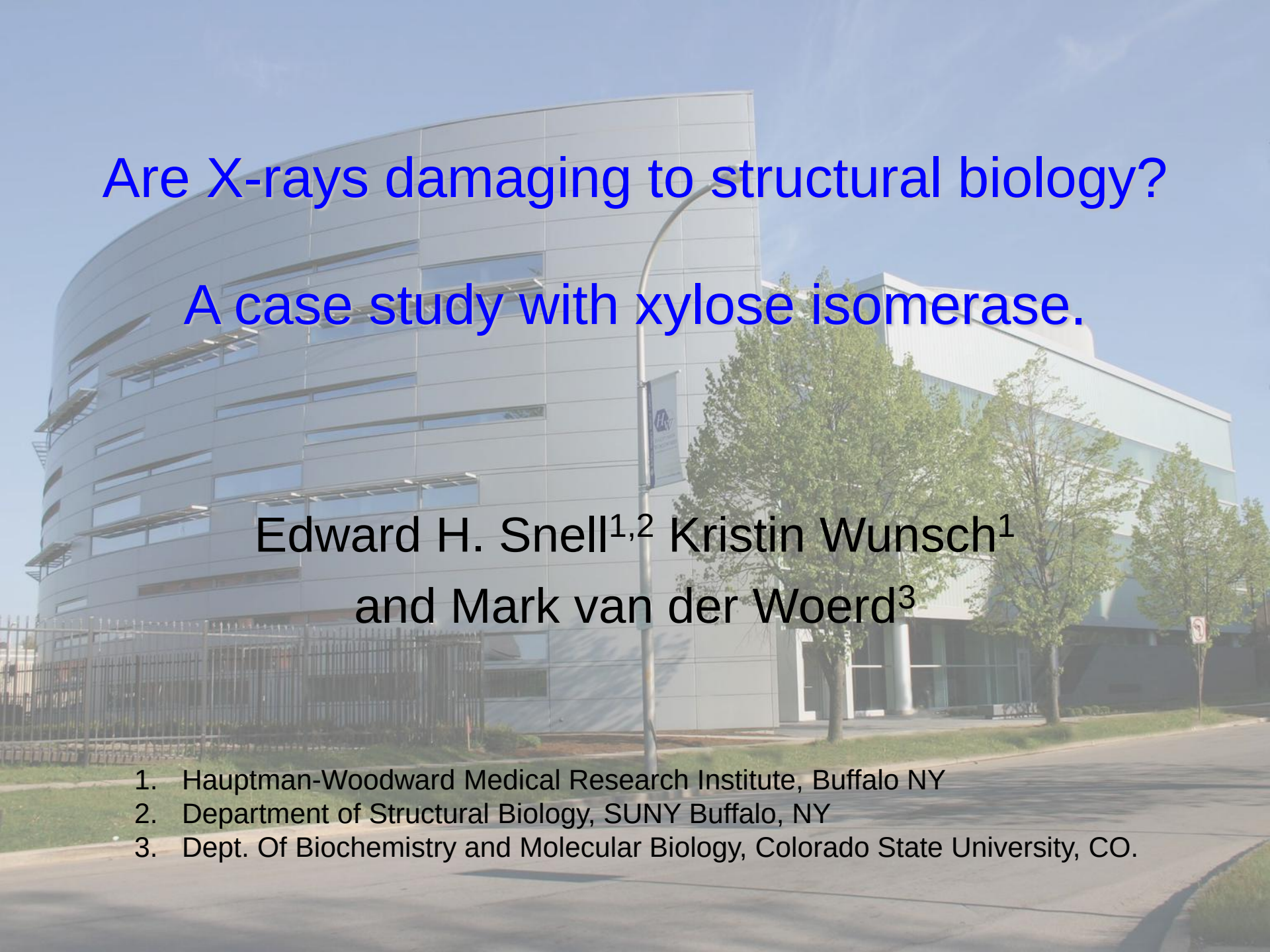


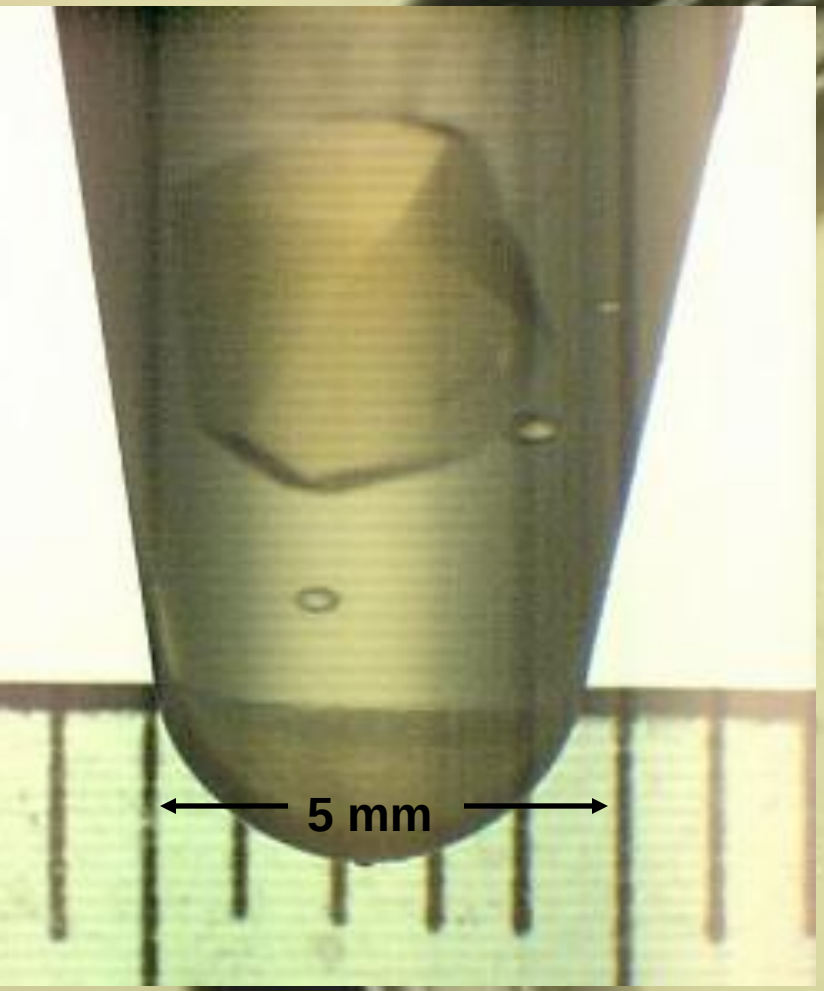
Are X-rays damaging to structural biology?

A case study with xylose isomerase.

Edward H. Snell[1,2] Kristin Wunsch[1]
and Mark van der Woerd[3]

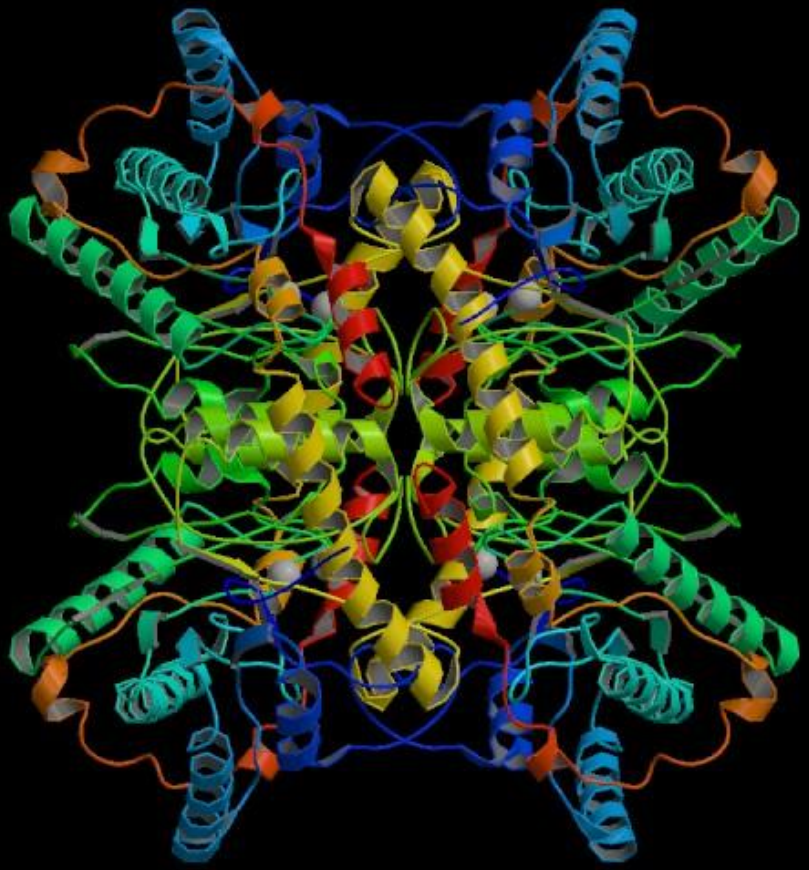
1. Hauptman-Woodward Medical Research Institute, Buffalo NY
2. Department of Structural Biology, SUNY Buffalo, NY
3. Dept. Of Biochemistry and Molecular Biology, Colorado State University, CO.

Xylose Isomerase is cheating ….



- Readily available in large quantities.
- Temperature stable; the enzyme is active at 70°C for up to two years.
- Can be reproducibly crystallized.
- Well studied biochemically.

But it’s interesting for us …



But it’s not cheating that much

Commercially interesting - used industrially to catalyze the conversion of glucose to fructose for the production of high-fructose corn syrup.

The enzymatic mechanism is a transfer of one H atom from one C atom of the substrate to an adjacent C atom. Three pathways for this mechanism have been proposed – a base-catalyzed proton transfer, a simple hydride shift or a hydride shift mediated by a metal ion.

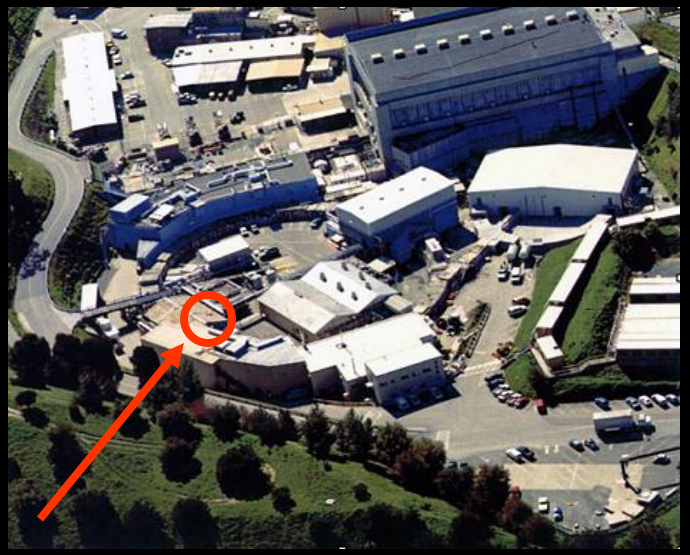
X-ray date eludes to one of these, neutron data confirms it.

Xylose isomerase is interesting with respect to radiation damage studies. It contains two metal ions surrounded by carboxylic groups in the active site (but no disulfide bridges). Extensive biochemical studies have been performed on the active site and it provides a good candidate for complementary biophysical techniques.

