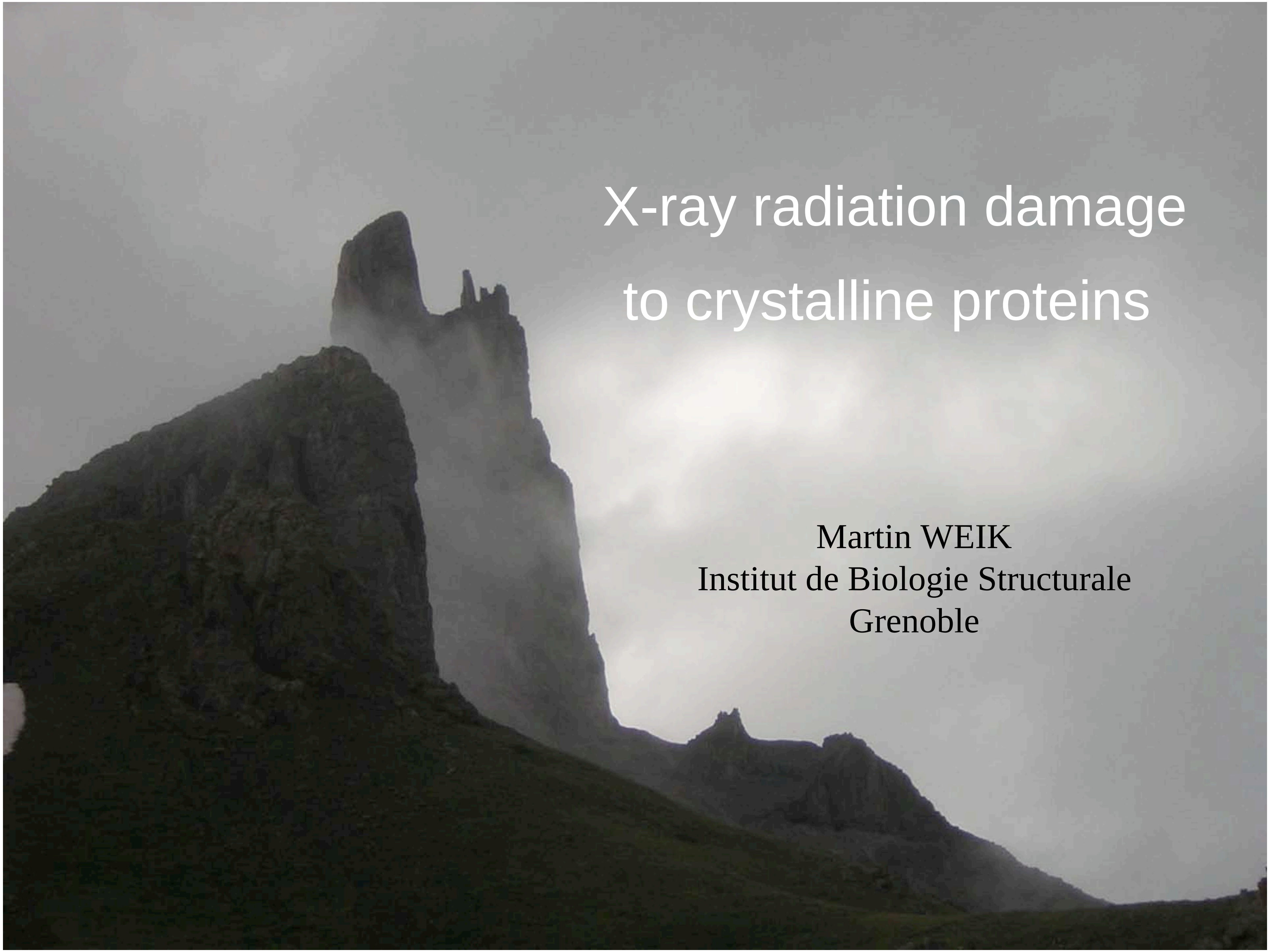
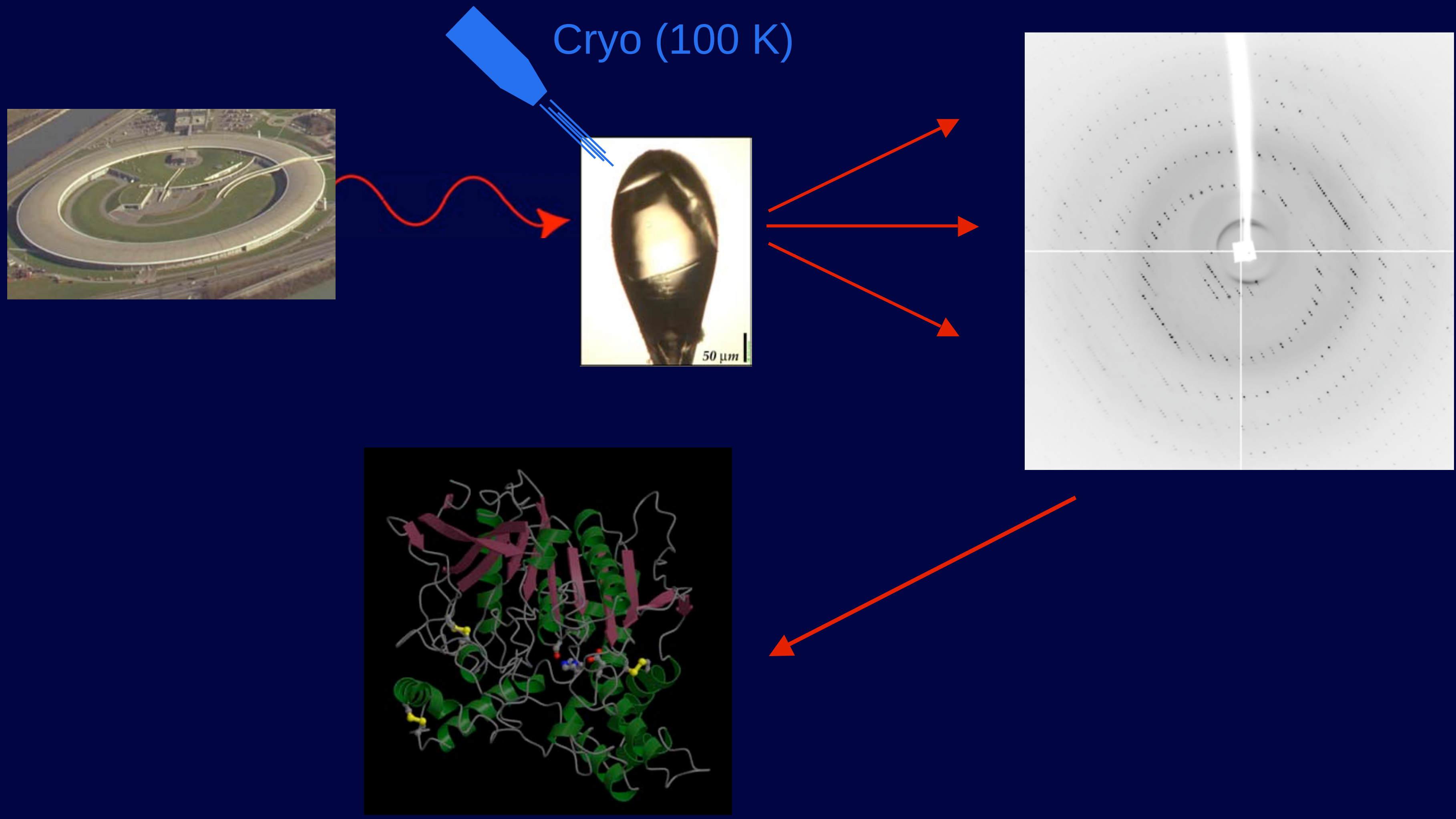


X-ray radiation damage to crystalline proteins

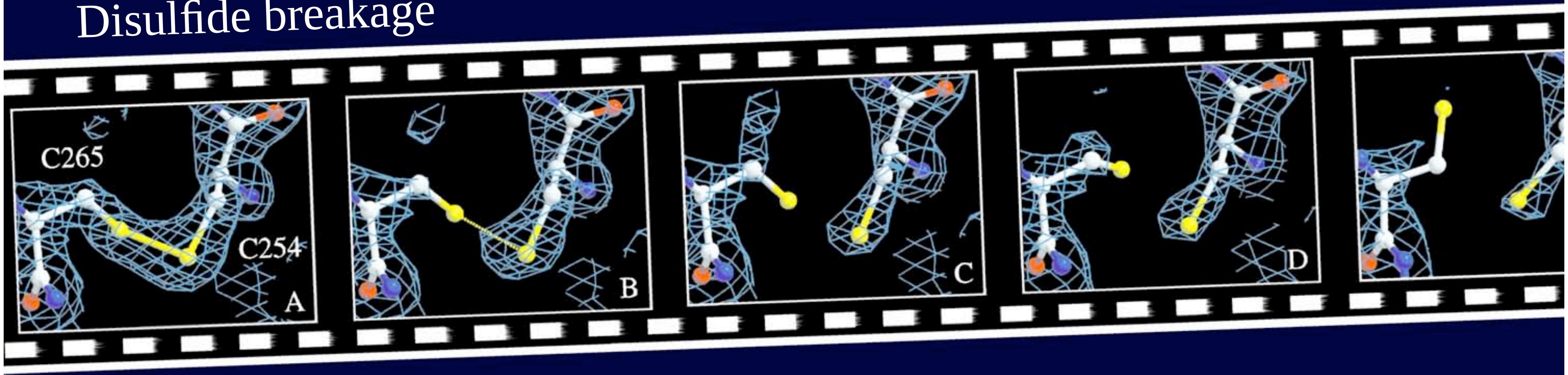
Martin WEIK
Institut de Biologie Structurale
Grenoble

Protein X-ray crystallography



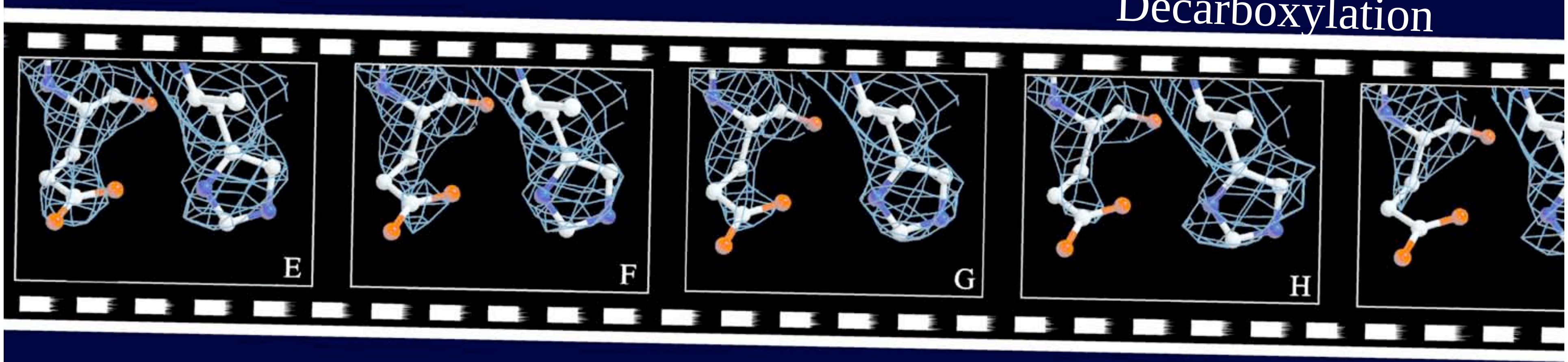
Synchrotron radiation is a powerful tool, but price must be paid

Disulfide breakage



Radiation damage to proteins

Decarboxylation



Outline

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

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  - bacteriorhodopsin, malate dehydr, cholinesterase, DNA photolyase, IrisFP
  - redox proteins (e.g. metalloproteins)
  - online spectroscopy complements crystallography

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- 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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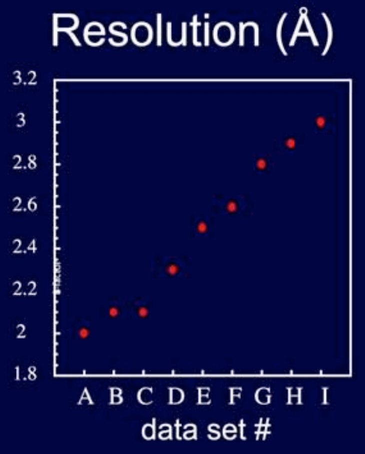
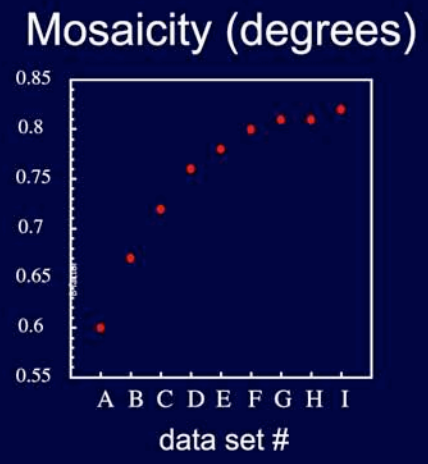
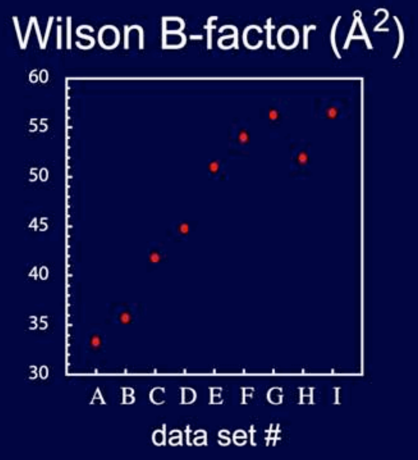
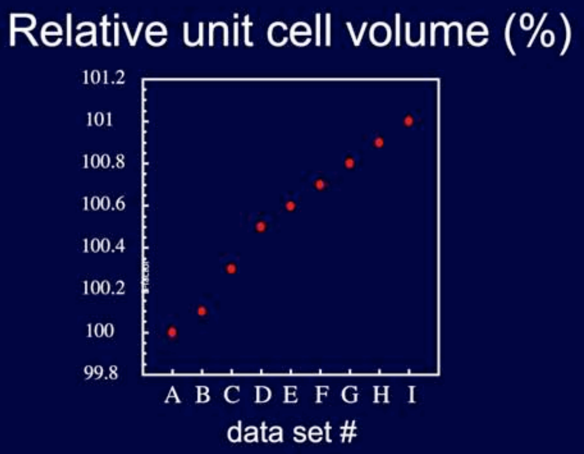
Global indicators of radiation damage

Gonzalez & Nave (1994) *Acta Crys. D* **50**, 874





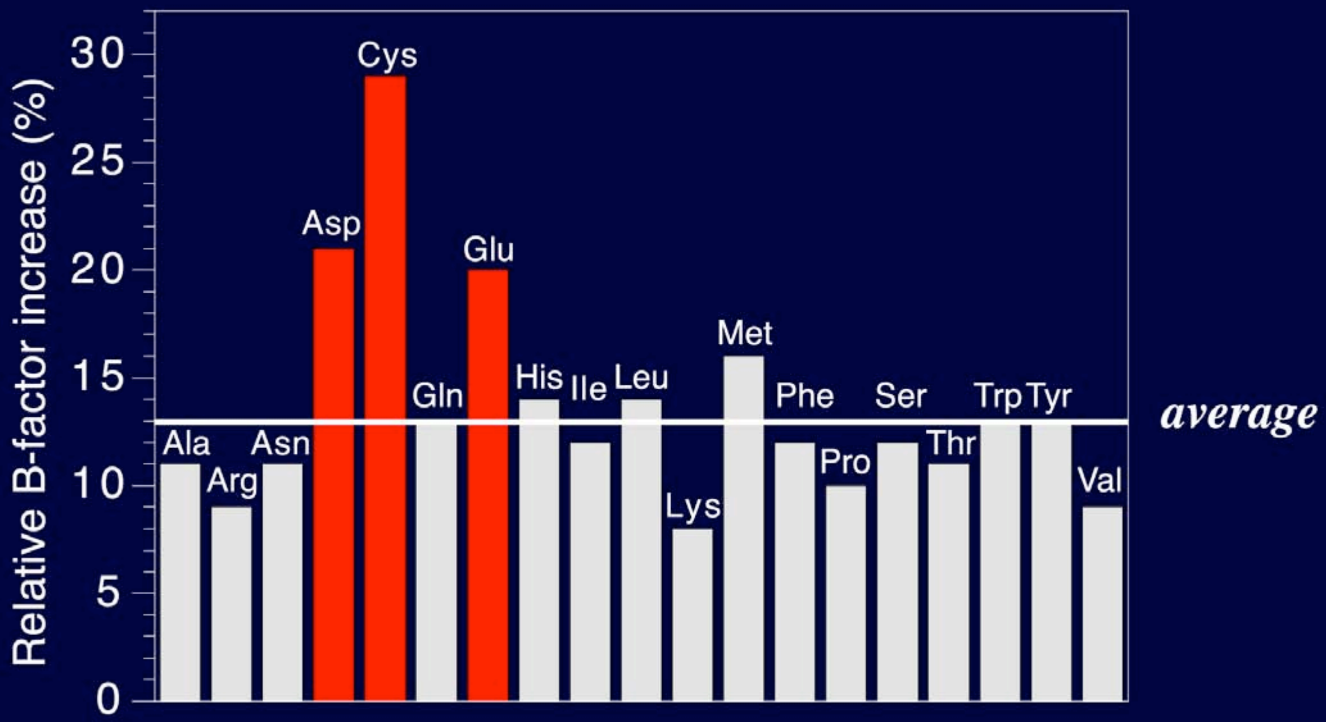




Ravelli & McSweeney (2000) *Structure* **8**, 315

Ravelli *et al.* (2002) *J. Synchrotron Rad.* **9**, 355

Relative B-factor increase per residue type



Most affected by radiation damage :

- Cysteine residues
- Acidic residues (Glu, Asp)

Specific structural damage ?

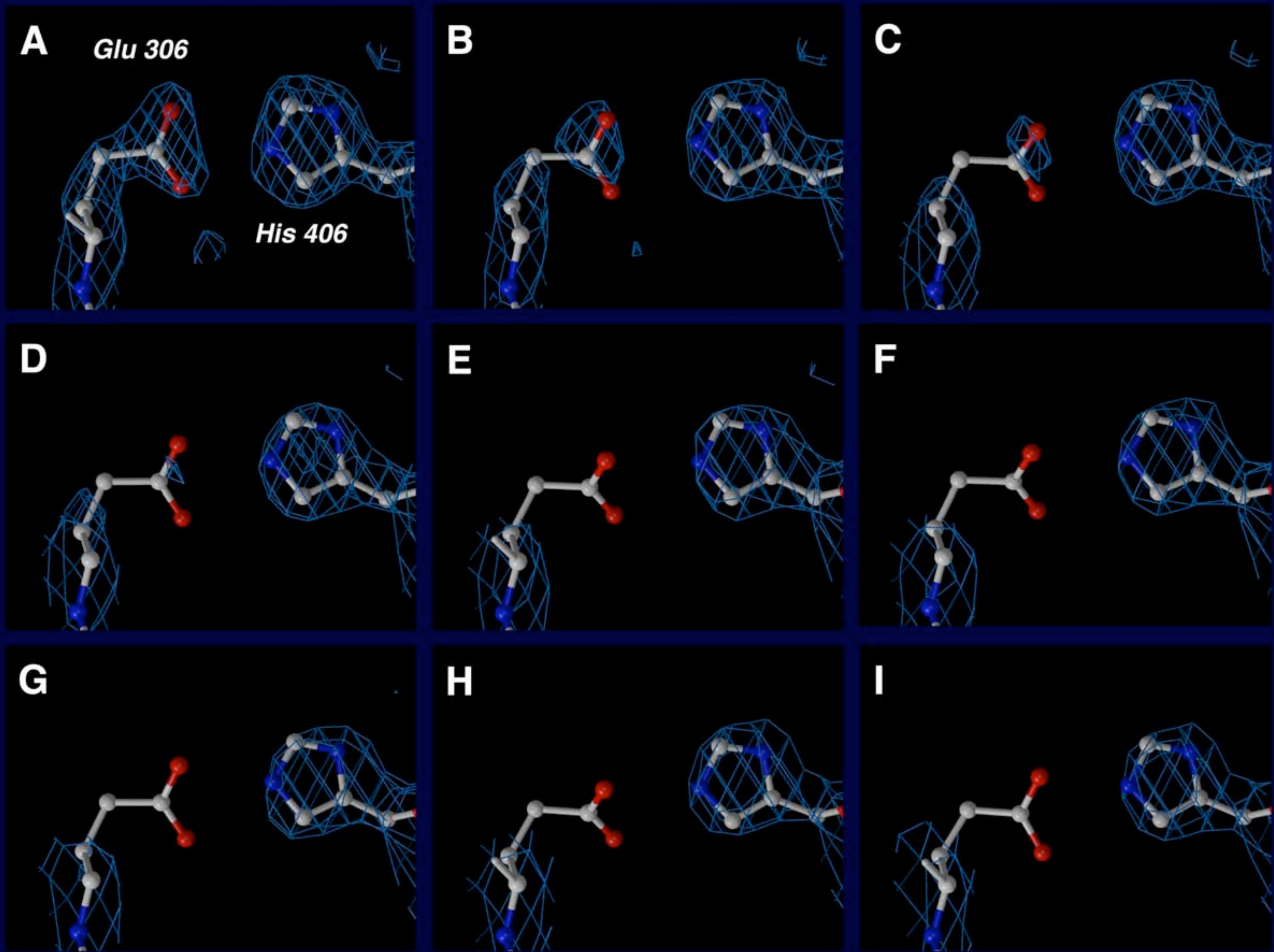
Data collection series

- 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)

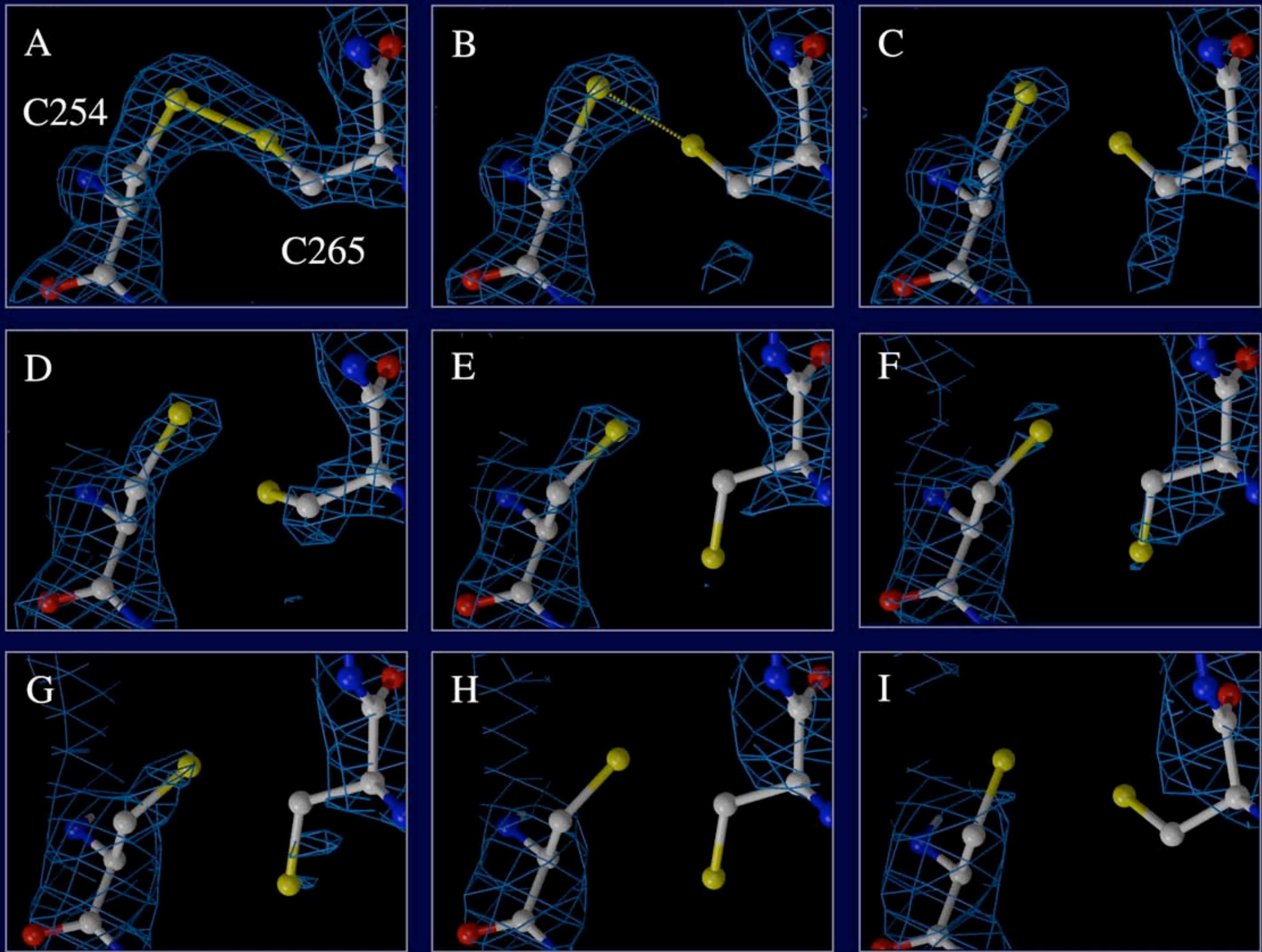
Weik, Ravelli, Kryger, McSweeney, Raves, Harel, Gros, Silman, Kroon & Sussman (2000) PNAS **97**, 623

Carboxyl groups lose definition (ACHE, Glu306)

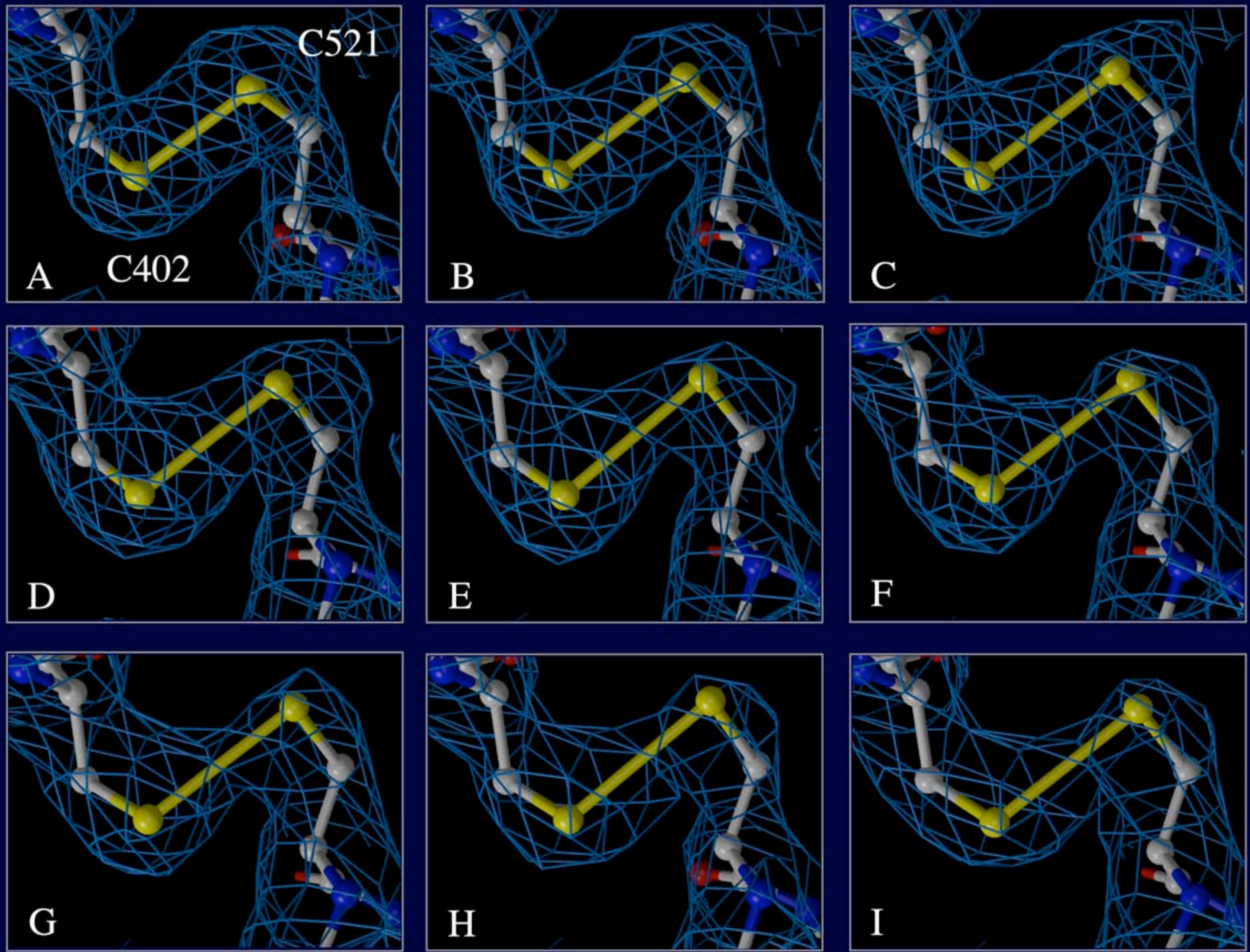


Decarboxylation ?

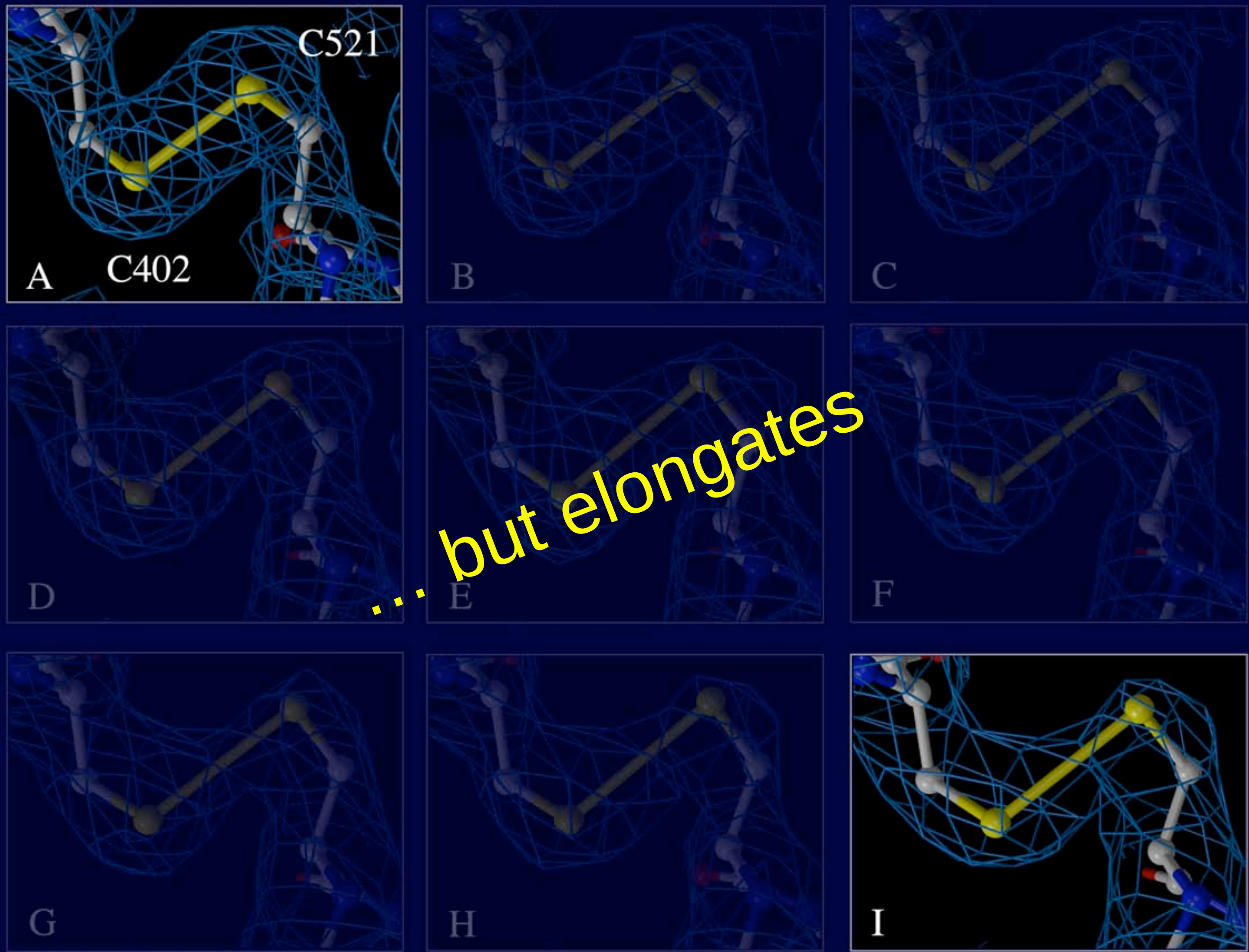
Disulfide bond C254 - C265 breaks in AChE



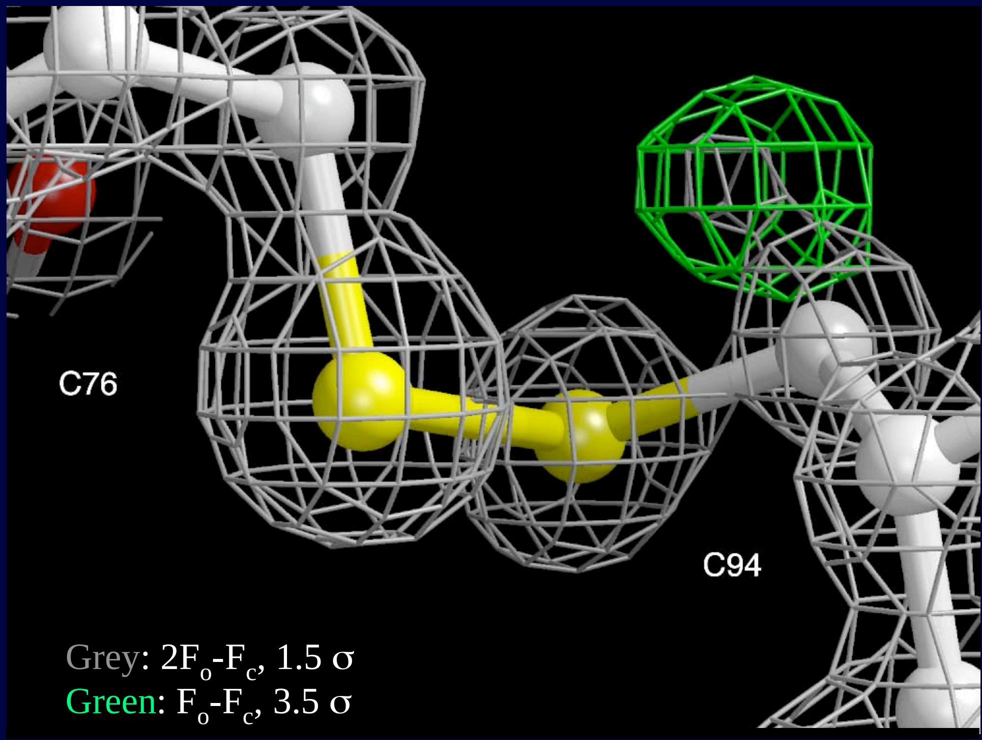
Disulfide bond C402 - C521 does not break ...