It diffracts to high resolution,

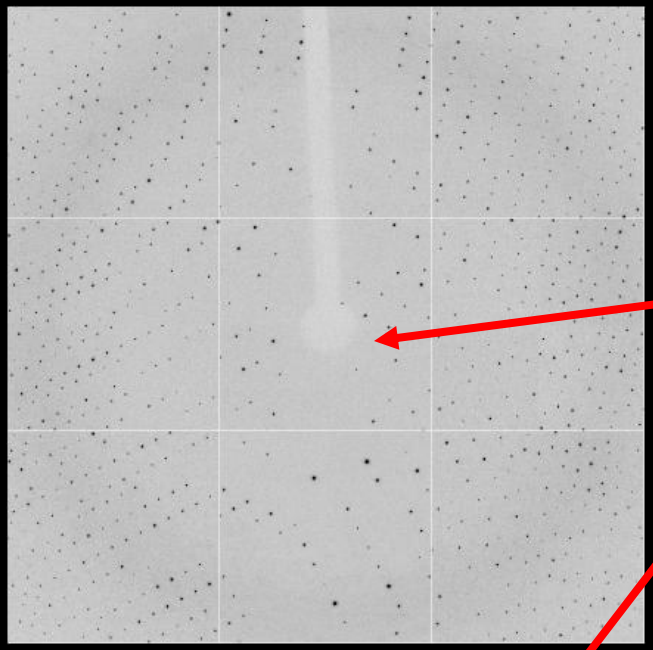
X-ray data collection

- Stanford Synchrotron Radiation Laboratory, beamline 9-1
- Beautiful diffraction to 0.86 Å



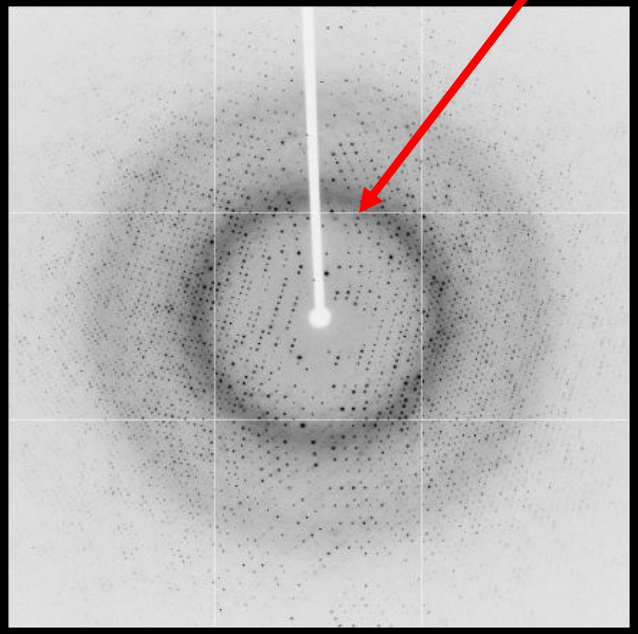
Experimental protocol

- Crystals grown at the ring in the presence of cryoprotectant.
- Collected a high resolution pass followed by a low resolution pass to cover the complete dynamic range.
  - Initial hypothesis: radiation damage would be seen in first the high resolution data and we could collect the 'undamaged' low resolution data last.
  - Oh bugger moment – The low resolution data would not scale to the high resolution data (which presented it's own processing problems). Each had been processed as it was collected but not scaled together (due to time constraints). The data had a low resolution hole and had to be discarded.
  - Lesson learned – Radiation damage is a global process. It is first observed in the high resolution data but it is affecting the data globally.
  - **New protocol – Low resolution data collection is very fast, collect it first with minimal exposure then extend the resolution with high resolution data collection.**

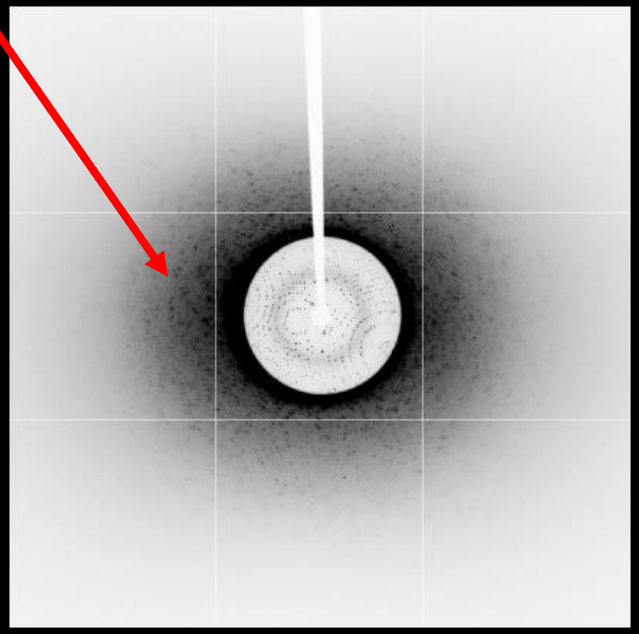


SSRL Beamline 11-1, ADSC Quantum-315 CCD detector
Low, medium and high-resolution data collection, 0.855 Å

- Low: Crystal-to-detector 600 mm, 2 degree rotation, 1s exposure, 80 images, 160 degrees of data.
- Medium: Crystal to detector 250 mm, 2 degree rotation, 2 s exposure, 75 images, 150 degrees of data.
- High: Crystal to detector 90 mm, 0.5 degree rotation, 4 s exposure, 720 images, 360 degrees of data

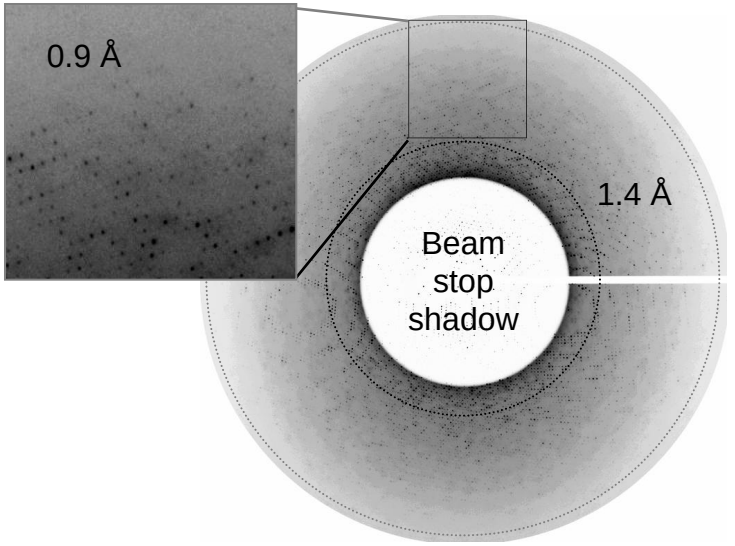


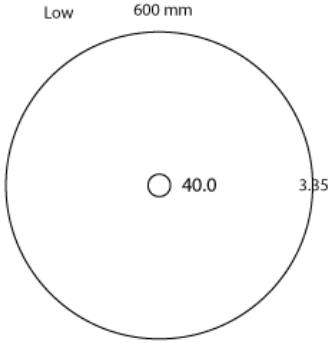
Total dose, 2.6×10^6 Gy

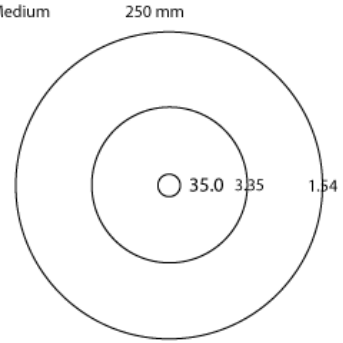


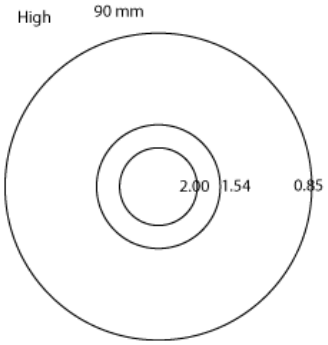
Data collection

	Low	Medium	High
Crystal to detector	600 mm	250 mm	90 mm
Oscillation	2.0°	2.0°	0.5°
Exposure time	1.0 s	2.0 s	4.0 s
(per degree)	0.5 s	1.0 s	8.0 s
Images	80	75	720
Coverage	160°	150°	360°

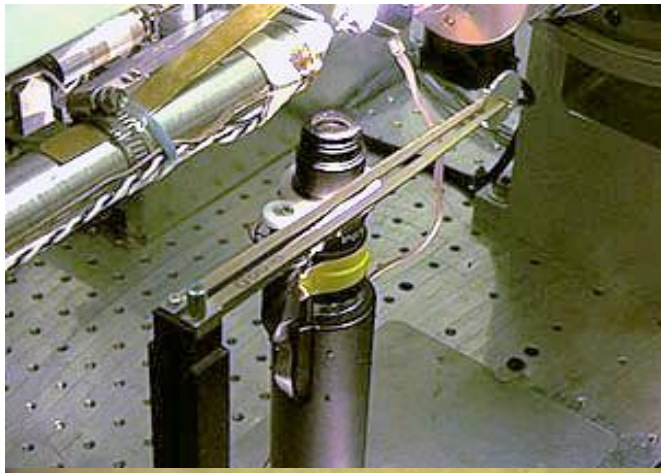


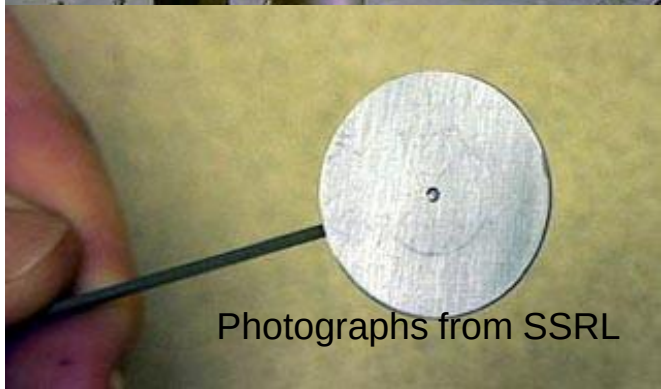






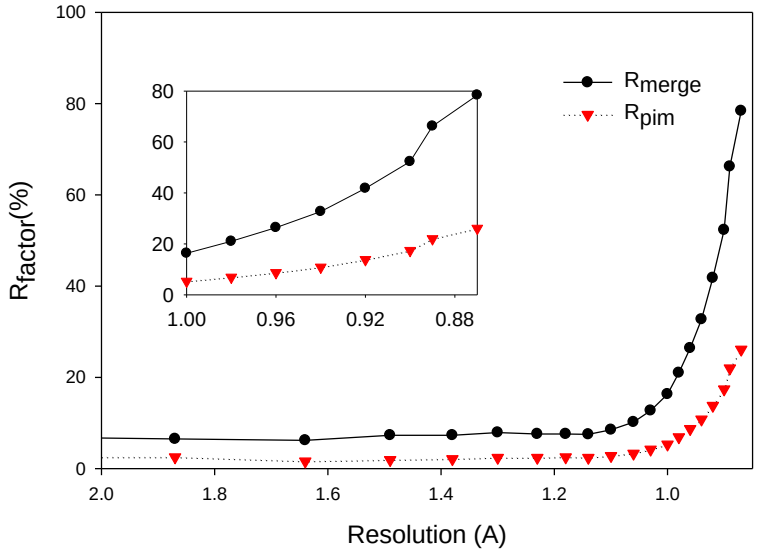
The data were integrated with both Mosflm and Denzo and reduced with Scala and Scalepack. There were no significant differences between the results from the two packages.