Disulfide bond C402 - C521 does not break ...



New rotamer position for C94 in HEWL



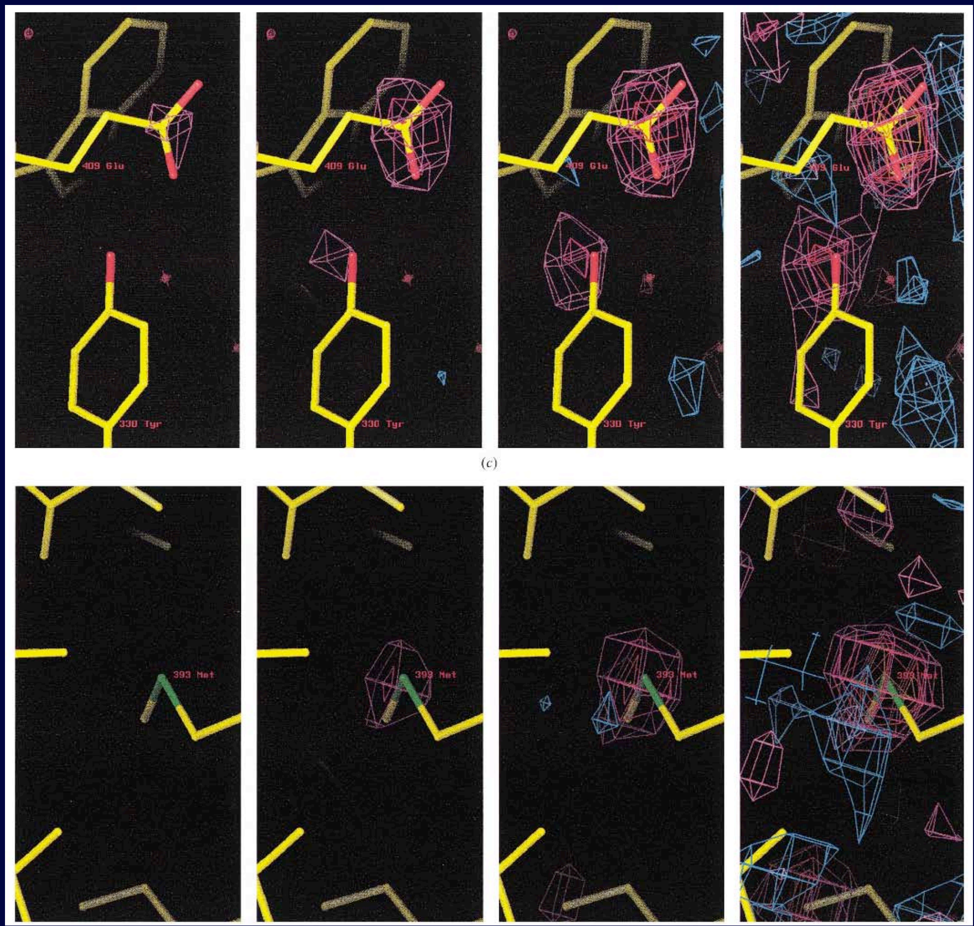
Ravelli & McSweeney (2000) Structure **8**, 315

Damage to a Tyr and a Met in myrosinase

Glu409

Tyr330

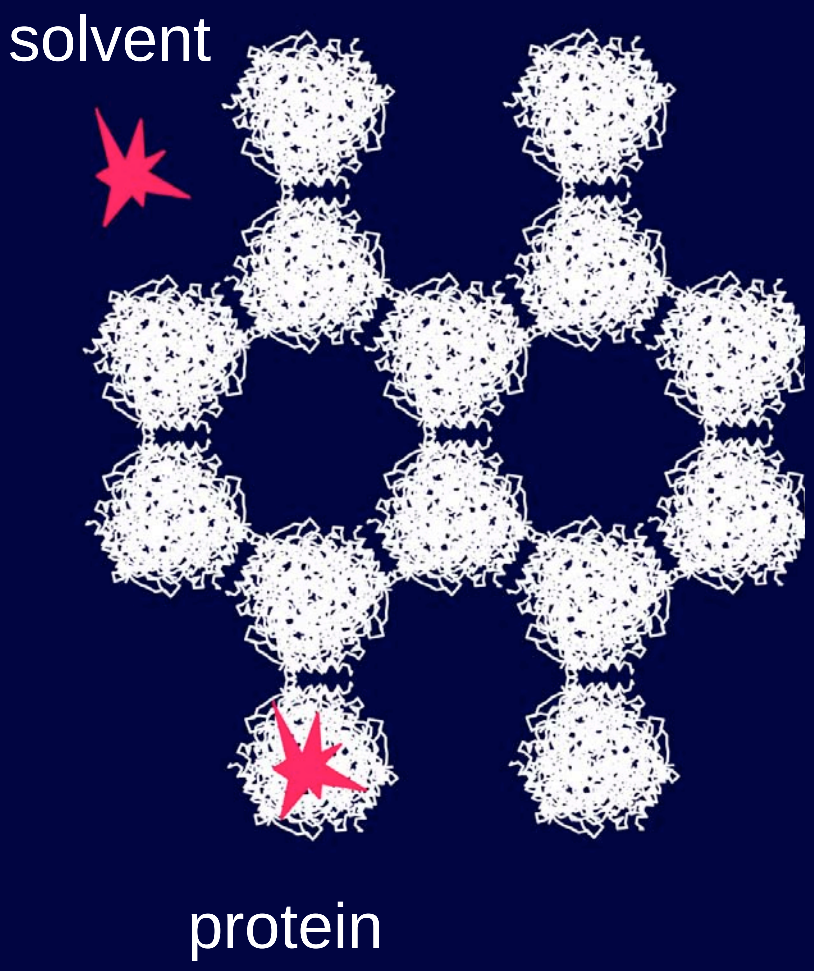
Met393



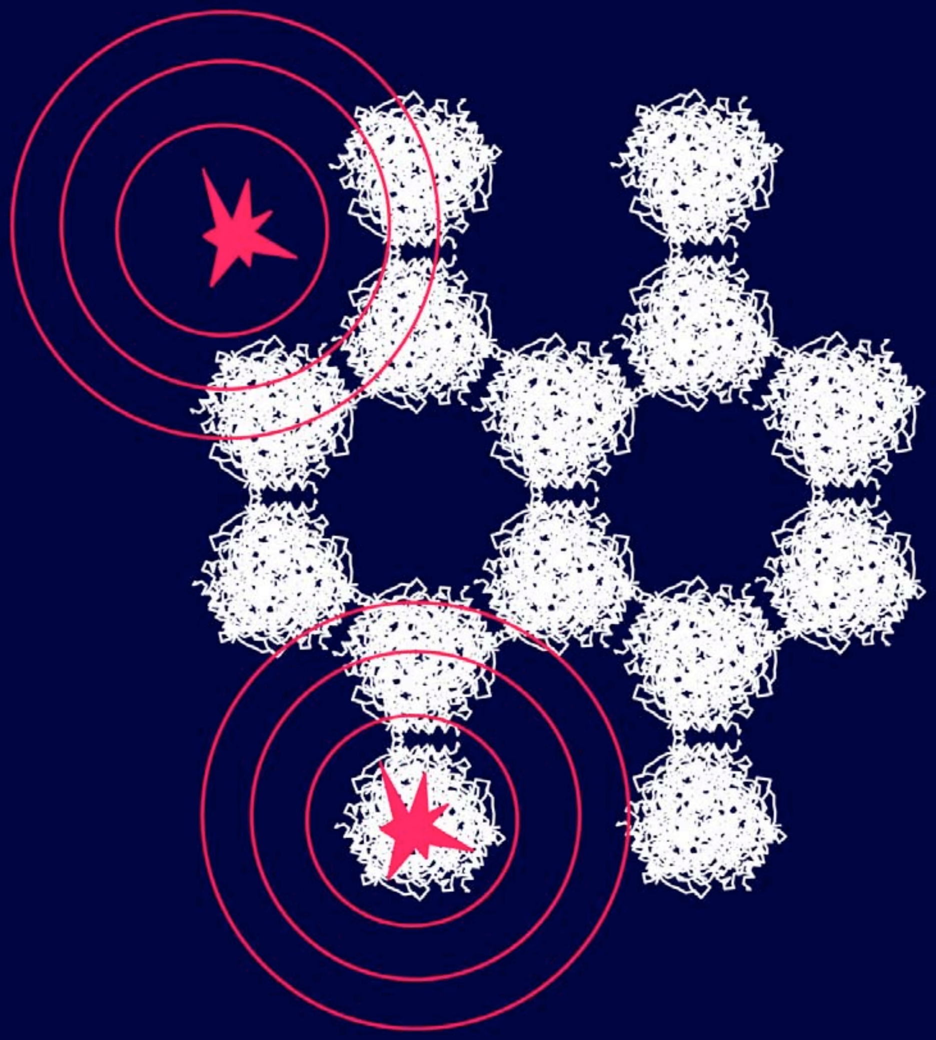
Burmeister (2000) *Acta Cryst.* D**56**, 328

Specific damage: primary or secondary event?

Primary damage



Secondary damage



Primary events

at 12.7 keV (λ=0.98 Å)

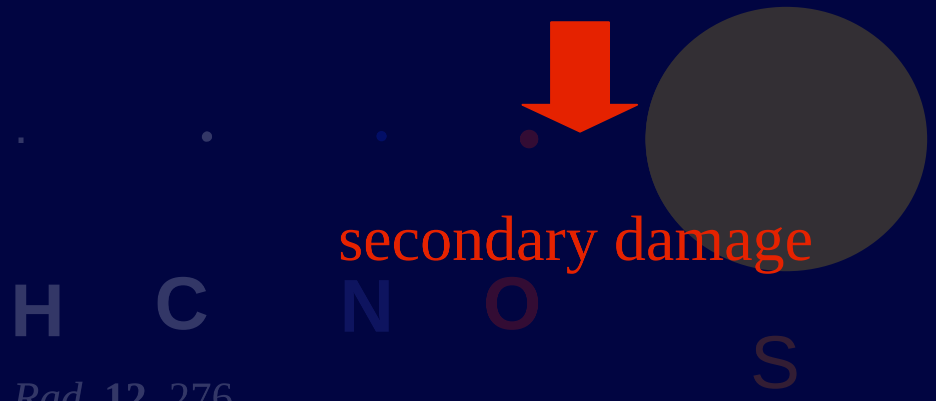
Murray *et al.* (2005) *J. Synchrotron Rad.* **12**, 268

- 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

Cross sections:



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

$$H_2O \xrightarrow{\text{Ionizing radiation}} H_2O^{+\bullet} + \boxed{e^-} \quad \text{(ionization)}$$

$$H_2O \xrightarrow{\text{Ionizing radiation}} H_2O^{*} \quad \text{(electronic ionization)}$$

$$H_2O^{+\bullet} + H_2O \longrightarrow H_3O^{+} + \boxed{{}^{\bullet}OH}$$

$$e^- + nH_2O \longrightarrow e^-_{aq}$$

$$H_2O^{*} \longrightarrow H^{\bullet} + {}^{\bullet}OH$$

$$e^-_{aq} + H^{+} \longrightarrow \boxed{H^{\bullet}}$$

Scheme from Southworth-Davies & Garman (2007) *JSR* **14**, 73

Temperature-dependence of radical mobility

T < 115 K : e^- are mobile in amorph. ice

T > 115 K : e- and H• are mobile in amorph. ice

Fisher and Devlin (1995) J. Phys. Chem. **99**, 11584

T > 130 K : e^-, H• and OH• are mobile in cryst. Ice

Symons (1999) Progr. Reaction Kinetics and Mechanisms **24**, 139

T > 110 K : e^-, H• and OH• 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

$$R - CH_2 - CO_2^- \longrightarrow R - CH_2 - CO_2^{\bullet} + e^-$$

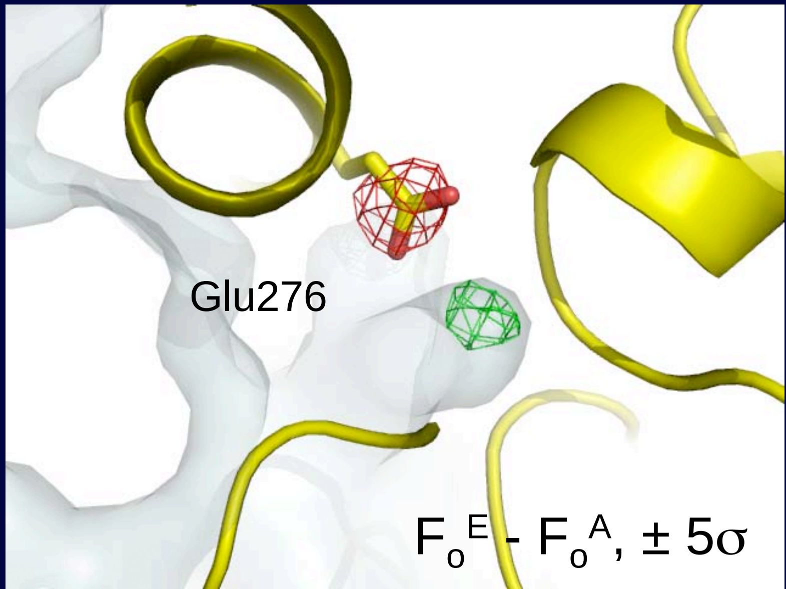
$$R - (CH_2) - CO_2^{\bullet} \longrightarrow R - {}^{\bullet}CH_2 + \boxed{CO_2}$$

Sevilla *et al*. (1979) *J. Phys. Chem*. **83**, 2887.
Ravelli & McSweeney (2000) *Structure* **8**, 315

CO_2 formation

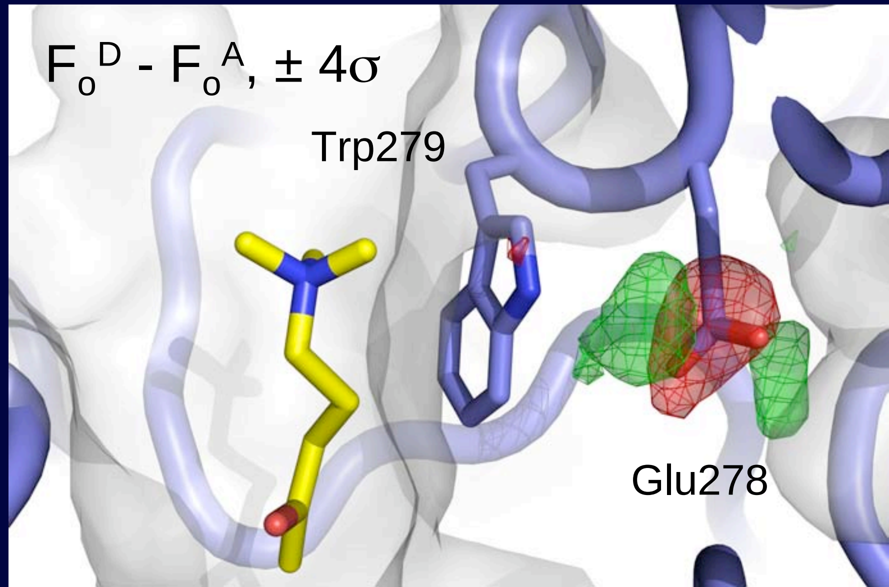
Can we see CO_2 in electron density maps ?

Human butyrylcholinesterase



J.-Ph. Colletier, PhD thesis

T. californica acetylcholinesterase



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

Possibly …

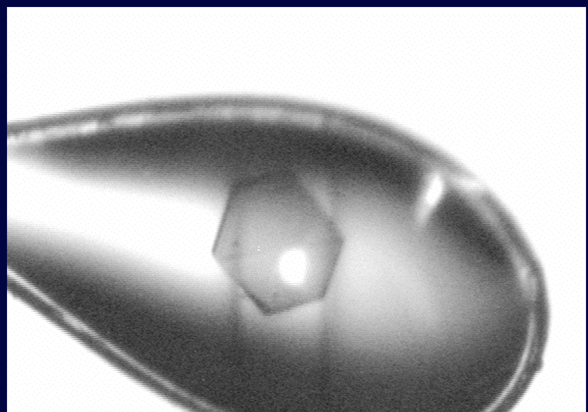
Gas formation after RT annealing

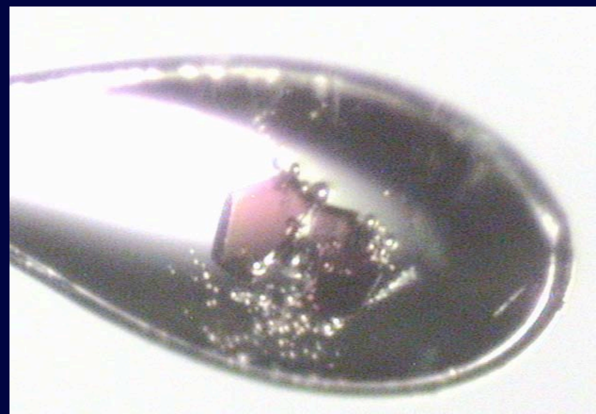
After irrad. at 100 K

After irrad. at 100 K
+ RT annealing

protein X

Mb





CO_2 ? H_2 ?

Evidence for H_2 formation during X-irradiation in protein crystals

Gas chromatography of bubbles: 80% of bubbles is H_2

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

Disulfide bond breakage

$$\mathrm{RSSR} + e^{-} \rightleftharpoons \mathrm{RSS}^{\bullet -}\mathrm{R}$$

$$\mathrm{RSS}^{\bullet -}\mathrm{R} + \mathrm{H}^{+} \rightleftharpoons \mathrm{RSH} + {}^{\bullet}\mathrm{SR}$$

$$\mathrm{RSS}^{\bullet -}\mathrm{R} + \mathrm{H}^{+} \xrightarrow{+\ \text{flexibility}} \mathrm{RSH} + {}^{\bullet}\mathrm{SR}$$

Favaudon *et al*. (1990) Biochemistry **29**, 10978

Disulfide-radical signatures

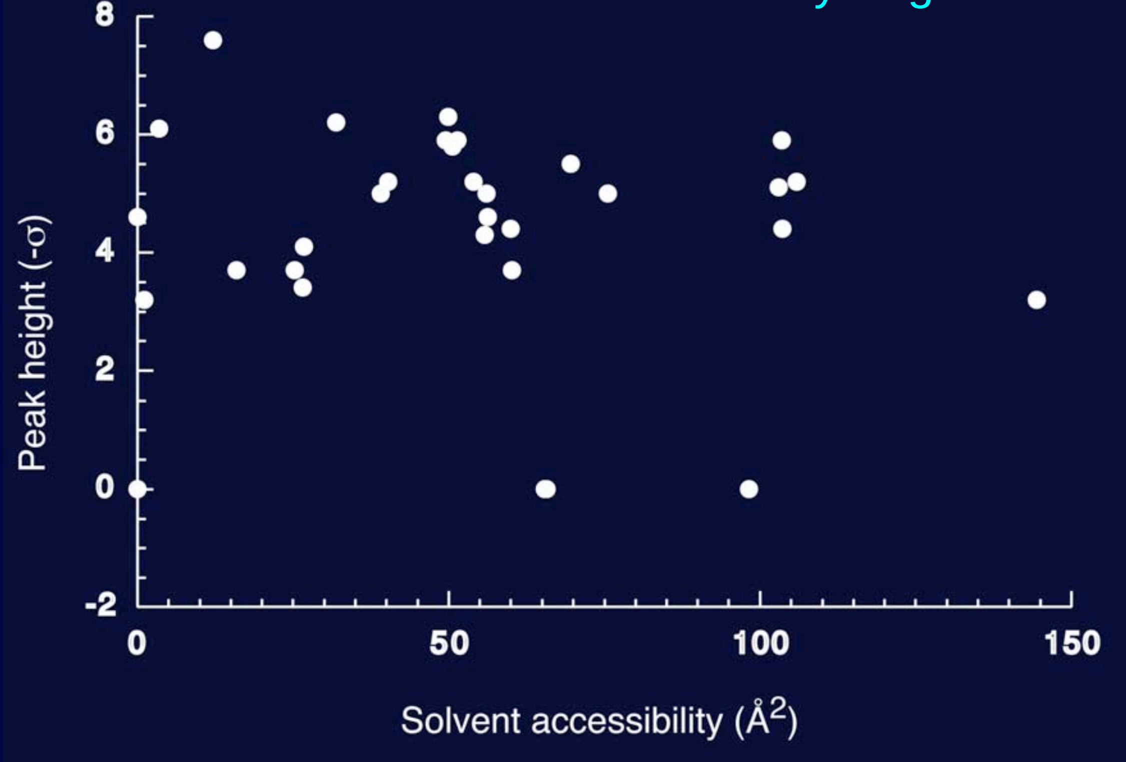
Entity	S-S distance (calculated)	Absorption max. (in proteins)
HSSH	2.1 Å	-
$HSSH^{\bullet -}$	2.8 Å	425 - 440 nm
$HSSH_2^{\bullet}$	3.5 Å	400 nm

Favaudon *et al*. (1990) Biochemistry **29**, 10978
Bergés *et al*. (2000) Nukleonika **45**, 23

Influence on specific damage of …

… solvent accessibility of carboxyl groups

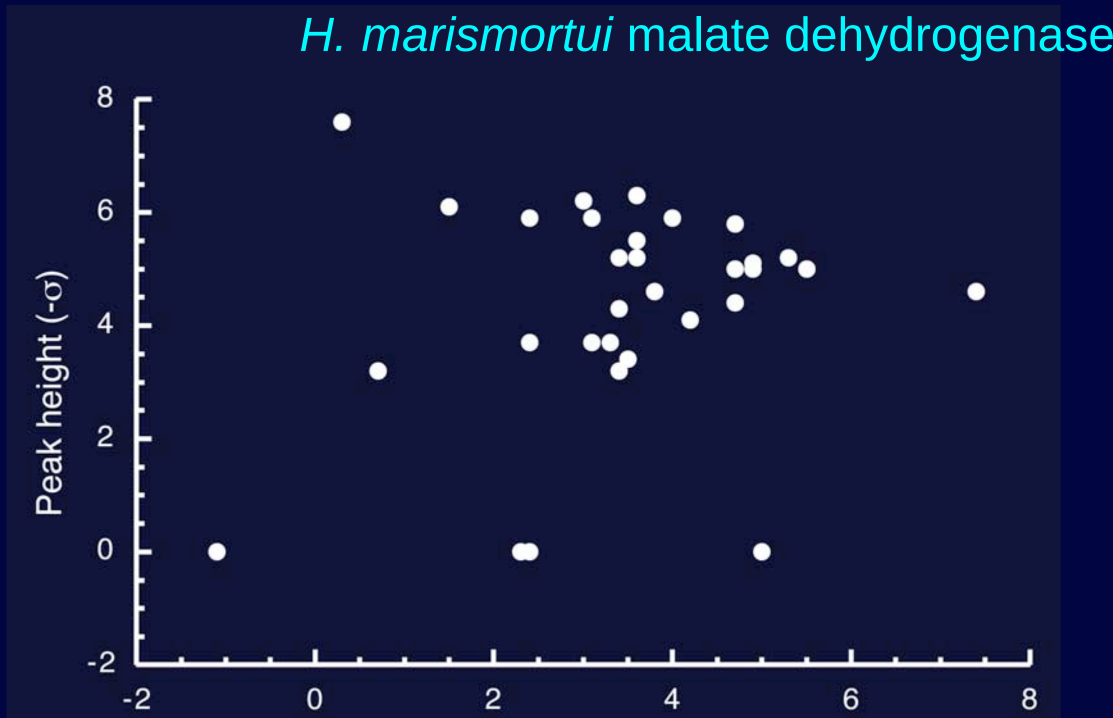
H. marismortui malate dehydrogenase



No correlation between solvent accessibility and radiation-sensitivity

Fioravanti *et al.* (2007) *J. Synchrotron Rad.* **12**, 84

… calculated pKa of carboxyl groups



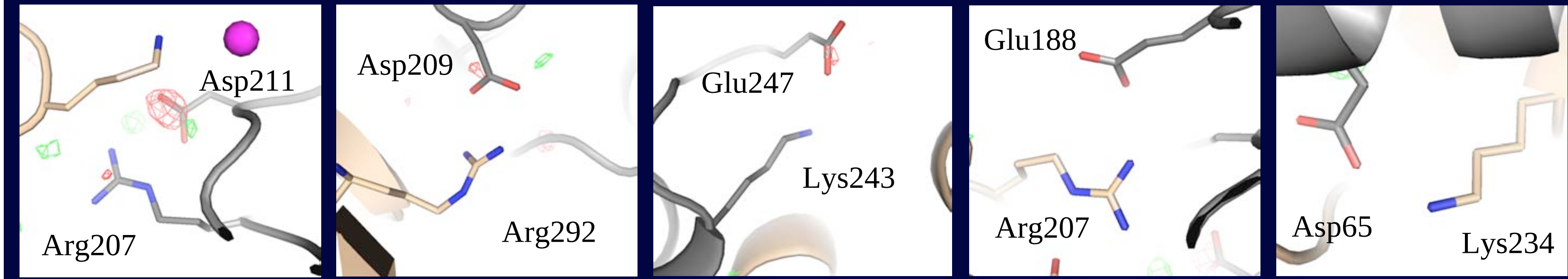
No correlation between pK_a and radiation-sensitivity

(see also Ravelli & McSweeney (2000) Structure **8**, 315)

Fioravanti *et al.* (2007) *J. Synchrotron Rad.* **12**, 84

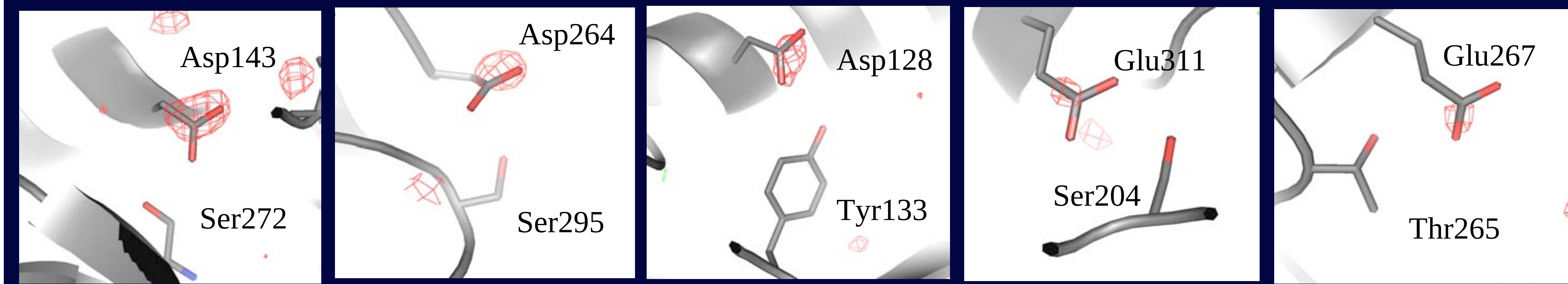
… chemical environment of carboxyl groups

Low damage to Glu/Asp involved in salt bridges



$F_o^C - F_o^A$, ± 3.5σ

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

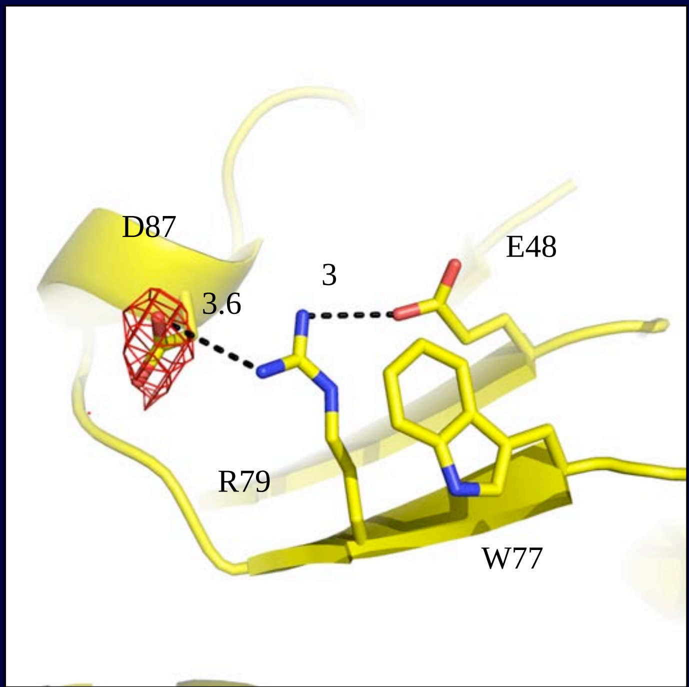


H. marismortui malate dehydrogenase Fioravanti *et al.* (2007) *J. Synchrotron Rad.* **12**, 84

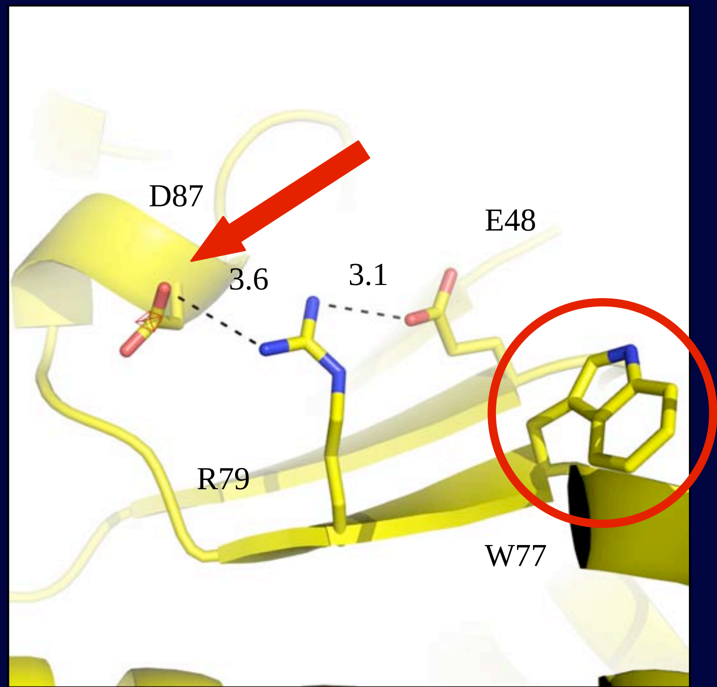
… chemical environment of carboxyl groups

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

Wild type



Ternary complex



Proximity of Trp
influences radiation damage to Asp

$F_o^4 - F_o^1$, ± 0.1 $e^-/Å^3$

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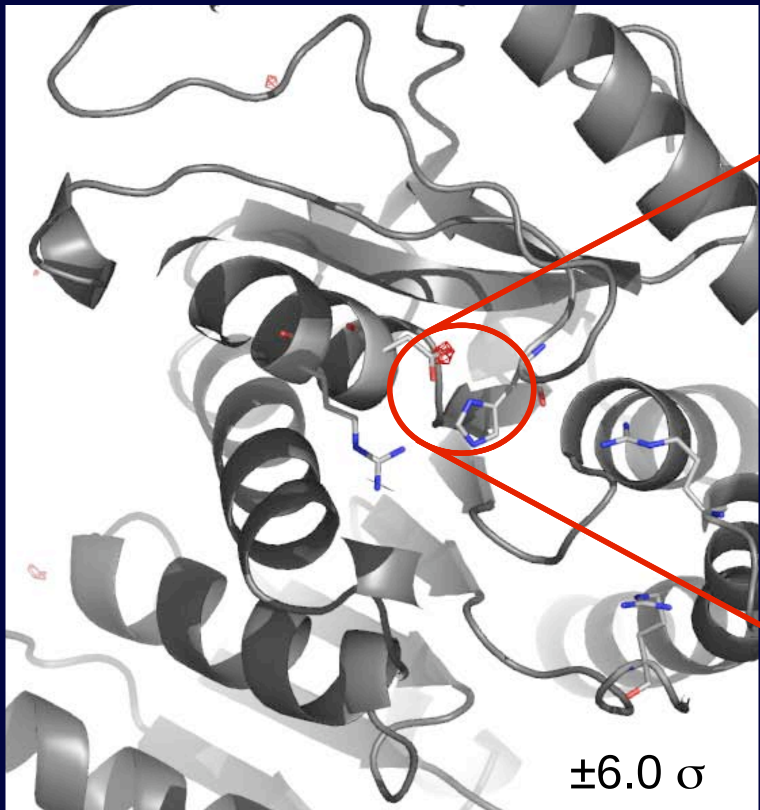
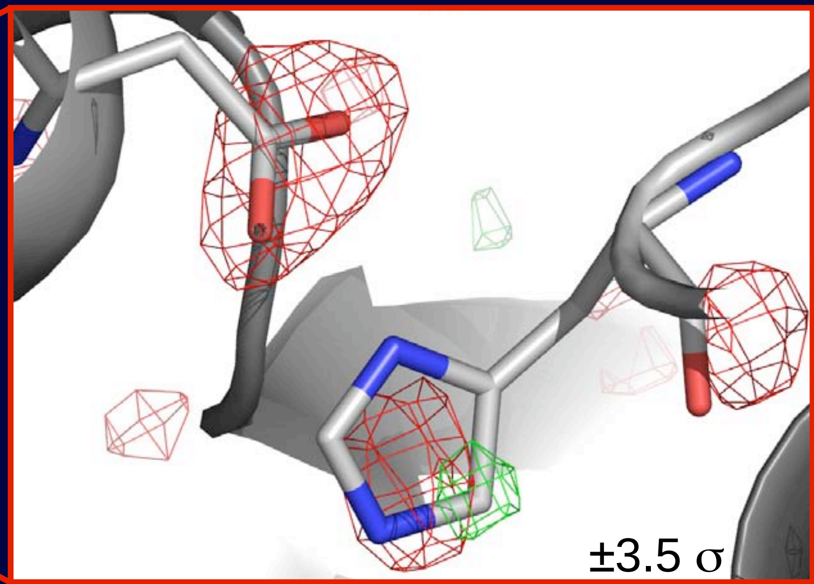
Active site damage: *Hm*MalDH