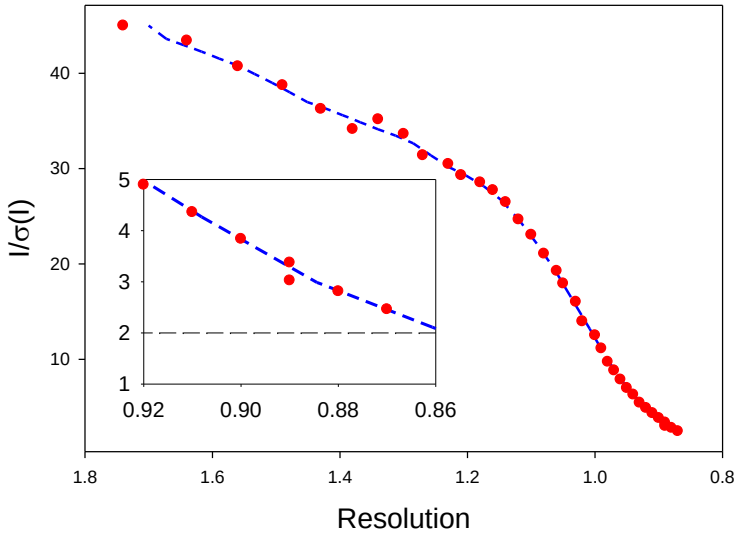




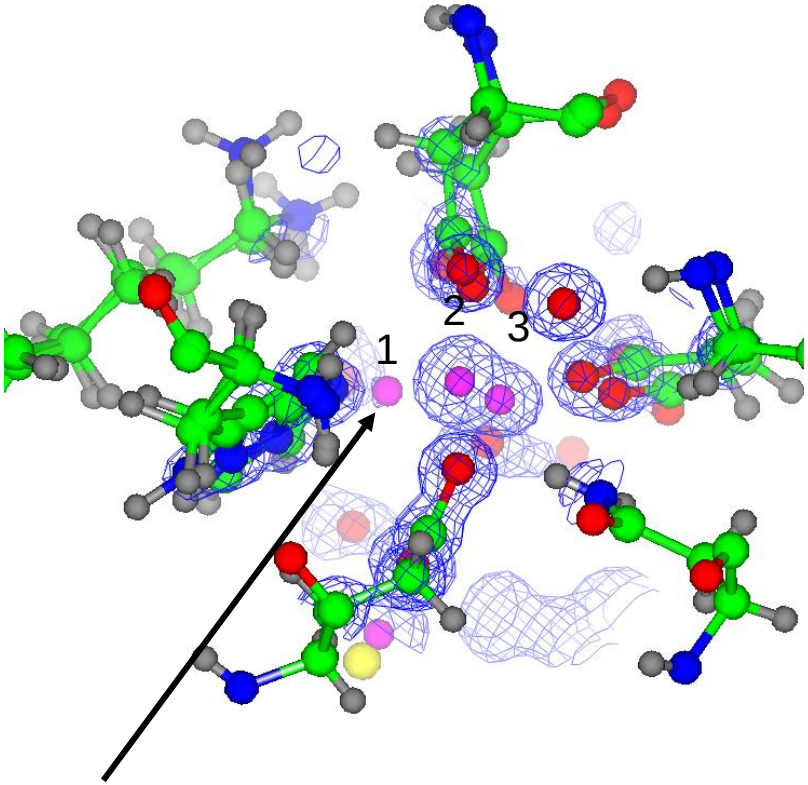
Processing statistics

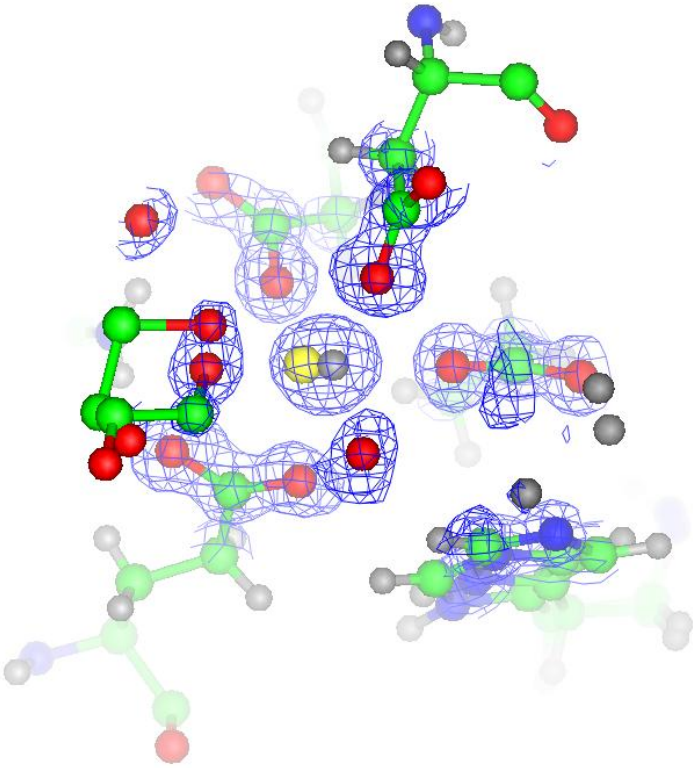
	Low	Medium	High	Overall
Resolution (Å)	30-2.50 (2.59-2.50)	30-1.30 (1.32-1.30)	1.97-0.87 (0.88-0.87)	30-0.87 (0.88-0.87)
Unique reflections	12,827	104,051	342,898	376,419
Observed reflections	64,460	524,073	4,776,450	3,991,720
Completeness	77.7(24.9)	89.5(43.4)	100.0(100.0)	99.5(88.1)
Redundancy	5.0(2.0)	5.0(2.1)	13.9(13.5)	10.6
<I/σ(I)>	23.6(15.4)	21.8(5.83)	31.7(3.1)	27.8(2.4)
Rmerge	0.070(0.052)	0.074(0.144)	0.085(0.907)	0.077(0.817)
Rpim				0.024(0.261)





The resulting structure



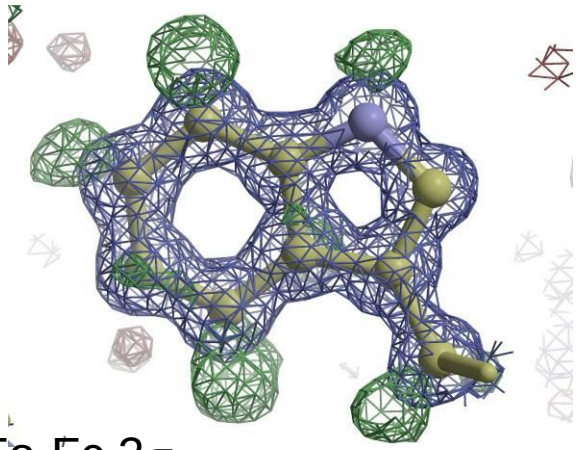


9%, 54%, and 37% occupancy respectively

R=9.0%

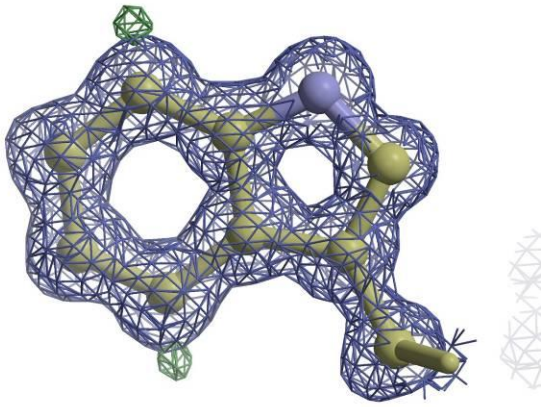
R_{free}=10.0%

The resulting structure

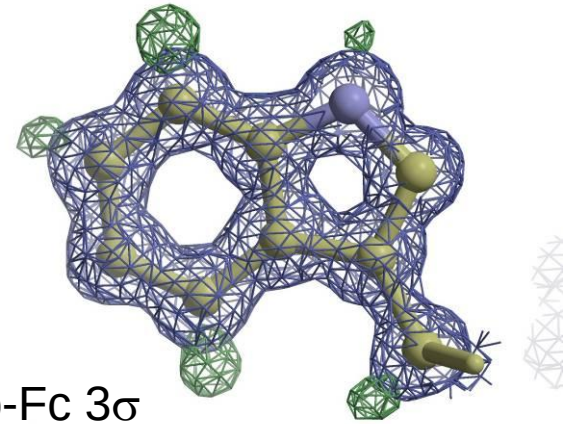


Fo-Fc 2σ

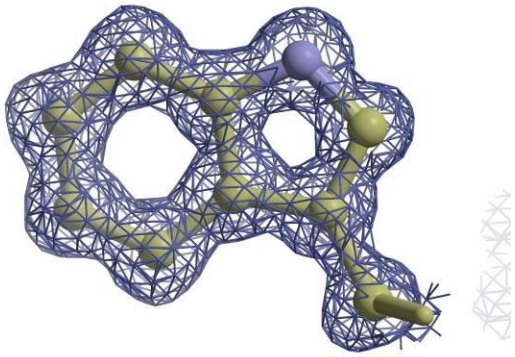
2Fo-Fc 1.0σ



Fo-Fc 4σ

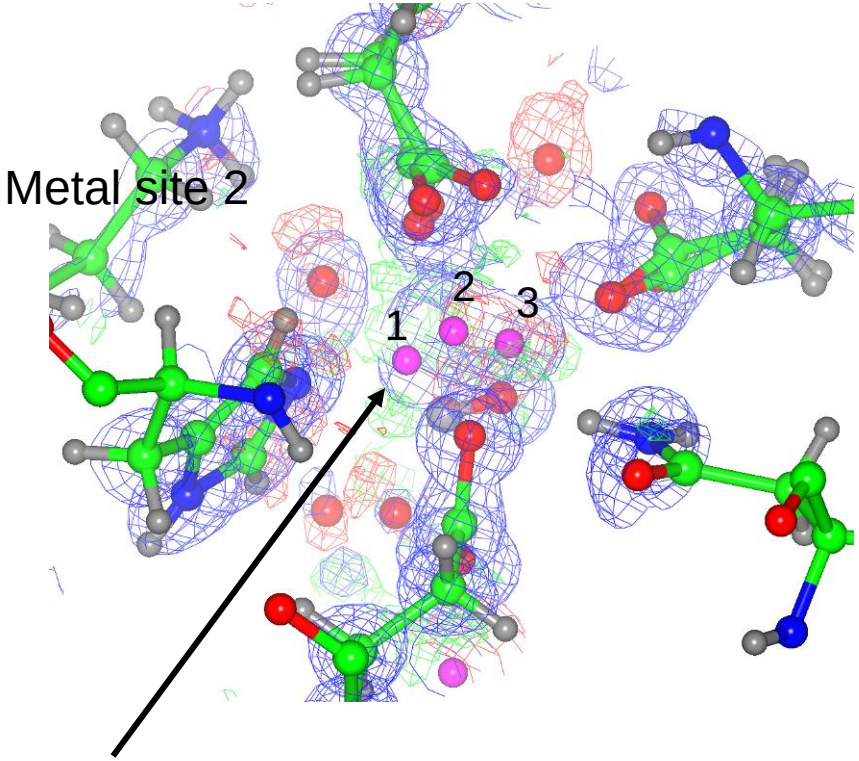


Fo-Fc 3σ



Fo-Fc 5σ

A related structure

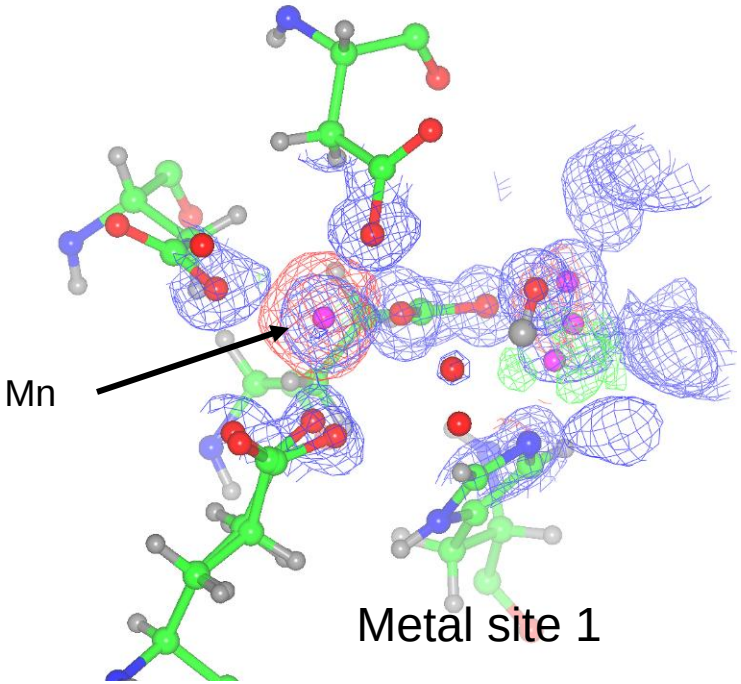


Three alternate Mn sites, occupancy, 16%, 69% and 48% respectively (133%)

Maps contoured at 2σ 2Fo-Fc (blue) and 4σ for the Fo-Fc, negative in red, positive in green.