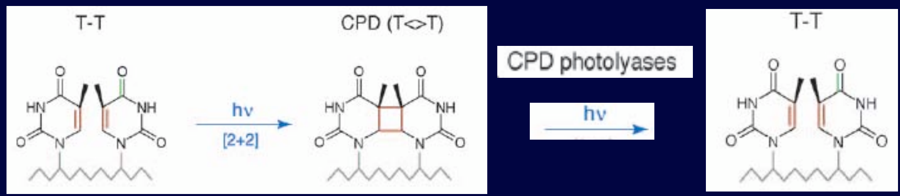
Asp168 (deprotonated) in active site most sensitive

Fioravanti *et al.* (2007) *J. Synchrotron Rad.* **12**, 84

DNA photolyase

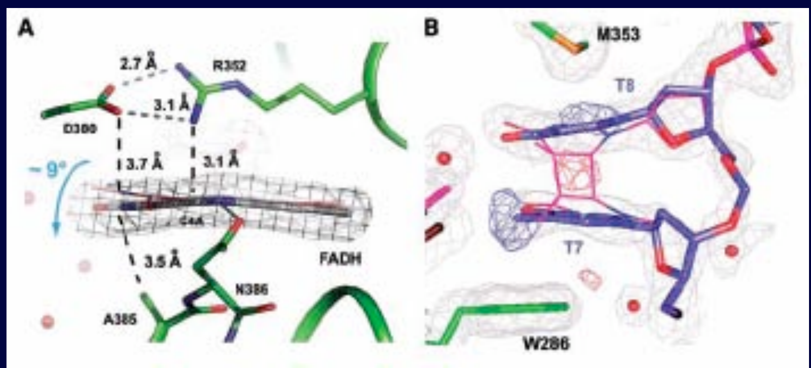
Mees *et al*. (2004) Science 306, 1789

UV induced DNA damage



Essen (2006) COSB 16, 1

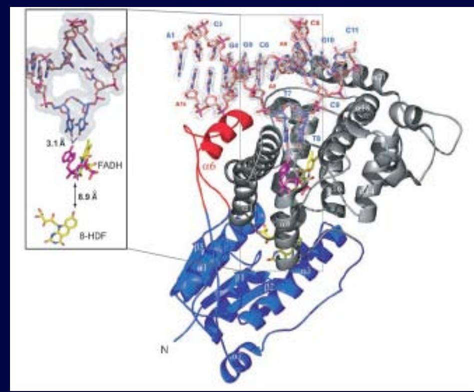
X-ray induced structural changes



FADH cofactor

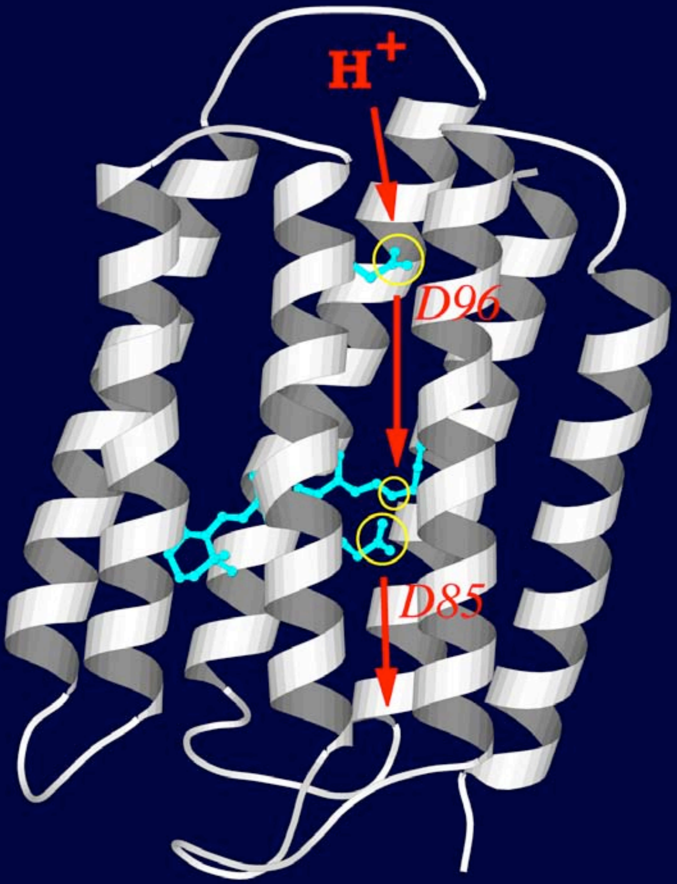
CPD repair

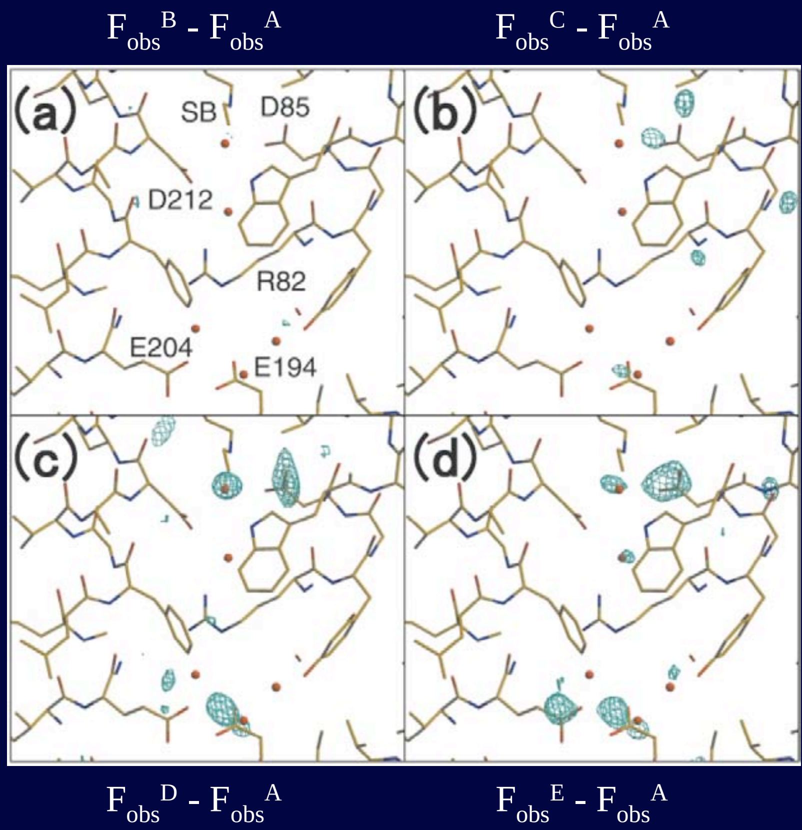
DNA photolyase/CPD-DNA complex



Active site damage: bacteriorhodopsin

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

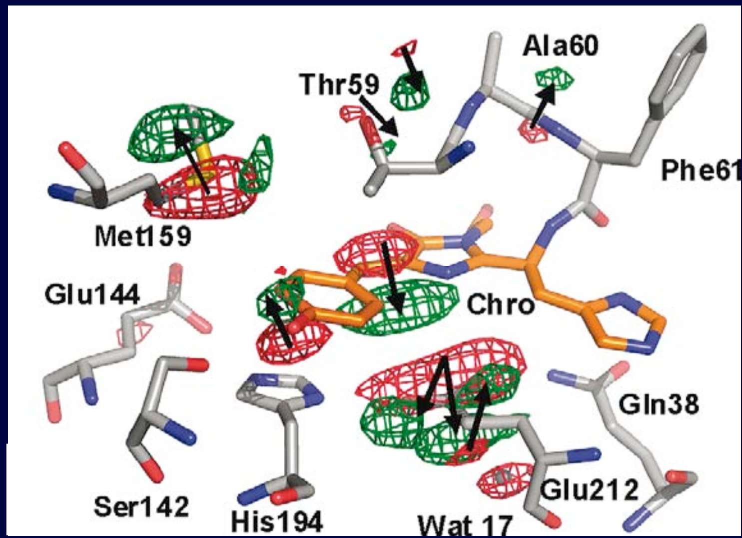




Asp85 (deprotonated) in active site most sensitive

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

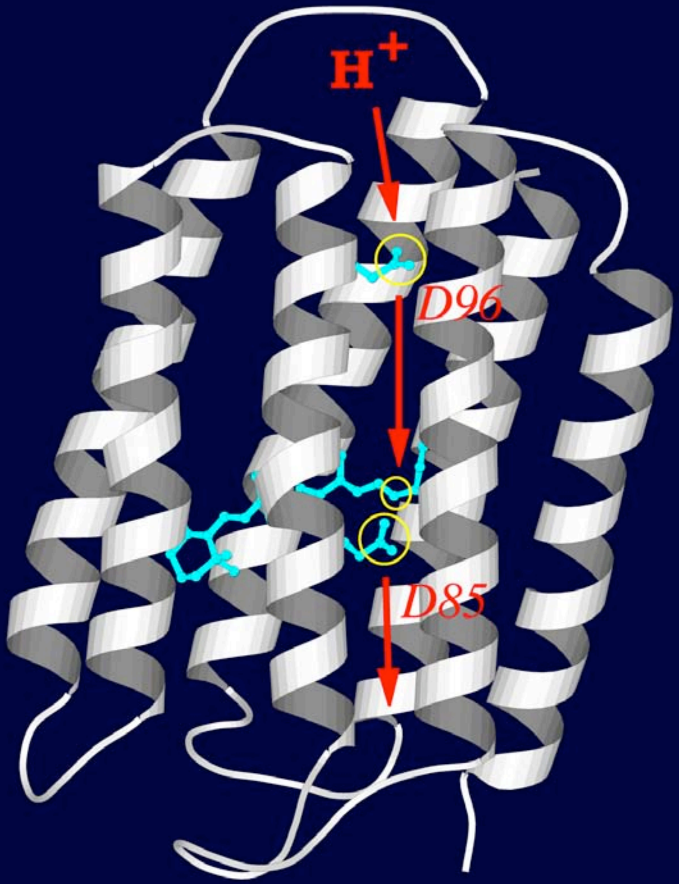
Active sites are very radiation-sensitive

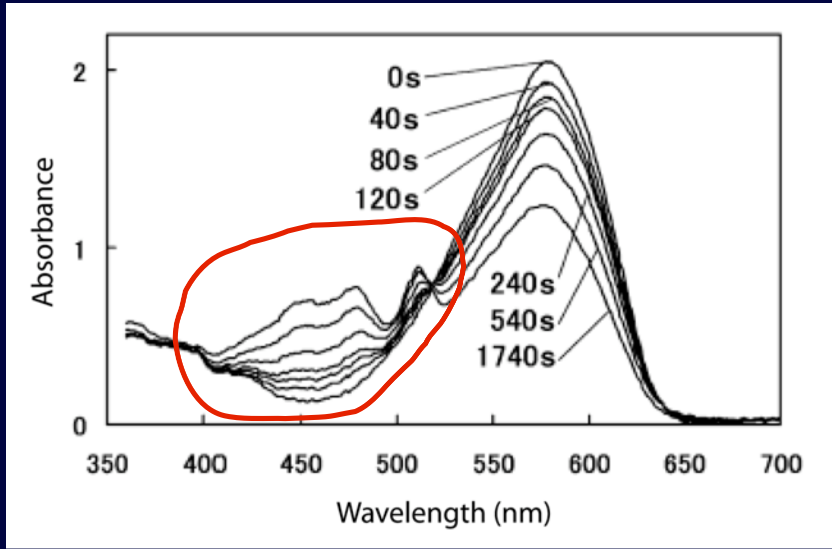
do careful control experiments

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:

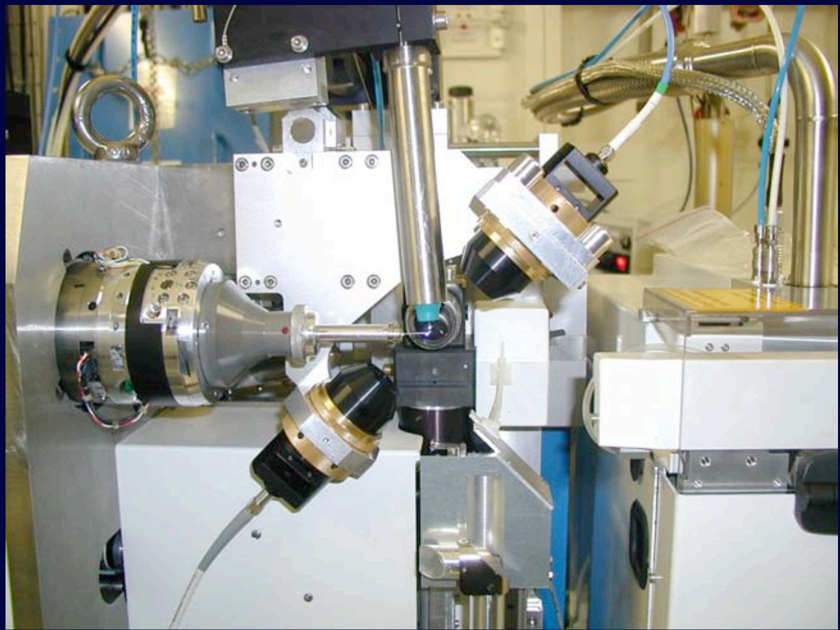
UV-vis

Raman

XAS

see talk by
Antoine Royant

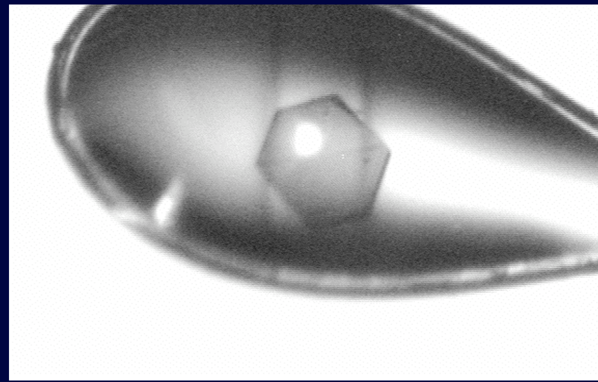
Online UV-vis microspectrophotometer on ID14-4 (ESRF)

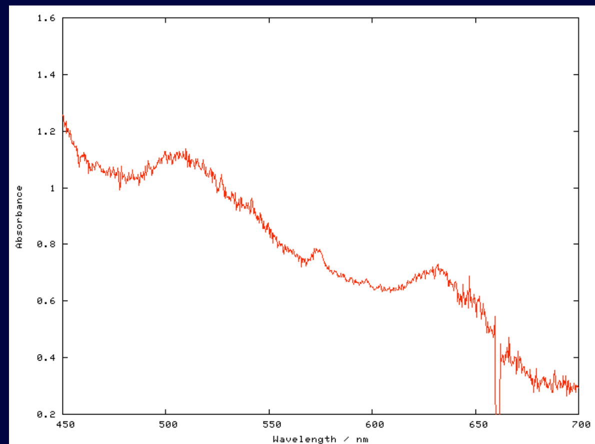


ESRF: McGeehan *et al.* (2009) *JSR* 16, 163

SLS: Owen *et al.* (2009) *JSR* 16, 173

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





Ostermann, Parak & Weik

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

X-ray induced reduction of metalloproteins

Schlichting *et al.* (2000) The catalytic pathway of cytochrome P450cam at atomic resolution. Science 287, 1615

Berglund *et al.* (2002) The catalytic pathway of horseradish peroxidase at high resolution. *Nature* **417**, 463

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

Beitlich *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 D***63**, 951

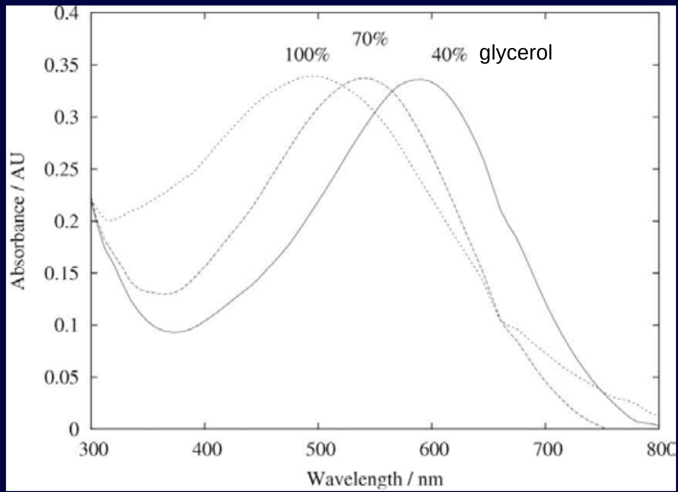
Hough *et al.* (2008) Crystallography with online optical and X-ray absorption spectroscopies. *J Mol Biol.* **378**, 353

At ESRF:

rolling access applications for beamtime with online microspec every 6 months

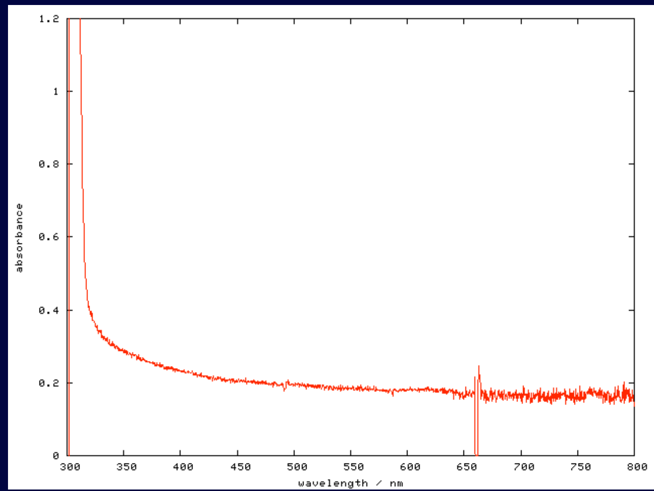
Two other spectroscopic radical signatures often observed with online microspectrophotometry

500 - 600 nm: Hydrated electrons



McGeehan *et al.* (2009) *J. Synchrotron Rad*; 16, 163

400 nm: Disulfide radical anion

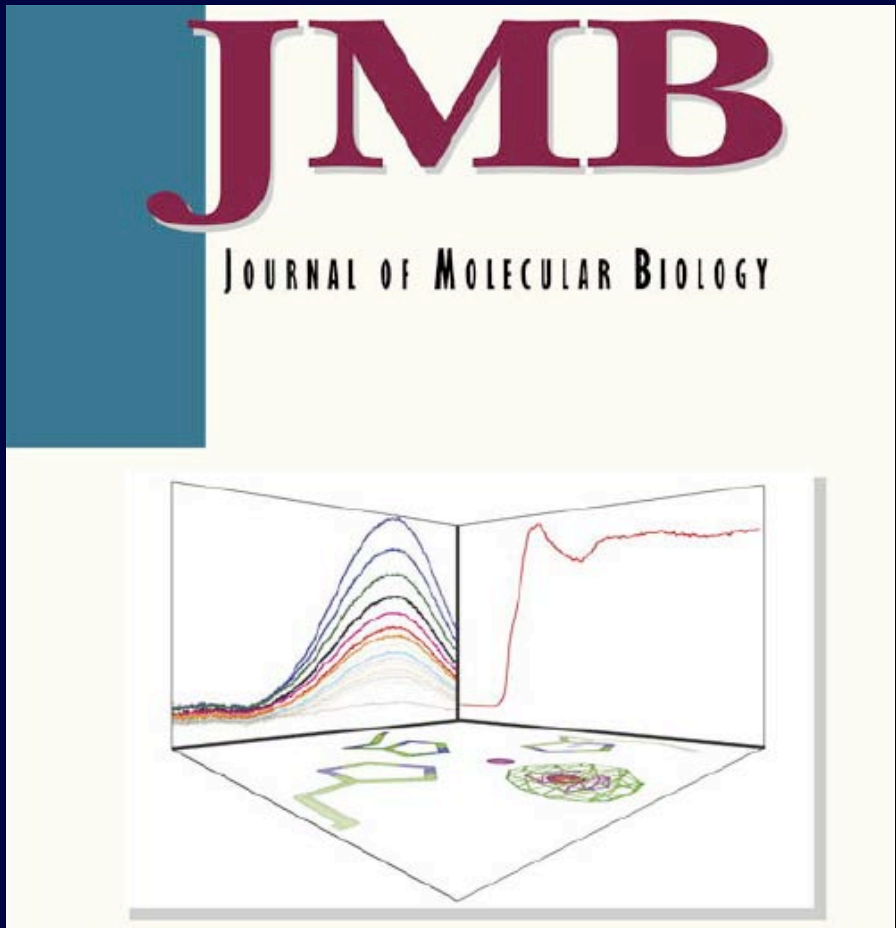


Weik *et al.* (2002) *J. Synchrotron Rad*. **9,** 342

peak: glycerol, PEG
no peak: MPD, glucose

Combining XAS, UV-vis and crystallograpy (SRS Daresbury, UK)

Hough *et al.* (2008) *J Mol Biol.* **378**, 353



'Crystallography with online optical and X-ray absorption spectroscopies demonstrates an ordered mechanism in copper nitrite reductase'

Outline

- 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 dehydrogenase, cholinesterase, DNA photolyase
  - 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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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:

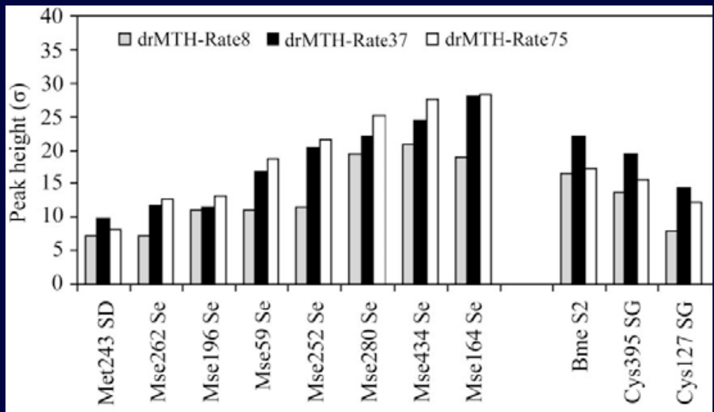
(mosaicity, unit-cell volume increase, R_{merge} …)

No: Sliz *et al.* (2003) *Structure* **11**, 13
S.-Leiros *et al.* (2006) *Acta Cryst.* D**62**, 125

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



S.-Leiros *et al.* (2006) *Acta Cryst.* D**62**, 12

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)

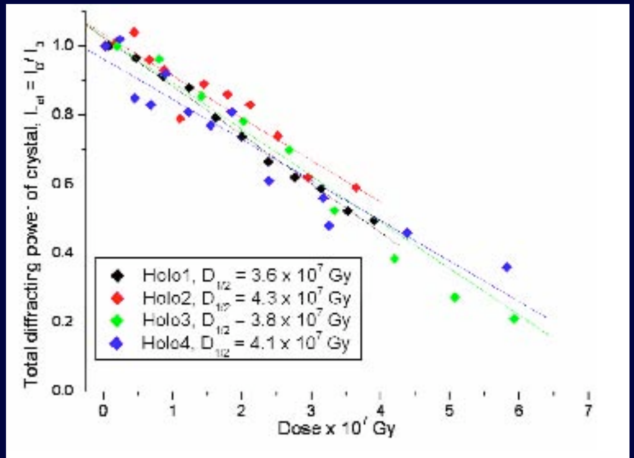
Experimental *Garman* limit

(determined from X-ray data)

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 x 10^7 Gy

$D_{1/2}$= 4.3 x 10^7 Gy

D_{ln2}= 3 x 10^7 Gy (recommended)

Henderson (1990) *Proc. R. Soc. Lond. B* **241**, 6

Owen *et al.* (2006) *PNAS 103, 4912*

Important: calculate absorbed dose …

… with RADDOSE

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

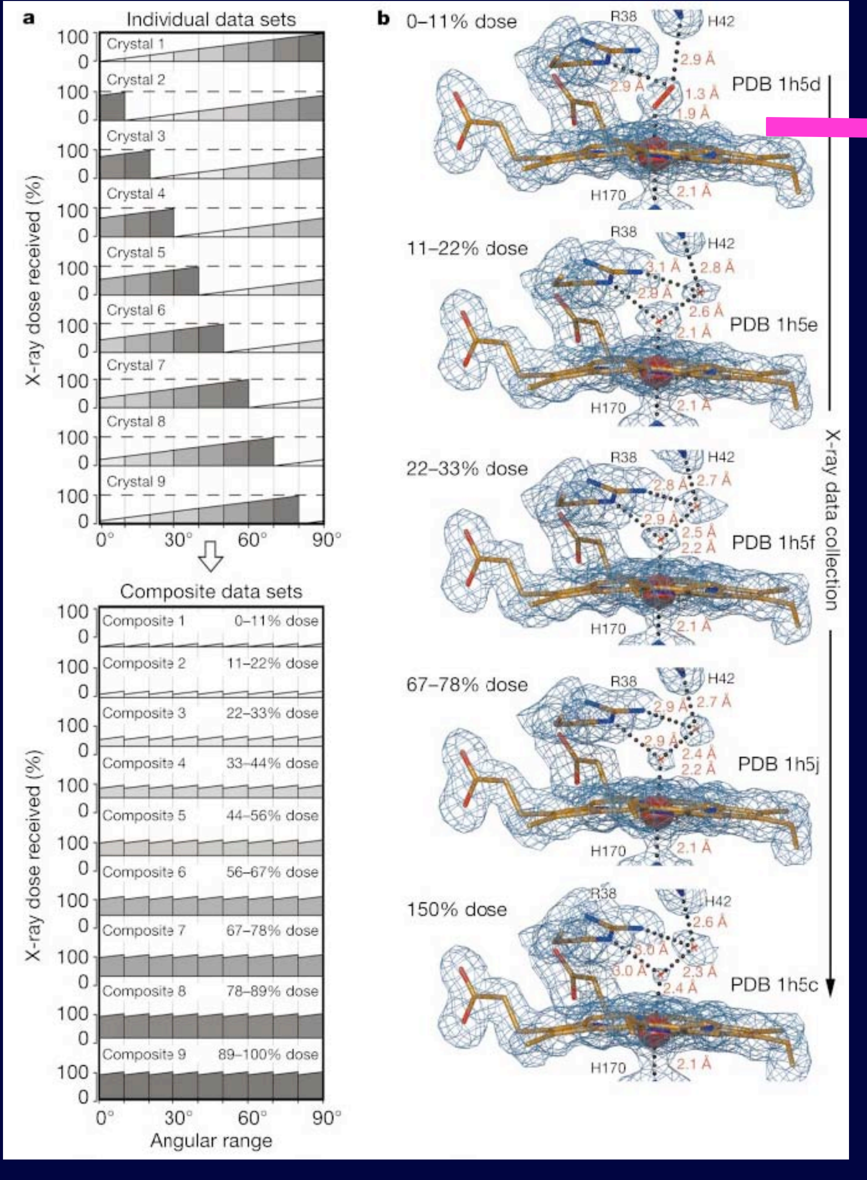
Output: - absorption coefficient
- exposure time after which a critical dose is absorbed

Murray *et al.* (2004) *J. Appl. Cryst.* **37**, 513
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



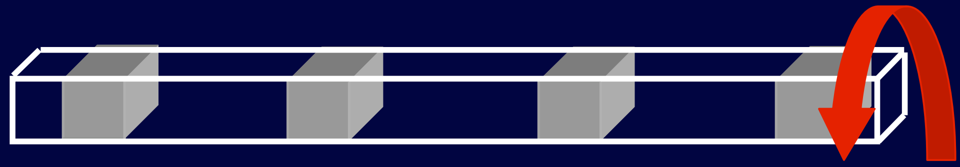
Oxidized redox intermediate in HRP

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



Adam *et al.* (2004) *Structure* **12**, 1729

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

Schulze-Briese *et al.* (2005) *J. Synchotron Radiat.* **12**, 261

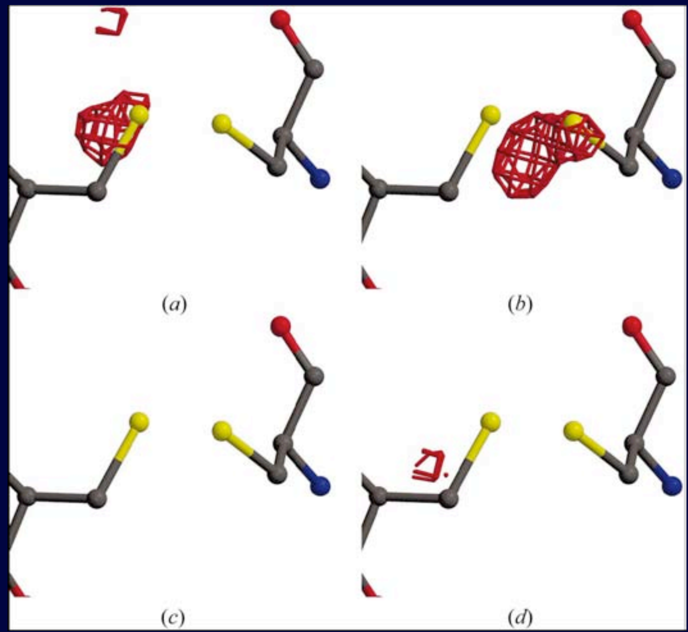
Scavenger: Vitamin C keeps your crystal fit

Murray & Garman (2002) *J. Synchrotron Rad.* **9**, 347

1st data set 6th data set

Crystal without ascorbate

Crystal with 1 M ascorbate



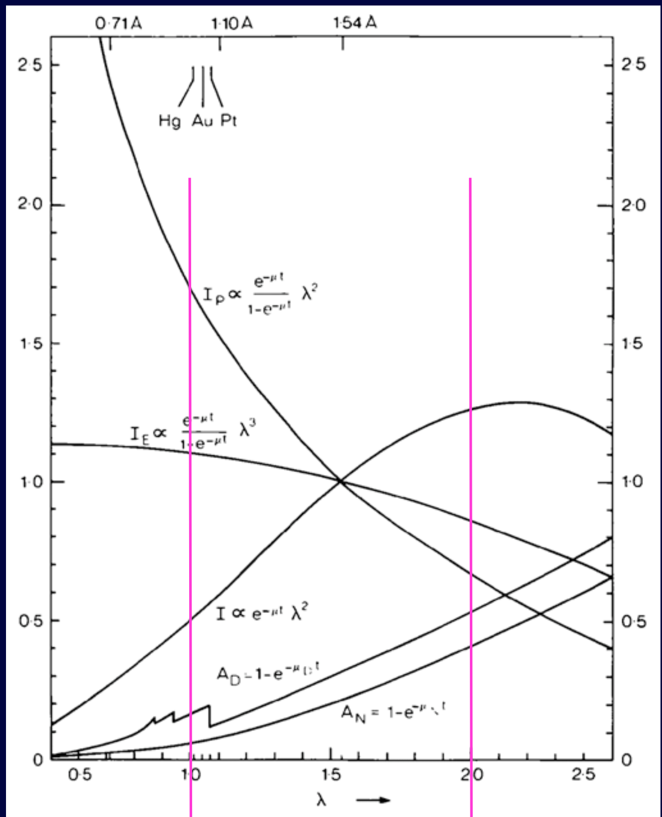
Cys76-Cys94 disulfide bond in HEWL

Further effective scavengers:

- Nicotinic acid and DTNB (Kauffmann *et al.* (2006) *Structure* **14**, 1099)
- 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)

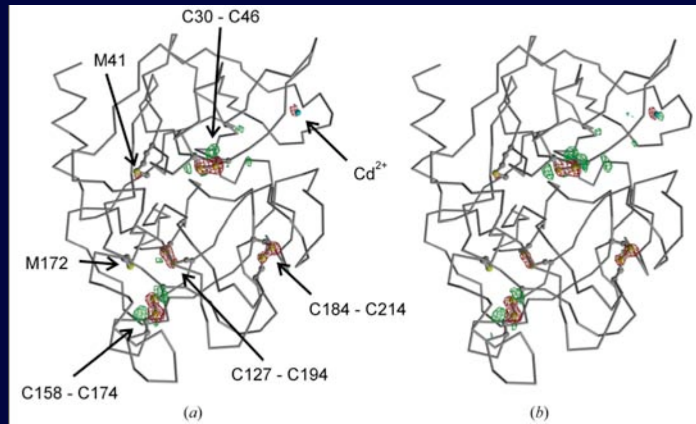


λ=1 Å 2 Å

Arndt (1984) *J. Appl. Cryst.* **17**, 118

Experimentally: no

λ=1 Å 2 Å

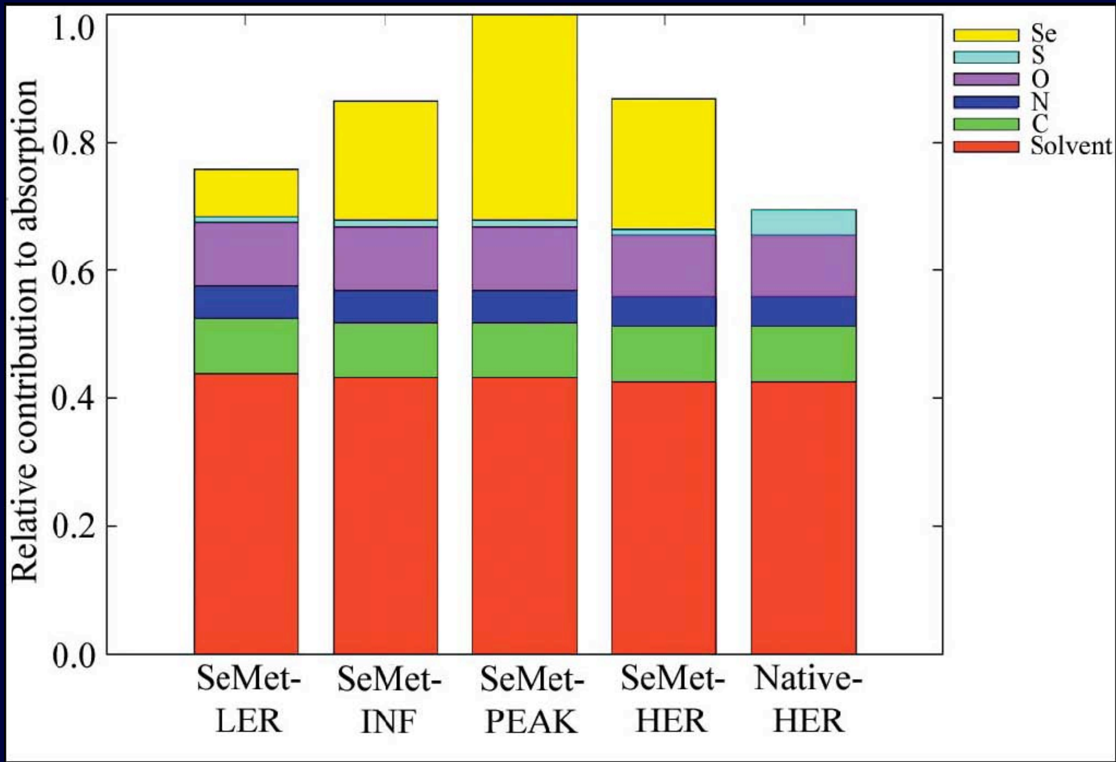


Weiss *et al.* (2005) *J. Synchrotron Rad.* **12**, 30
Shimizu *et al.* (2007) *J. Synchrotron Rad.* **14**, 4

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

Rice *et al.* (2000) *Acta Cryst. D***56**, 1413
Gonzalez *et al.* (2005) *J. Synchrotron Rad.* **12**, 285
Ravelli *et al.* (2005) *J. Synchrotron Rad.* **12**, 276
Olieric *et al.* (2007) Acta D63, 759.