1MUW, 0.86 Å X-ray structure of xylose isomerase from Streptomyces olivochromogenes (Fenn *et al.* Biochemistry 2004).

$R=12.5\%$, $R_{free}=14.3\%$



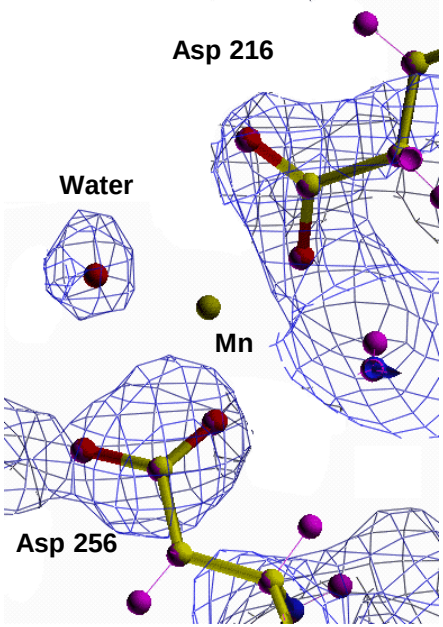
Questions from the structures

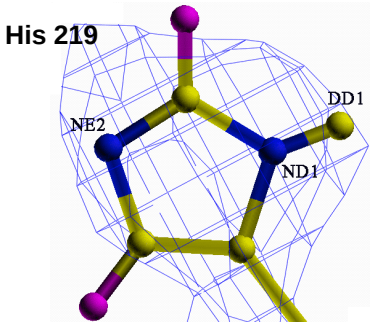
- In the data from Streptomyces *rubiginosus* xylose isomerase the metal site is shared between Magnesium and Manganese. For Streptomyces olivochromogenes it is modelled as a 100% occupied Manganese.
- For the second metal site the occupancies for the Streptomyces *rubiginosus* xylose isomerase are different from the related structure from Streptomyces olivochromogenes.
- The occupancy has been reported to be critical to the turnover rate of the enzyme.
- Are the occupancies accurate, is there any effect from radiation damage on the active site and therefore interpretation of biological mechanism.
- Is the metal conformation a function of the biochemistry involved in crystallization or an artifact of the radiation used to look at the structure?

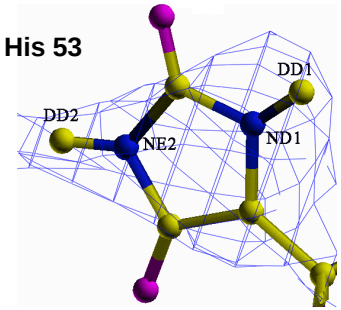
Two experiments

- The crystals grow large, they can be studied them with neutrons.
- Or we can design an X-ray experiment look specifically at radiation effects.

Neutrons







Neutron data

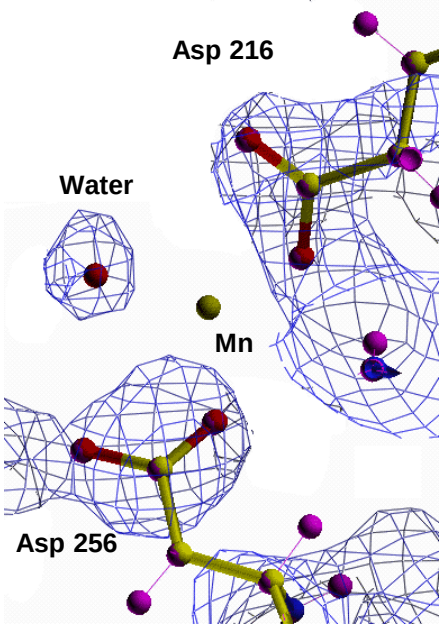
Advantages:

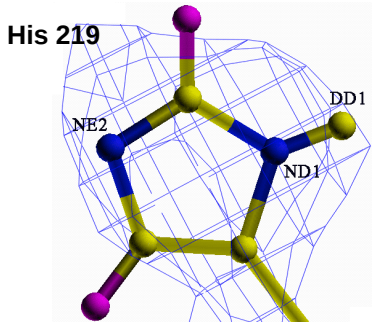
- Non-ionizing.
- The scattering factors are monotonic, sensitive to hydrogen and deuterium (opposite scattering signs) and can be used to study protonation state.

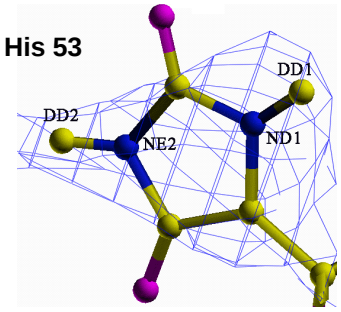
Disadvantages:

- Sources are extremely weak compared to synchrotron X-ray sources
- Neutron scattering efficiency is small
- D_2O chemistry is required for best results.

In the case of Xylose Isomerase, neutron data the data showed that none of the active site carboxlyic acids were protonated. However His 219 is singly protonated where it is ligated to the catalytic metal ion and His 53 is double protonated. His 53 has been postulated to be involved in the opening of the substrate ring. This is in agreement with results suggested by high-resolution X-ray studies.







But!

The neutron structure did not agree with either high-resolution X-ray structure. No multiple confirmations were seen in the active site. Metals were invisible.

The neutron data was collected at room temperature. There is no need to cryocool the crystals as the neutrons are non-ionizing and radiation damage is not a concern. The X-ray data was collected at 100K in the presence of a cryoprotectant, ethylene glycol.

The neutron data diffracted to 2.4 Å, significantly less than the X-ray data.

The neutron data confirmed the mechanism but did not reveal any information on the metal sites.

X-rays – Back to SSRL

Does radiation damage come into play?

- Fresh crystals of xylose isomerase were grown at the beamline.
- Initially, 2 images to index and determine a strategy.
- A high-resolution swathe with 20 images, 30s equivalent photon exposure (using Blue Ice dose mode), 100 mm crystal-to-detector distance 0.5 degree oscillation at 0.855A wavelength (total dose 0.47×10^6 Gy).
- A complete data set with 180 images, 2s equivalent photon exposure, 100 mm crystal to detector distance, 0.5 degree oscillation at 0.954A wavelength (total dose 0.35×10^6 Gy).
- Alternating data collection between high-resolution and complete data (0.82×10^6 Gy for each set).
- Dose was calculated using Raddose with the flux calculated from the ion chamber reading (a calibrated pin diode was not available).

Radiation Damage

- General Effects:
  - Change in unit cell dimensions
  - Increase in B-factors
  - Decreased diffraction power from the crystal
  - Loss of high resolution data
  - Increase in mosaicity

Seen in the diffraction data

- Chemical Effects:
  - Disulfide bond breakage
  - Decarboxylation of Asp and Glu
  - Loss of OH group on Tyr
  - C-S bond cleavage in Met
  - Reduction of metal center

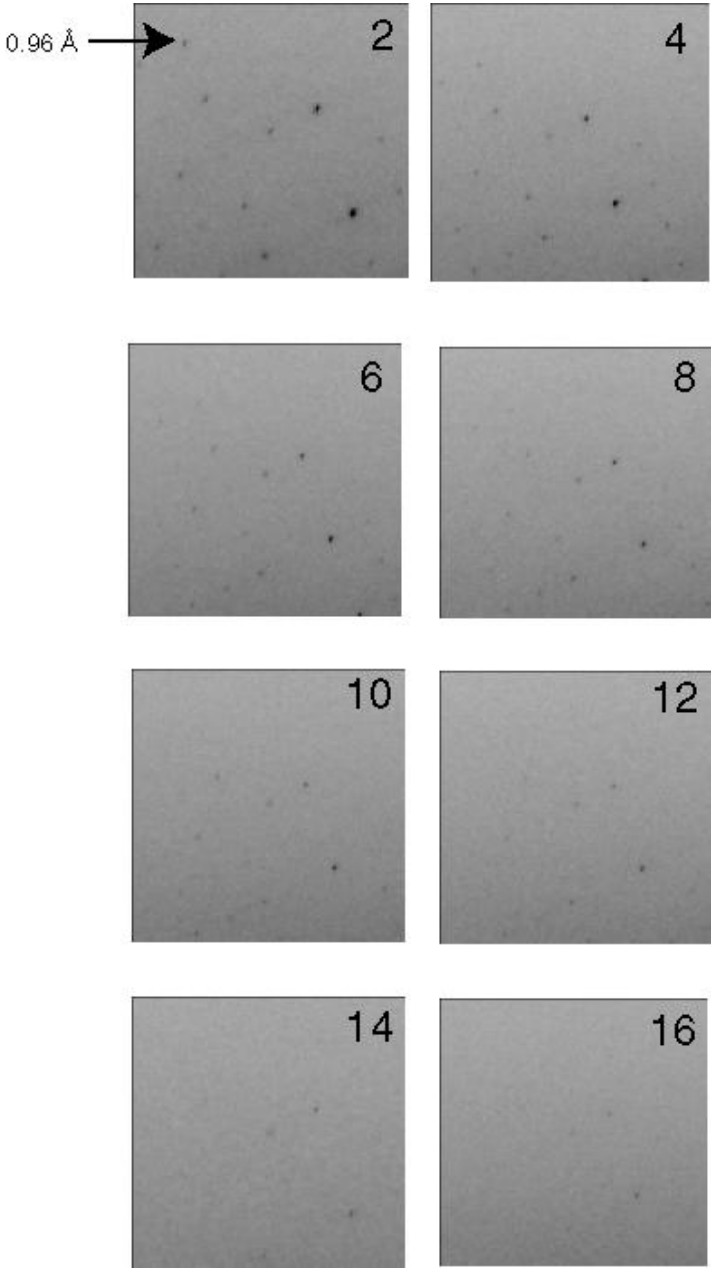
Seen in the structure

Diffraction data – Radiation Damage Datasets

High resolution partial data set (0.9 Å)								
Data set	2	4	6	8	10	12	14	16
R_{factor}	6.7(45.8)	6.7(54.7)	6.9(57.5)	7.2(59.4)	7.5(85.2)	8.0(68.7)	8.0(73.5)	8.0(-)
I/σ(I)	8.9(1.6)	8.5(1.2)	8.6(1.0)	8.3(0.8)	8.3(0.7)	8.0(0.6)	7.8(0.6)	7.7(0.5)
Completeness (%)	24.8(24.8)	24.8(23.2)	24.5(19.6)	24.1(15.3)	23.6(10.9)	23.0(7.0)	22.2(3.1)	21.7(1.4)
Redundancy	1.4(1.4)	1.4(1.3)	1.4(1.2)	1.3(1.2)	1.3(1.1)	1.3(1.1)	1.3(1.0)	1.3(1.0)
Mosaicity (º)	0.17	0.17	0.17	0.16	0.16	0.16	0.16	0.16
B_{factor}	6.04	6.35	6.70	6.85	7.25	7.54	7.85	8.13
Medium resolution complete data set (1.2 Å)								
Data set	3	5	7	9	11	13	15	
R_{factor}	7.5(22.5)	7.5(24.7)	7.7(27.3)	7.6(30.1)	7.9(33.4)	7.9(37.3)	7.8(41.7)	
I/σ(I)	16.8(5.0)	16.6(4.7)	16.4(4.3)	16.6(3.9)	16.1(3.3)	15.4(2.8)	15.3(2.4)	
Completeness (%)	99.7(99.3)	99.7(99.4)	99.7(98.9)	99.7(99.1)	99.7(98.4)	99.6(96.8)	99.4(93.7)	
Redundancy	3.6(3.2)	3.6(3.3)	3.5(3.2)	3.5(3.1)	3.5(2.8)	3.5(3.0)	3.5(2.8)	
Mosaicity (º)	0.14	0.14	0.14	0.14	0.14	0.14	0.14	
B_{factor}	8.77	8.83	9.07	9.61	9.83	10.31	10.86	