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 ($1\ 10^7$ Gy) → Low-dose data set B

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

See also UV-RIP : UV-induced damage used for phasing

Nanao & Ravelli (2006) *Structure* **14**, 791

Radiation damage and crystallographic software

XDS: Zero dose extrapolation (Diederichs *et al*. (2003) *Acta Cryst. D* **59**, 9033)

SHELX: RIP (Nanao *et al.* (2003) *Acta Cryst. D* **61**, 1227)

BEST: Data collection strategy (Bourenkov & Popov (2006) *Acta Cryst. 62, 58)*

see talk by
Sasha Popov

Temperature dependence of radiation damage

	T < 100 K (He - cooling - 40 K)	T > 100 K (N_2 - cooling)
Specific and non-specific protein damage	small/no dependence (max 2 - 4-fold reduction)	large dependence
Reduction of metal sites	large dependence (30-fold reduction)	? ?

Chinte *et al.* (2007) *Acta Cryst. D* 63, 486
Meents *et al.* (2007) *Acta Cryst. D* 63, 302
Corbett *et al.* (2007) *Acta Cryst. D* 63, 951
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

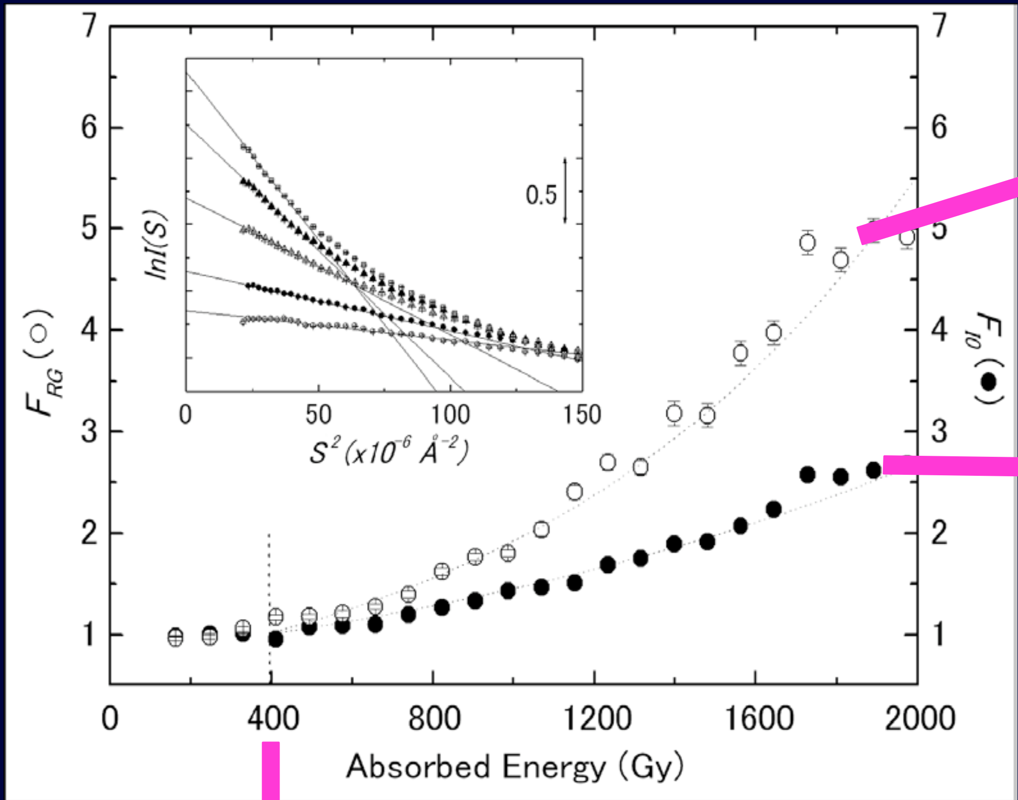
Crystal heating by X-ray beam?

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



Radius of gyration increases

Aggregation occurs

400 Gy: critical dose

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

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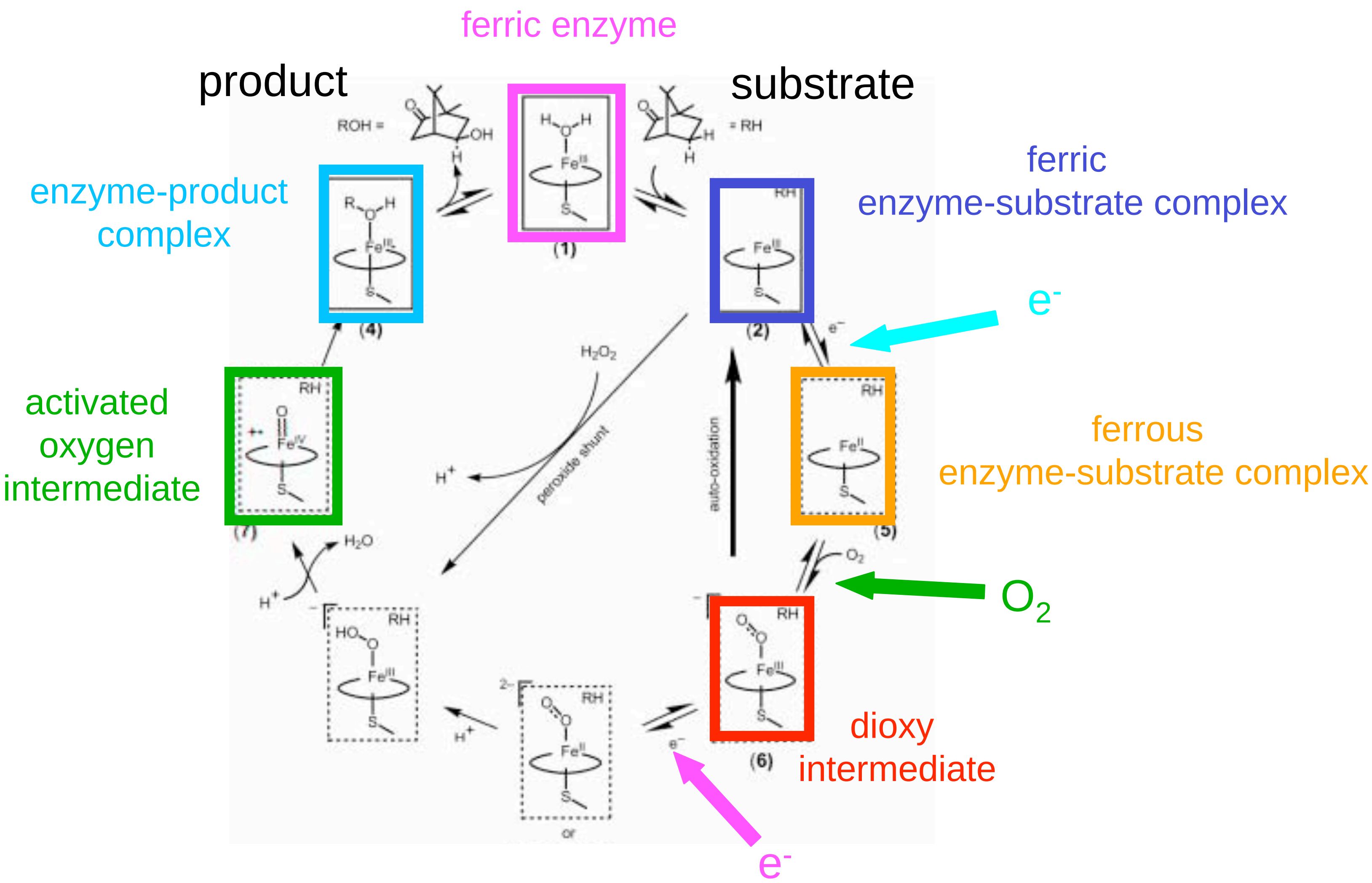
Metastable species on the P450cam reaction pathway

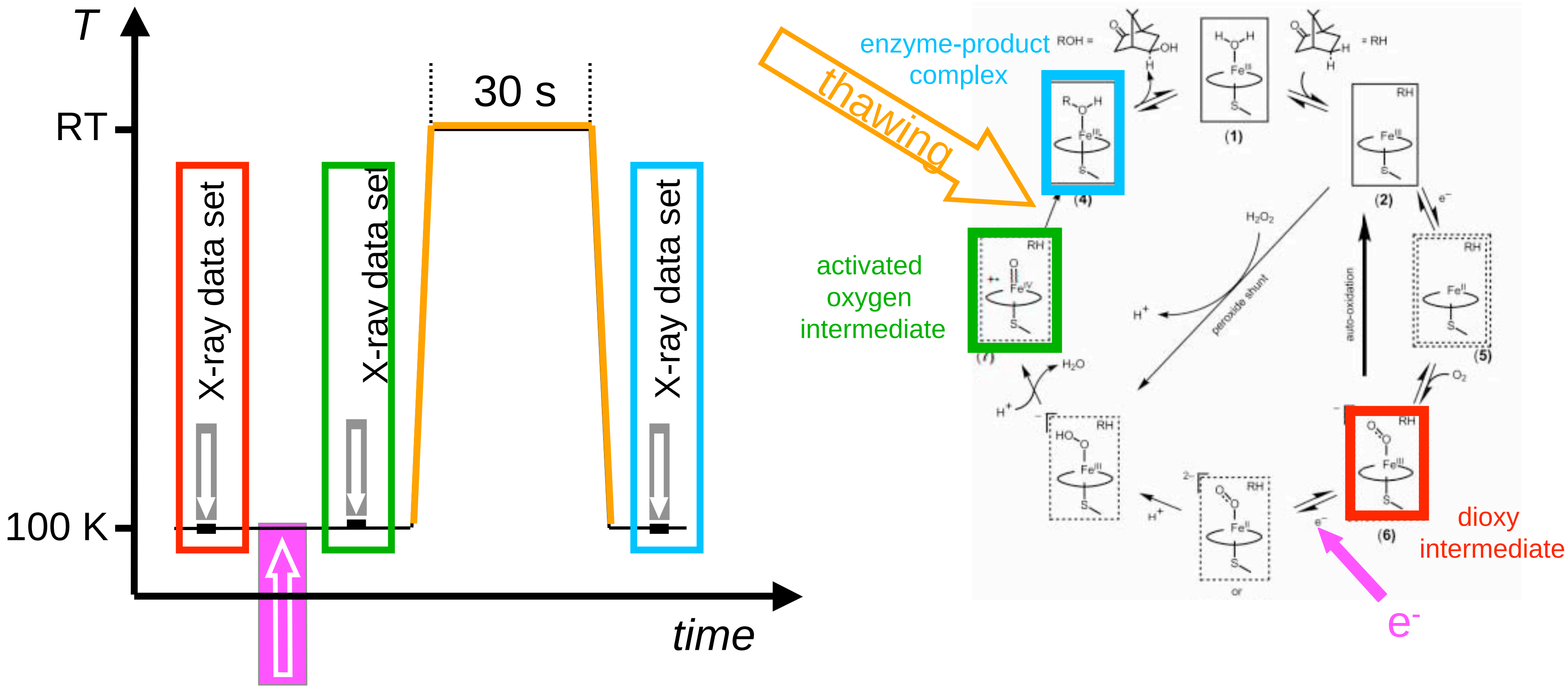
Schlichting, Berendzen, Chu, Stock, Maves, Benson, Sweet, Ringe, Petsko & Sligar (2000) Science 287, 1615



Cytochrome P450cam catalyses hydroxylation of camphor: $2e^-$ redox reaction

Metastable species on the P450cam reaction pathway

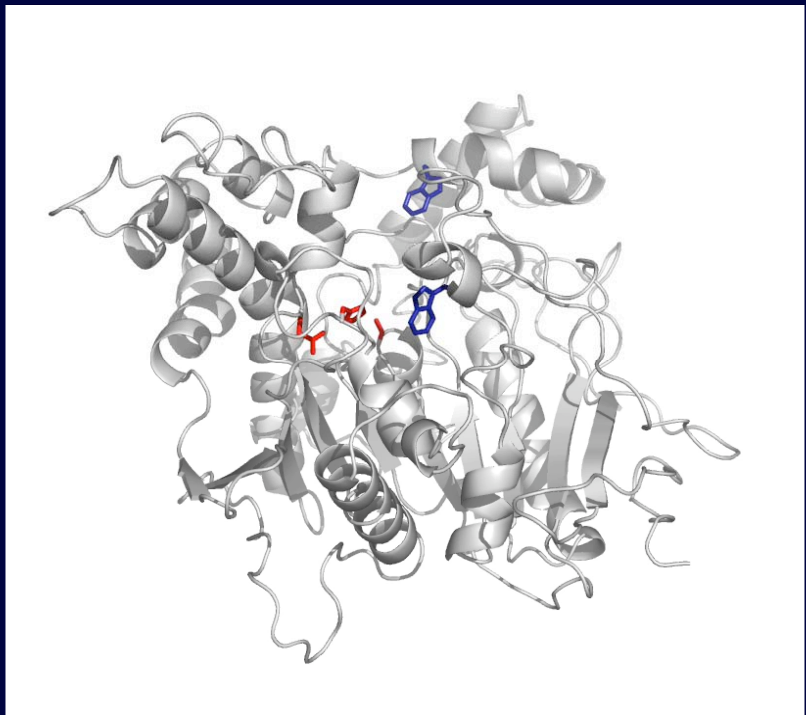
Schlichting, Berendzen, Chu, Stock, Maves, Benson, Sweet, Ringe, Petsko & Sligar (2000) Science 287, 1615

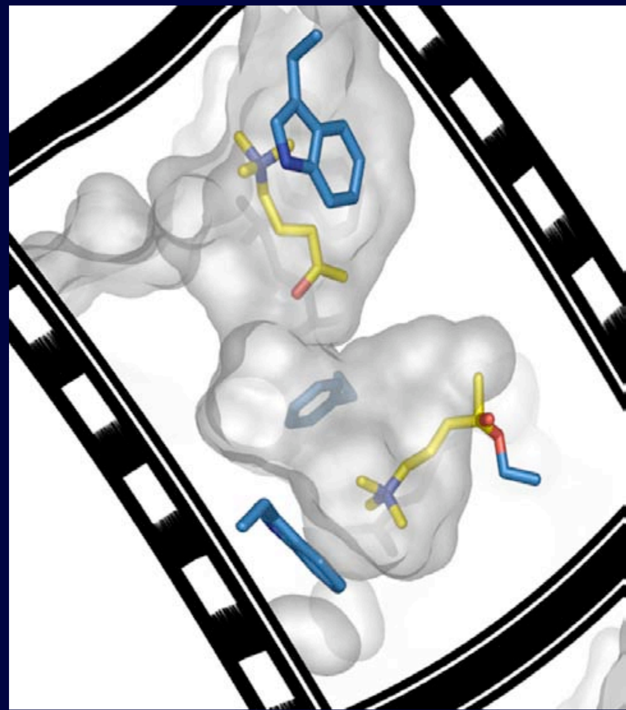


Electron generation by X-irradiation (1.5 Å) for 3h

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

Radiolysis of substrate analogue in acetylcholinesterase

Consecutive data sets at 100 and at 150 K :

(ID14-4, ESRF)

100 K: A, B, C, D

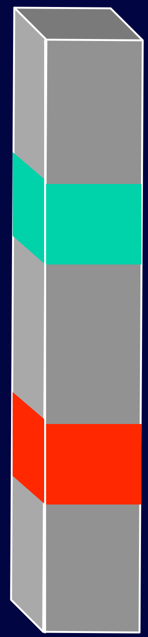
cumul. dose: 0.92 10^7 Gy

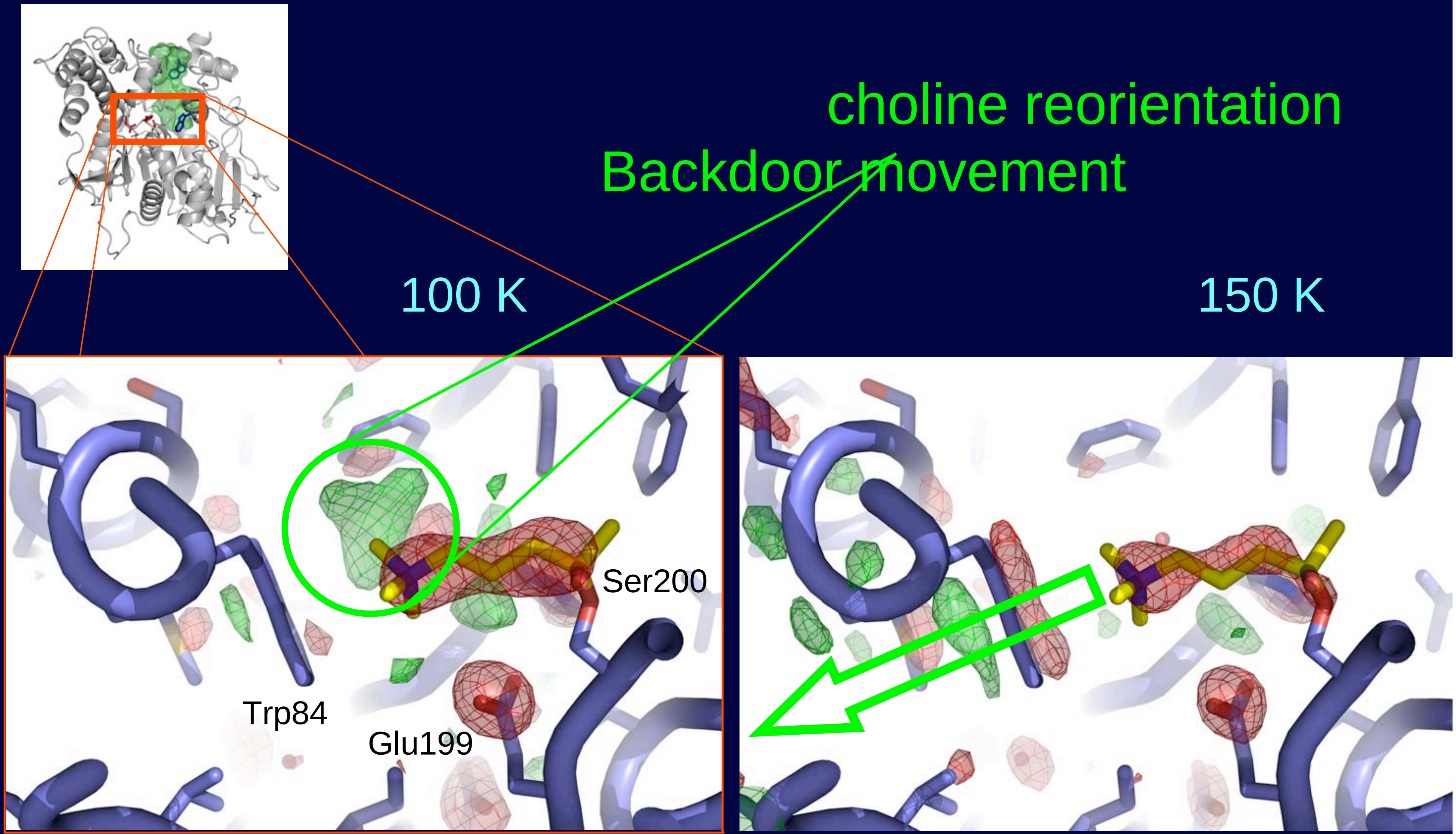
150 K: A, B, C

cumul. dose: 0.95 10^7 Gy

= 1/3 *Garman limit (*3 10^7 Gy)

Owen *et al.* (2006) PNAS 103, 4912



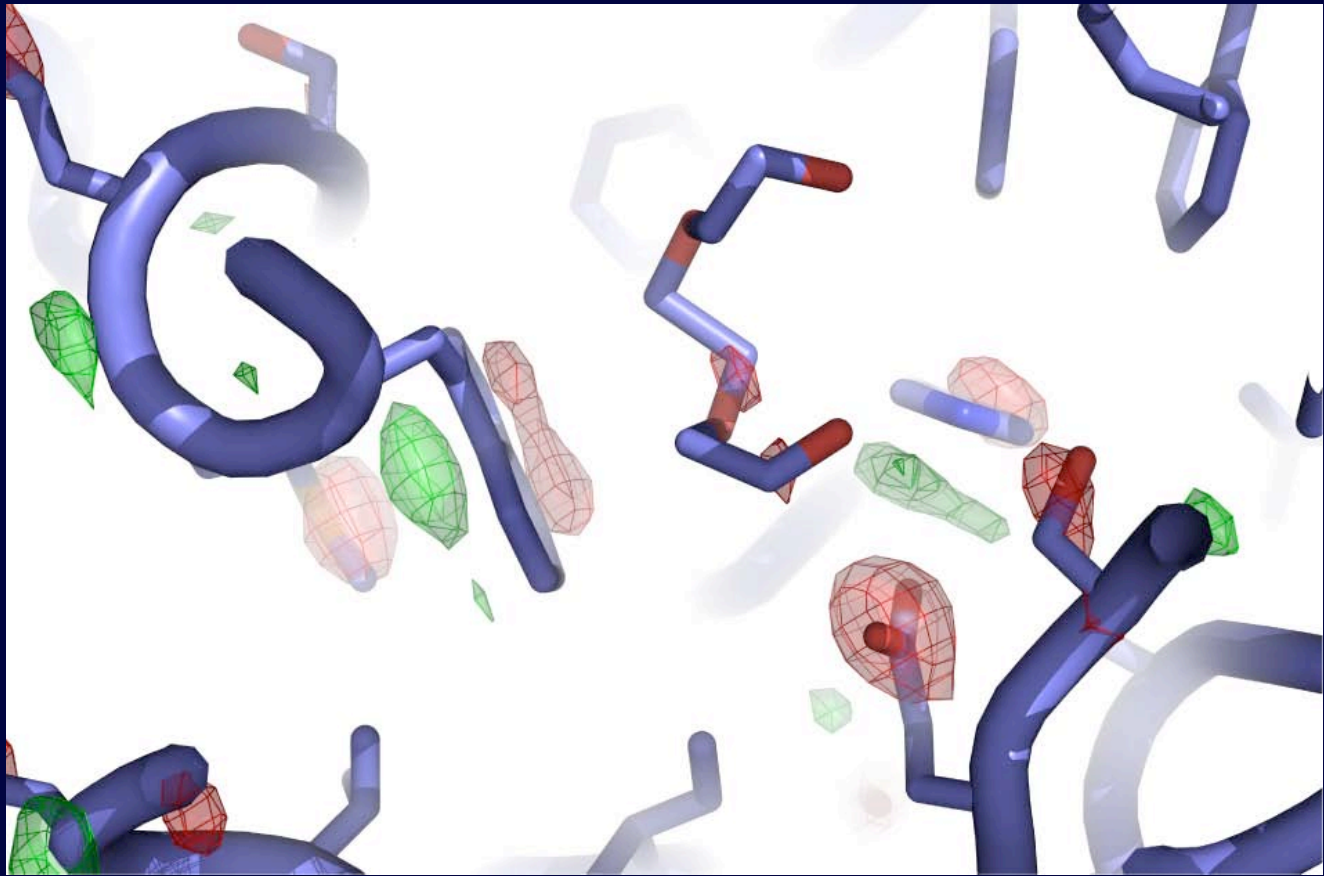


$F_o^{100\,D} - F_o^{100\,A}$ (+, - 4σ)

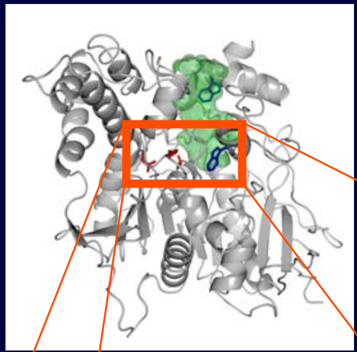
$F_o^{150\,C} - F_o^{150\,A}$ (+, - 4σ)

Colletier, Bourgeois, Sanson, Fournier, Sussman, Silman & Weik (2008) *PNAS*, 105, 11742

Radiation damage control - native AChE at 150 K



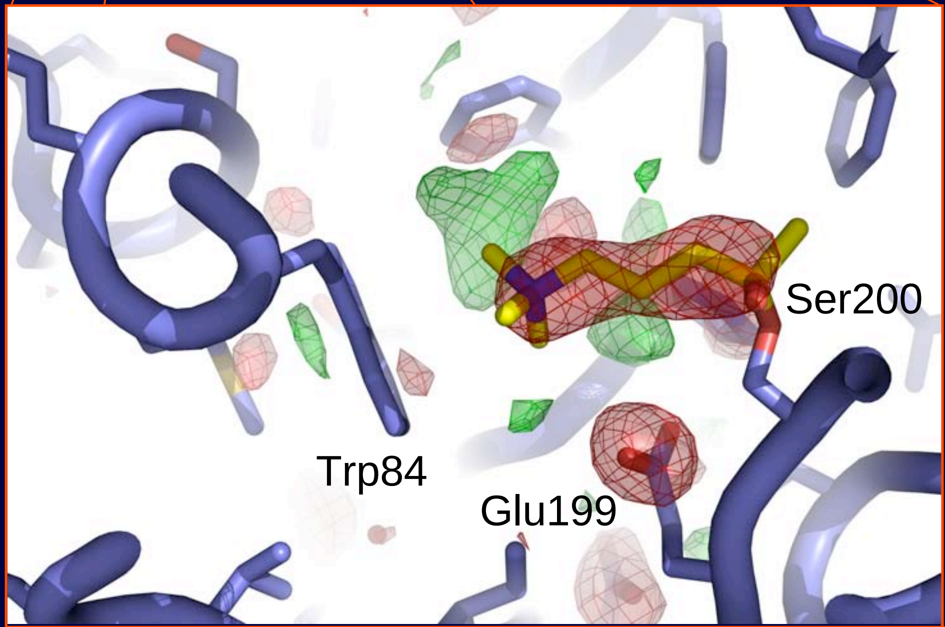
$F_o^{150\ C} - F_o^{150\ A}$ (+, - 4σ)

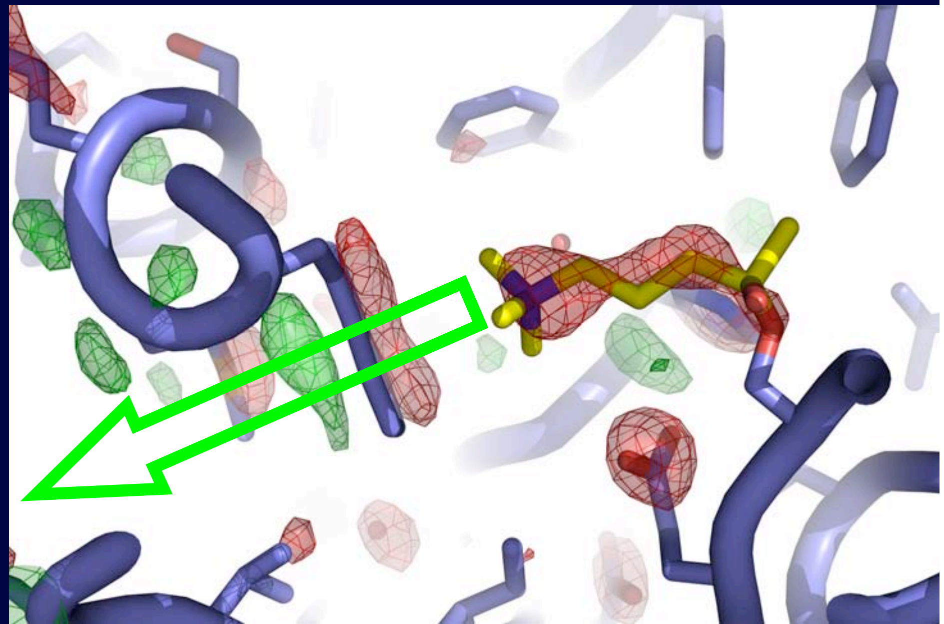


Backdoor movement

100 K

150 K





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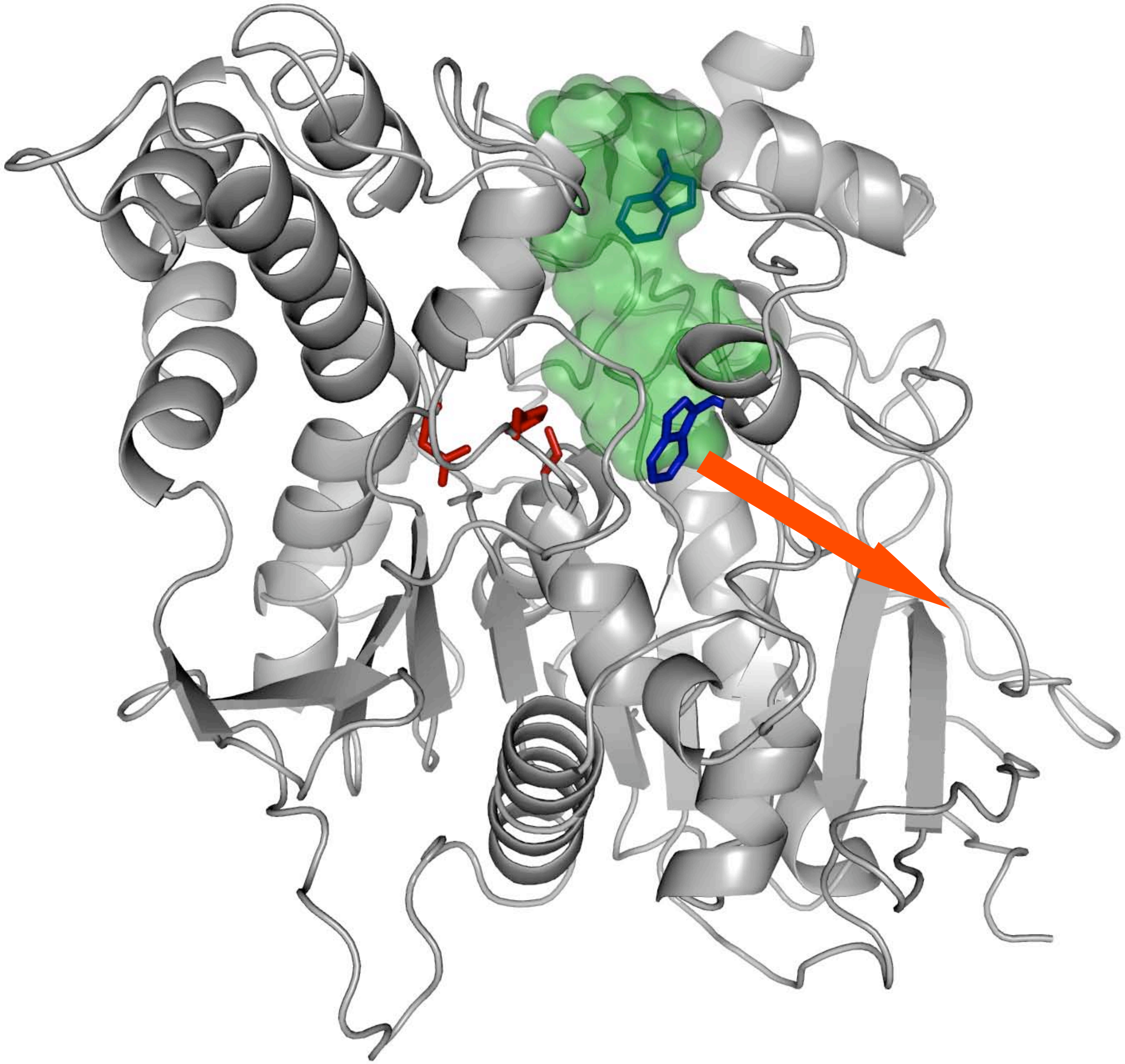
Colletier, Bourgeois, Sanson, Fournier, Sussman, Silman & Weik (2008) *PNAS*, 105, 11742



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, λ (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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LMB, Oxford

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Univ. Portsmouth

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Univ. Leiden

Raimond Ravelli

Univ. Notre Dame

Ian Carmichael

Univ. Paris Sud

Chantal Houée-Levin
Jacqueline Bergès

ESRF: Raddam BAG

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Benoit Sanson

Dominique Bourgeois

Antoine Royant

Philippe Carpentier