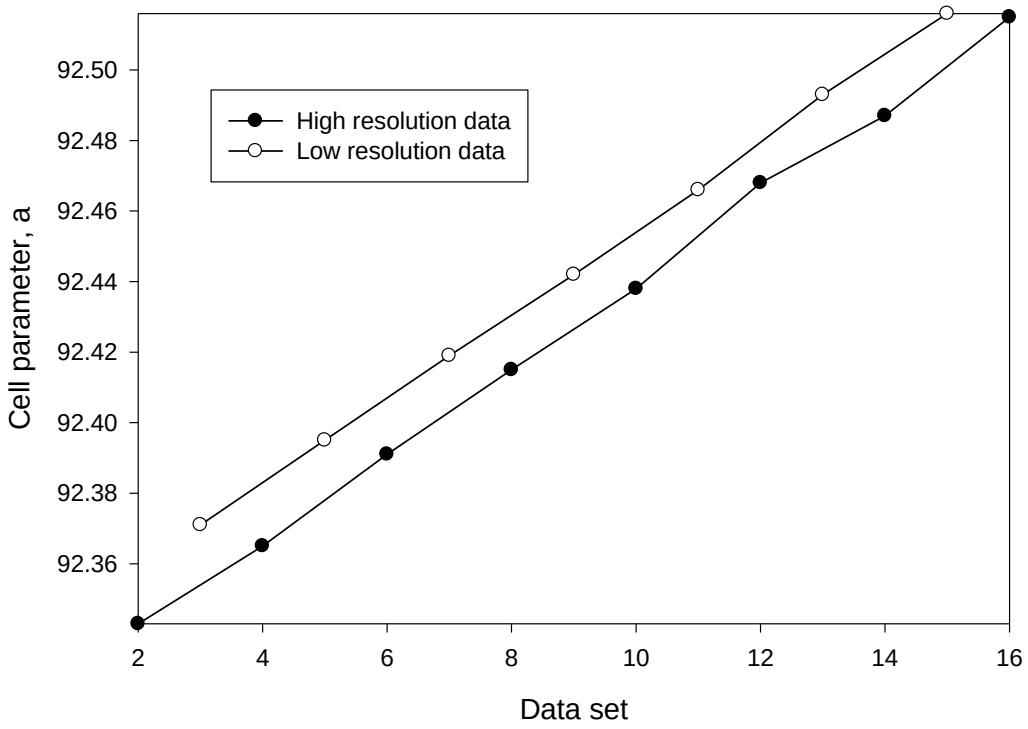
With each data set R_{factor} increases, signal-to-noise, completeness, and redundancy decreases. The mosaicity is unchanged, we are just seeing the beam contributions. The B_{factor} increases.

Odd numbers refer to the partial data sets and even to the complete data sets.



The Images

Same portion of high resolution data showing gradual decay of reflections.

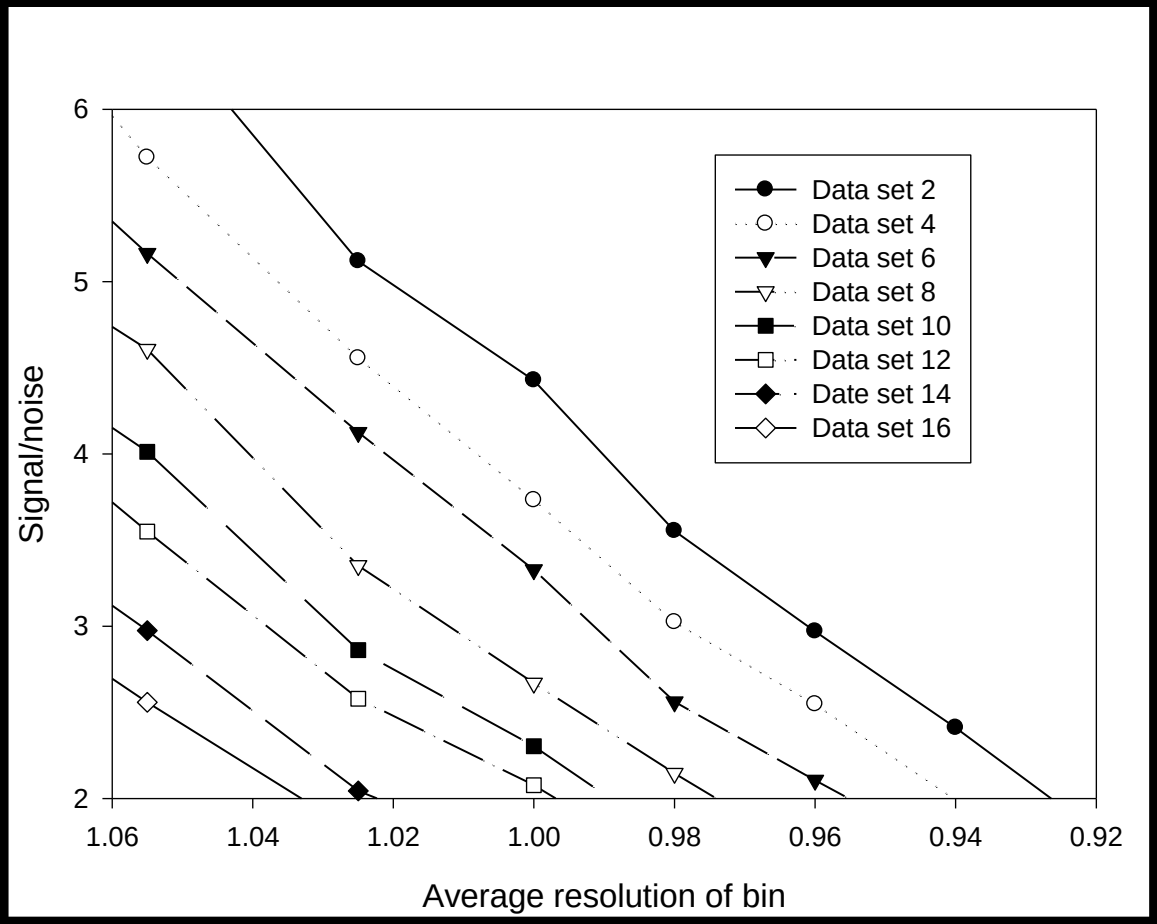


Diffraction data – Radiation Damage Datasets

High resolution partial data set (0.9 Å)								
Data set	2	4	6	8	10	12	14	16
R_{factor}	6.7(45.8)	6.7(54.7)	6.9(57.5)	7.2(59.4)	7.5(85.2)	8.0(68.7)	8.0(73.5)	8.0(-)
I/σ(I)	8.9(1.6)	8.5(1.2)	8.6(1.0)	8.3(0.8)	8.3(0.7)	8.0(0.6)	7.8(0.6)	7.7(0.5)
Completeness (%)	24.8(24.8)	24.8(23.2)	24.5(19.6)	24.1(15.3)	23.6(10.9)	23.0(7.0)	22.2(3.1)	21.7(1.4)
Redundancy	1.4(1.4)	1.4(1.3)	1.4(1.2)	1.3(1.2)	1.3(1.1)	1.3(1.1)	1.3(1.0)	1.3(1.0)
Mosaicity (º)	0.17	0.17	0.17	0.16	0.16	0.16	0.16	0.16
B_{factor}	6.04	6.35	6.70	6.85	7.25	7.54	7.85	8.13
Medium resolution complete data set (1.2 Å)								
Data set	3	5	7	9	11	13	15	
R_{factor}	7.5(22.5)	7.5(24.7)	7.7(27.3)	7.6(30.1)	7.9(33.4)	7.9(37.3)	7.8(41.7)	
I/σ(I)	16.8(5.0)	16.6(4.7)	16.4(4.3)	16.6(3.9)	16.1(3.3)	15.4(2.8)	15.3(2.4)	
Completeness (%)	99.7(99.3)	99.7(99.4)	99.7(98.9)	99.7(99.1)	99.7(98.4)	99.6(96.8)	99.4(93.7)	
Redundancy	3.6(3.2)	3.6(3.3)	3.5(3.2)	3.5(3.1)	3.5(2.8)	3.5(3.0)	3.5(2.8)	
Mosaicity (º)	0.14	0.14	0.14	0.14	0.14	0.14	0.14	
B_{factor}	8.77	8.83	9.07	9.61	9.83	10.31	10.86	

With each data set R_{factor} increases, signal-to-noise, completeness, and redundancy decreases. The mosaicity is unchanged, we are just seeing the beam contributions. The B_{factor} increases.

Odd numbers refer to the partial data sets and even to the complete data sets.



Linear dependence of I/σ(I) on dose,

Without solving the structure can we tell if there will be something for us to see?

The Normal Probability Plot

- The distribution of any set of magnitudes may be compared with any assumed distribution in a probability plot (Abrahams & Keve, 1971).
- Consider independent measurements of the same i^{th} structure factor, $F(1)_i$ and $F(2)_i$. The statistic δm_i is defined as

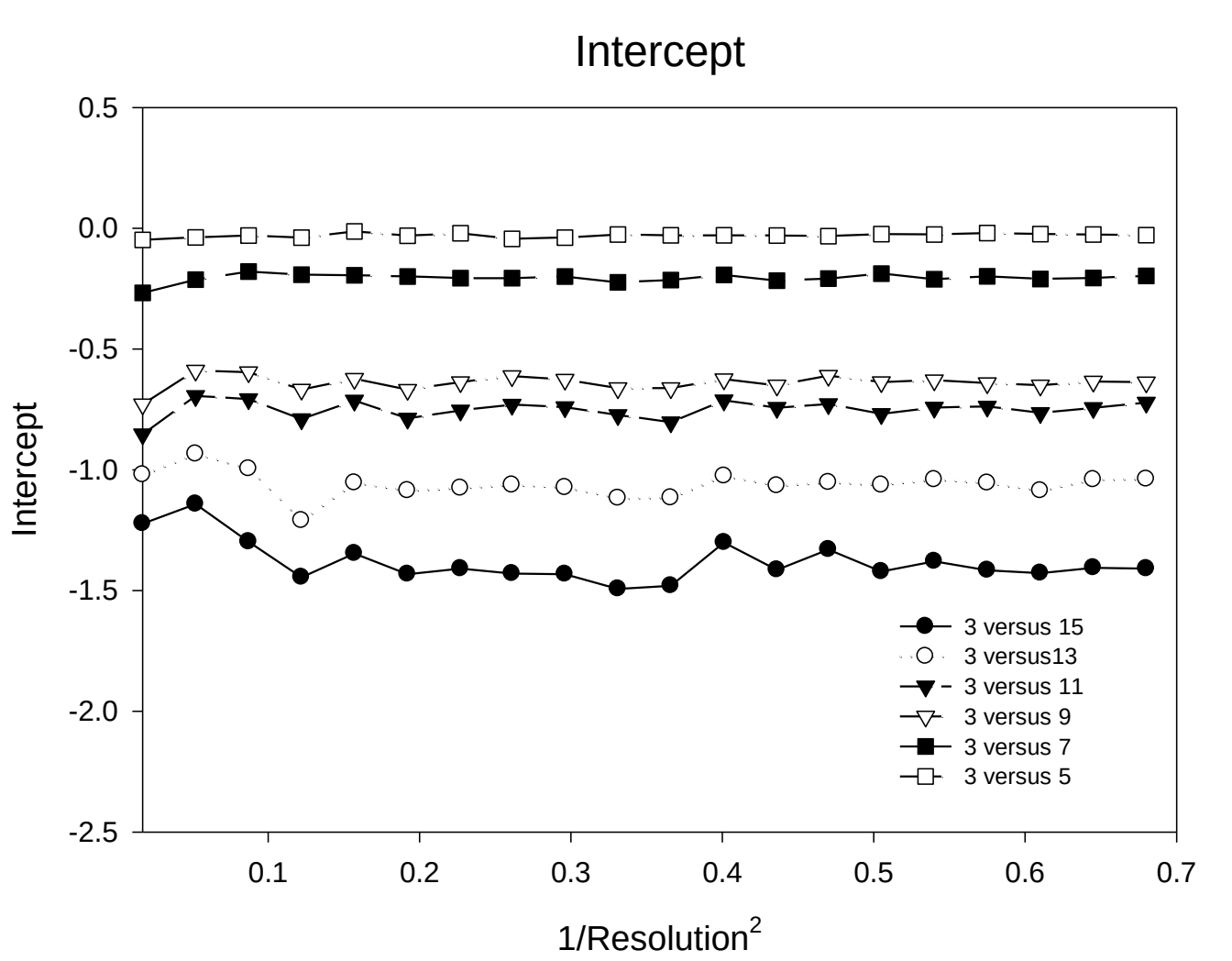
$$dm_i = [F(1)_i - KF(2)_i]/[s^2F(1)_i + K^2s^2F(2)_i]^{\frac{1}{2}}$$

- Where K is a scale factor that minimizes the sum of δm_i^2
- The distribution of δm_i *is Gaussian if* $F(1)_i$ and $F(2)_I$ contain only random error and $sF(1)_i$ and $sF(2)_i$ are correct.
- Deviations from this can be examined with great sensitivity using the probability plot.
- The normal probability plot is constructed by arranging δm_i in order of magnitude against xi, the values (quantiles) expected for a normal distribution (tabulated).

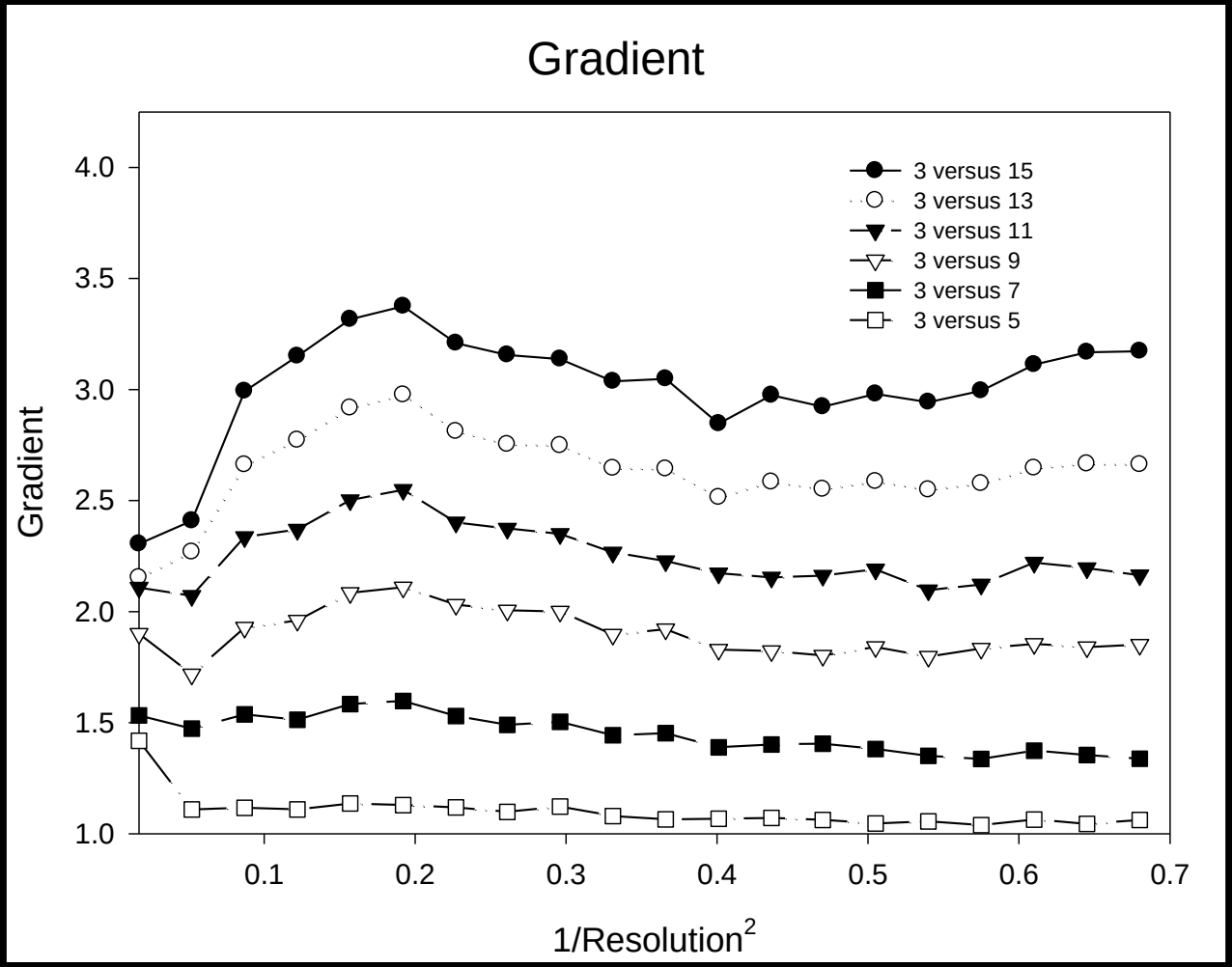
Used to identify heavy atom derivatives

- Used by Howell & Smith (1992) to identify data with a significant heavy atom contribution.
- Deviations from intercept of zero and slope of unity indicate significant structural difference.
- For the same sample on two different instruments the slope was 1.41 with intercept near zero. For heavy atom derivatives the intercept and slope were significantly increased. In one case as much as 4.6 for the intercept and 20.7 for the slope.

Applied to successive data sets



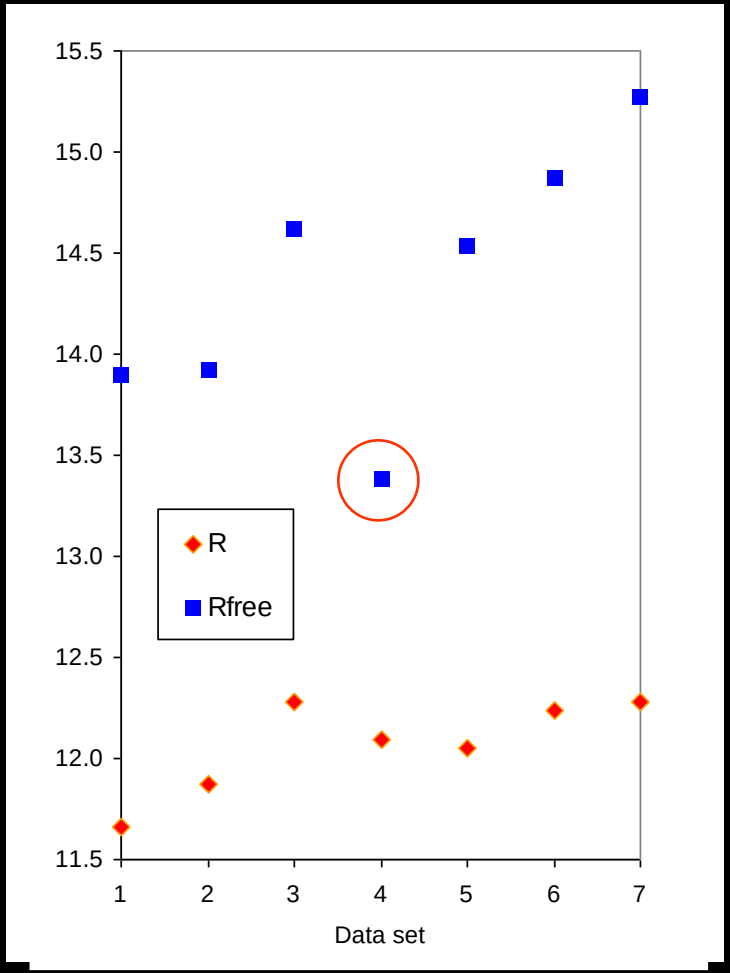
Applied to successive data sets



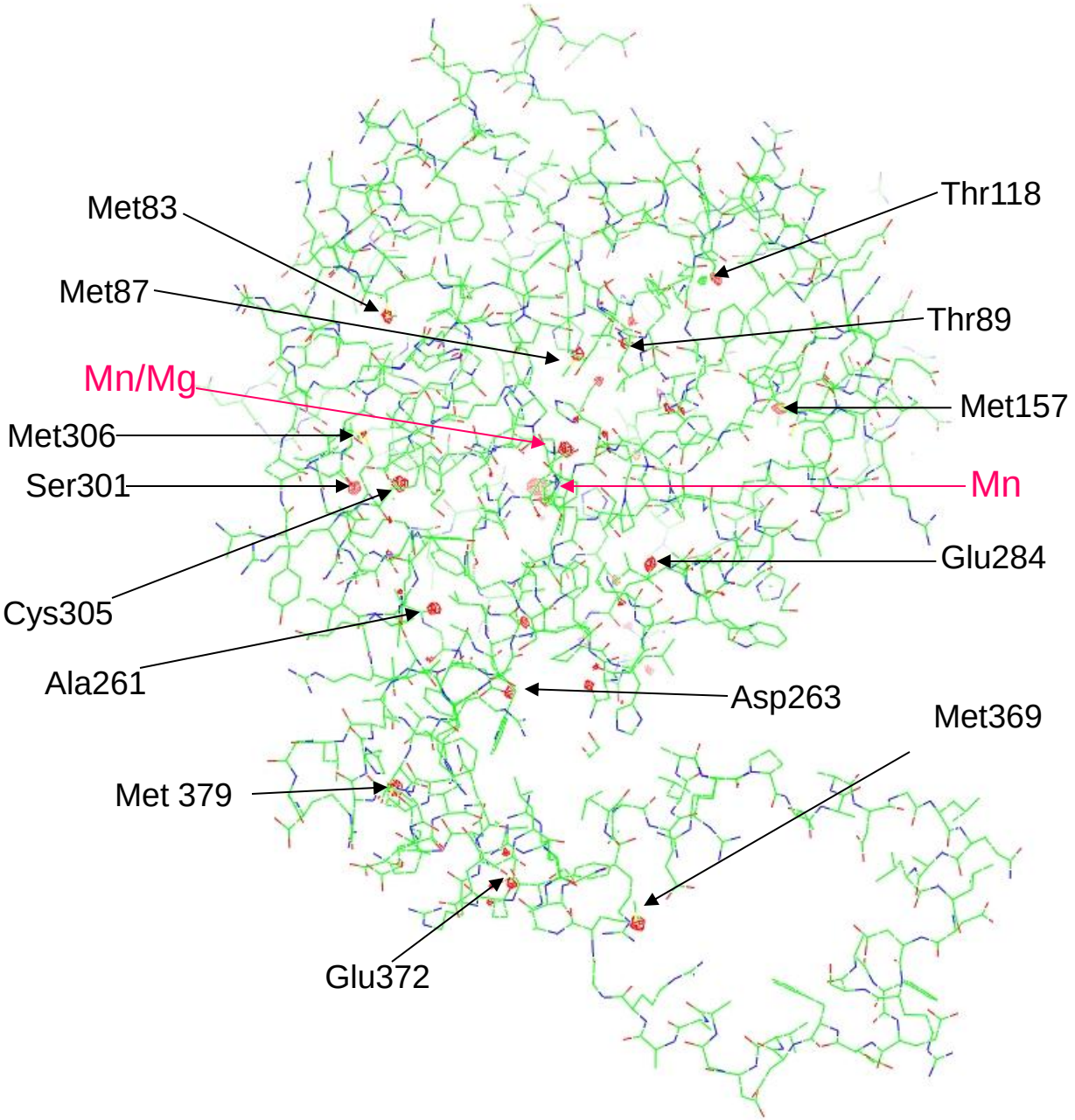
Structural refinement

- The complete, 1.2 Å data sets were renumbered 1 to 7.
- Arp/Warp was used to remove potential model bias
- Refmac was used initially for refinement followed by Shelx (for occupancy determination).
- We have now switched completely to Phenix for all refinement.

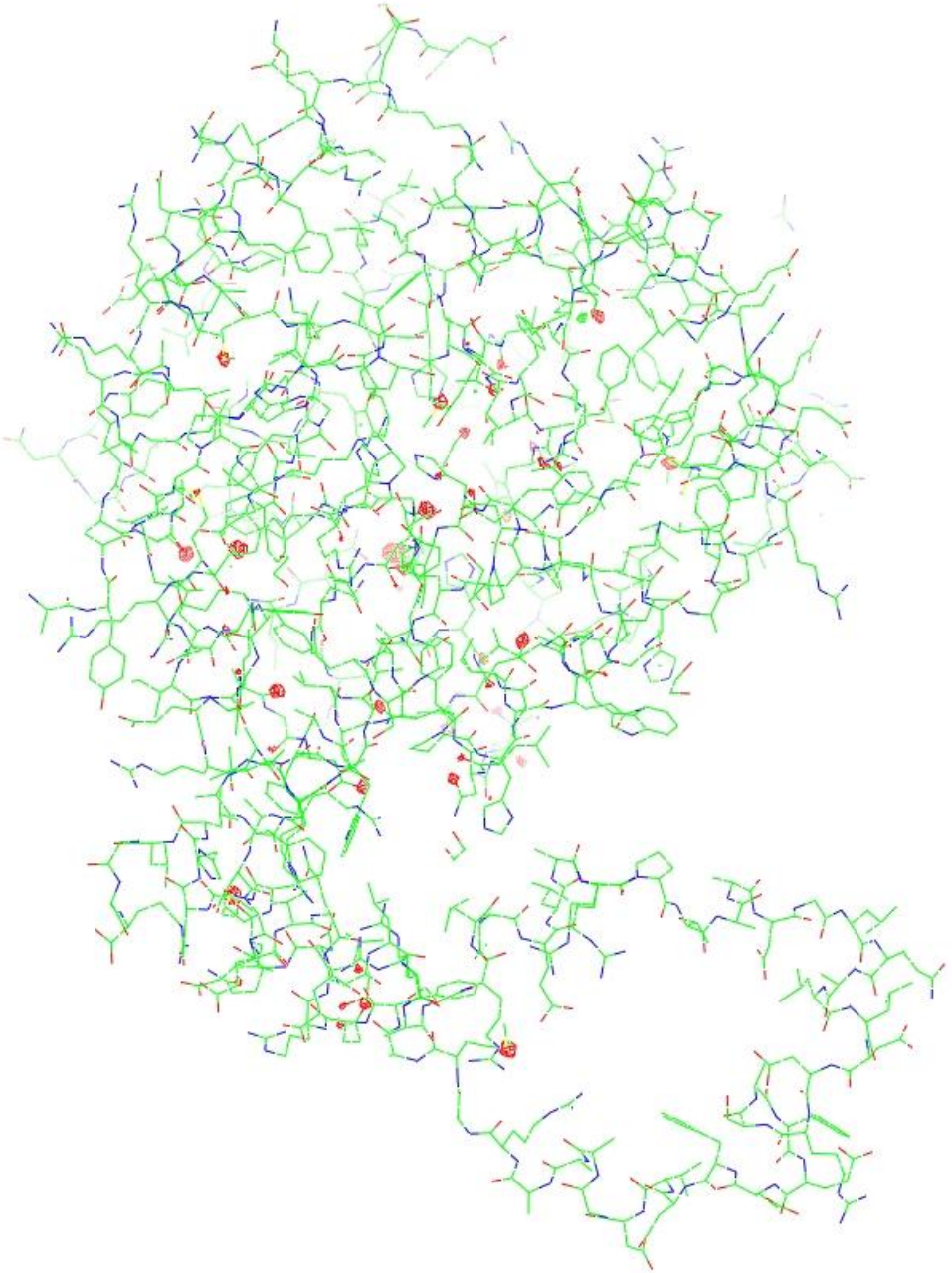
- Fo_1-Fo_n maps were calculated using CNS



Fo_i-Fo_n for structural data set 1 and 2

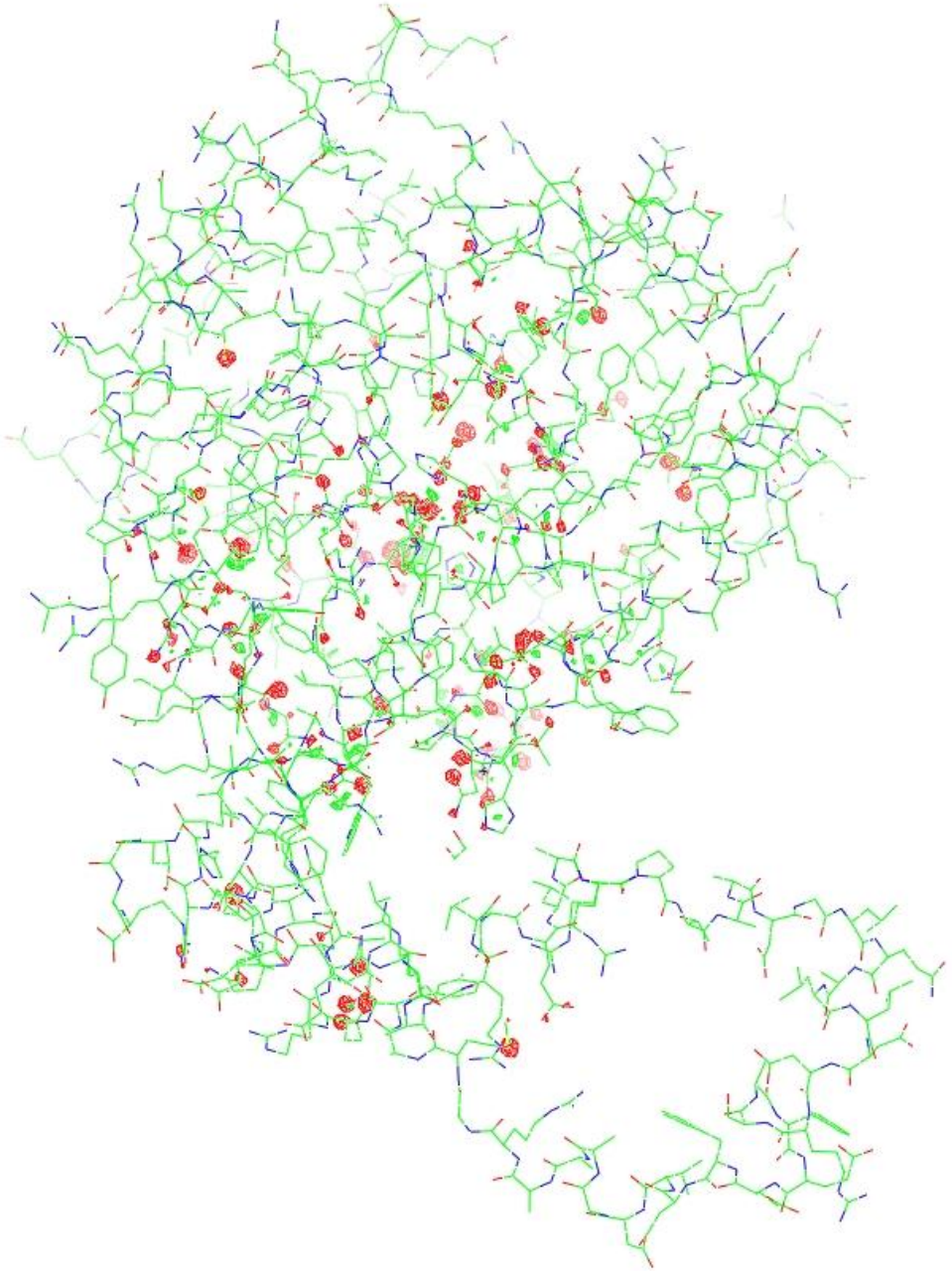


Fo_i-Fo_n for structural data set 1 and 2



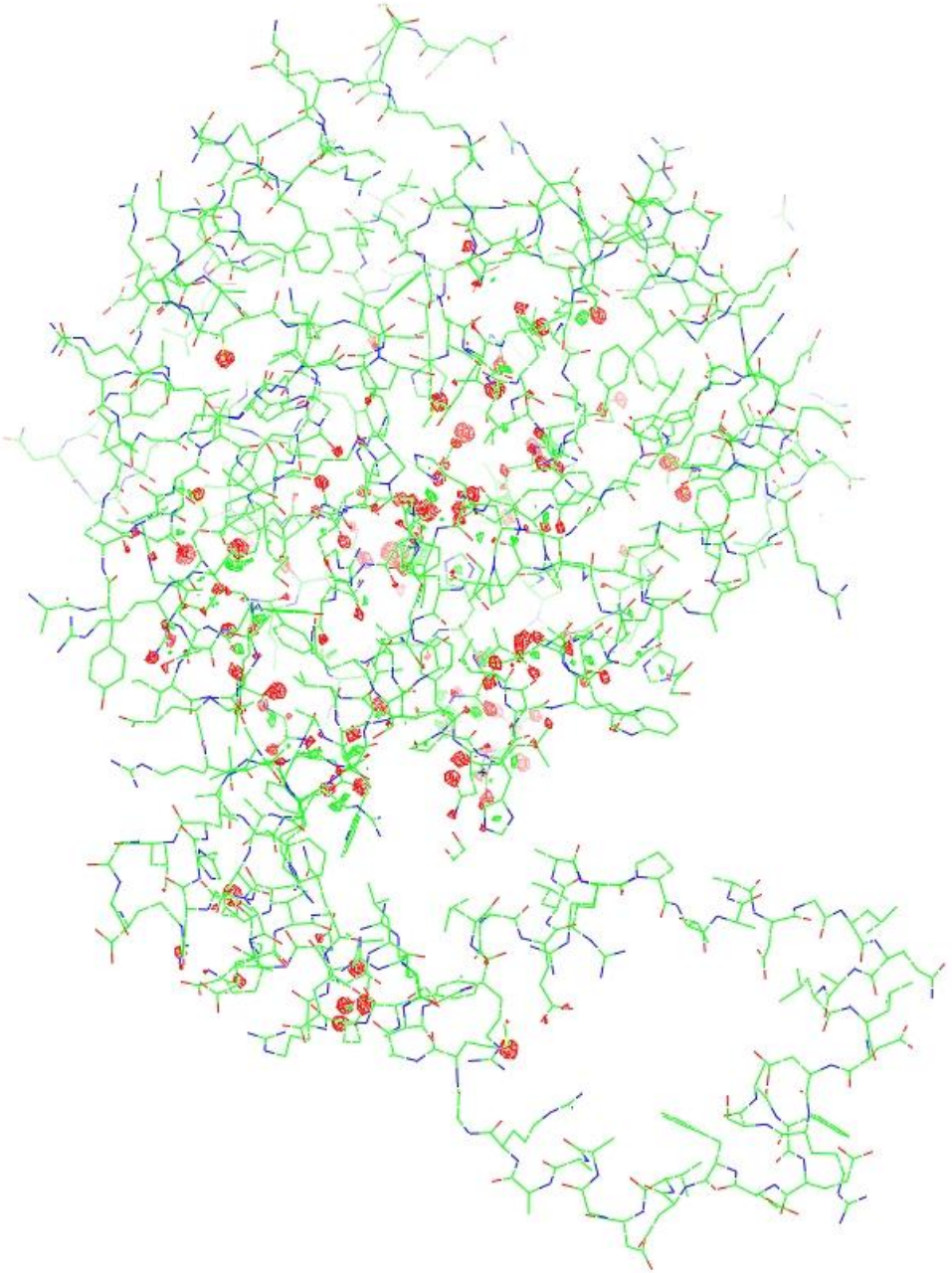
1.64×10^6 Gy

Fo_i-Fo_n for structural data set 1 and 3



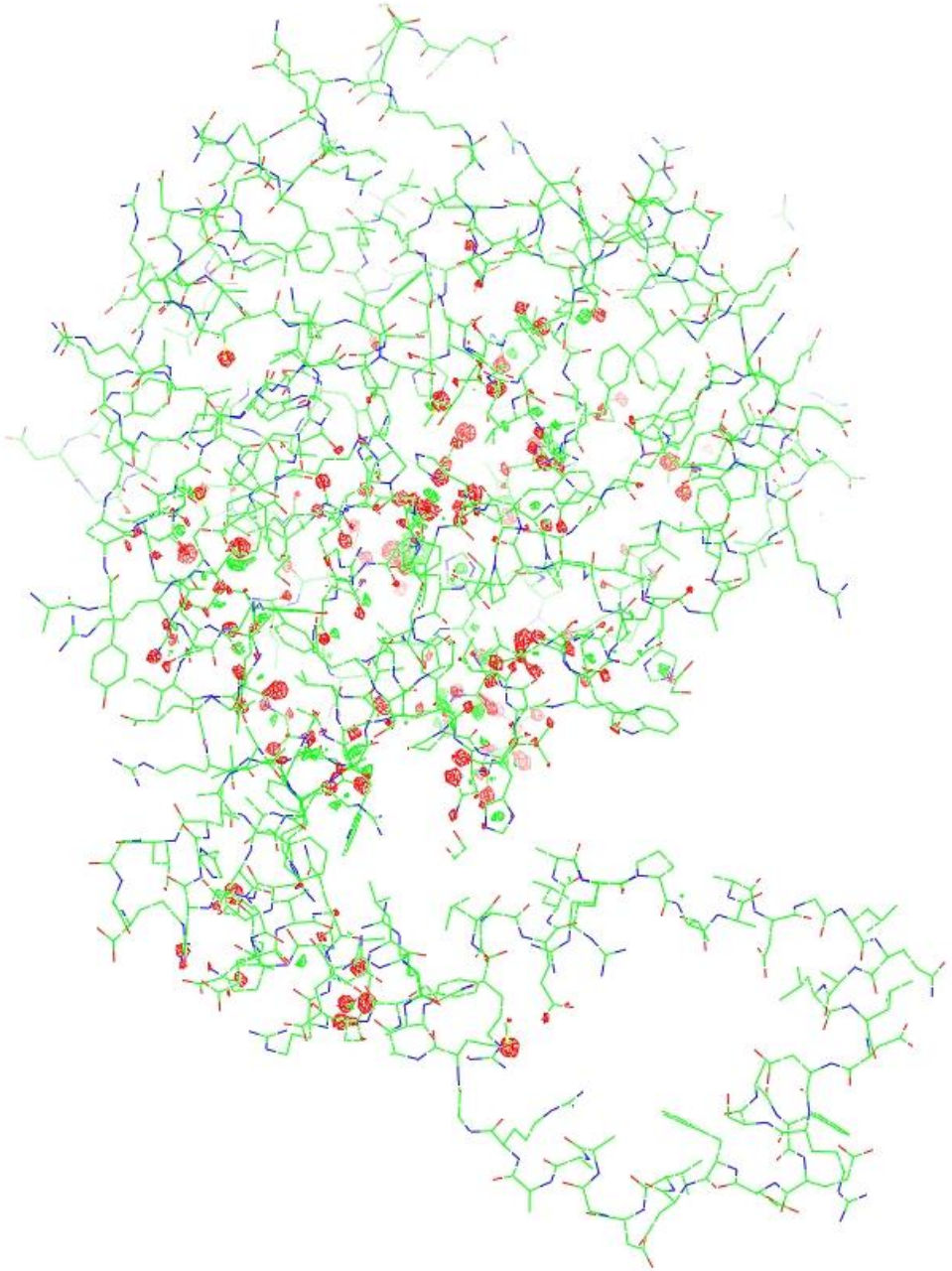
2.46×10^6 Gy

Fo_i-Fo_n for structural data set 1 and 4



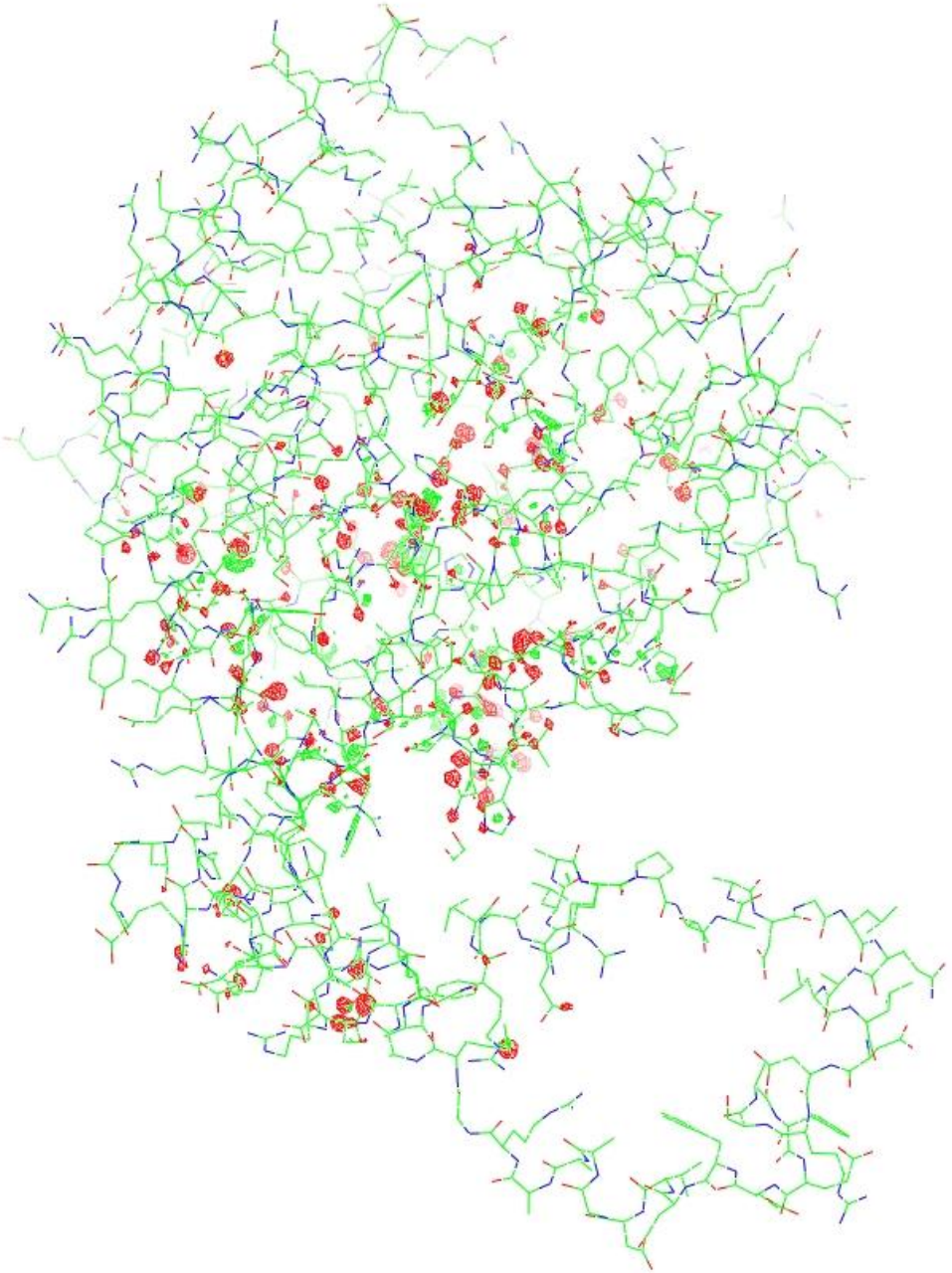
3.28×10^6 Gy

Fo_i-Fo_n for structural data set 1 and 5



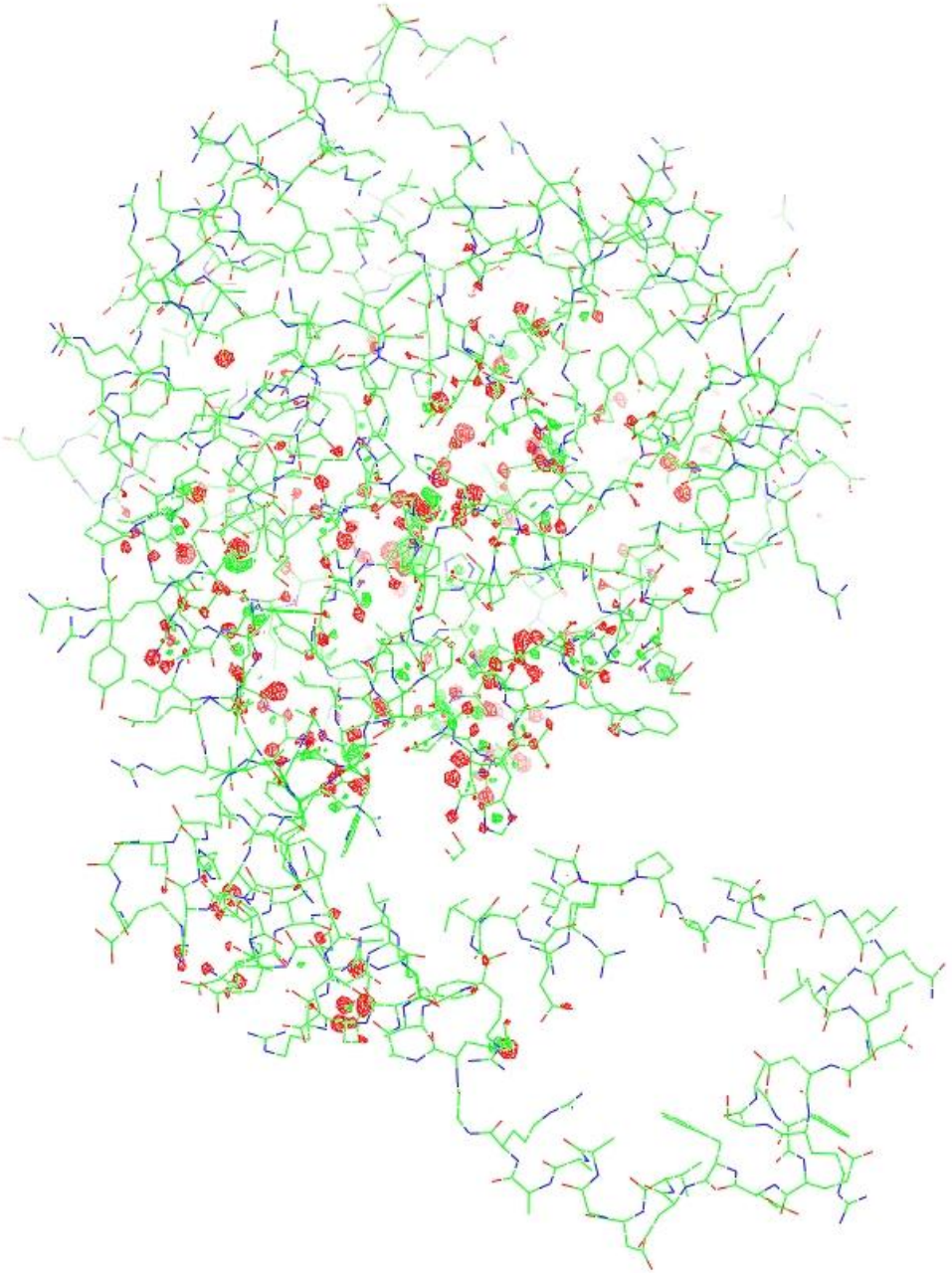
4.10×10^6 Gy

Fo_i-Fo_n for structural data set 1 and 6



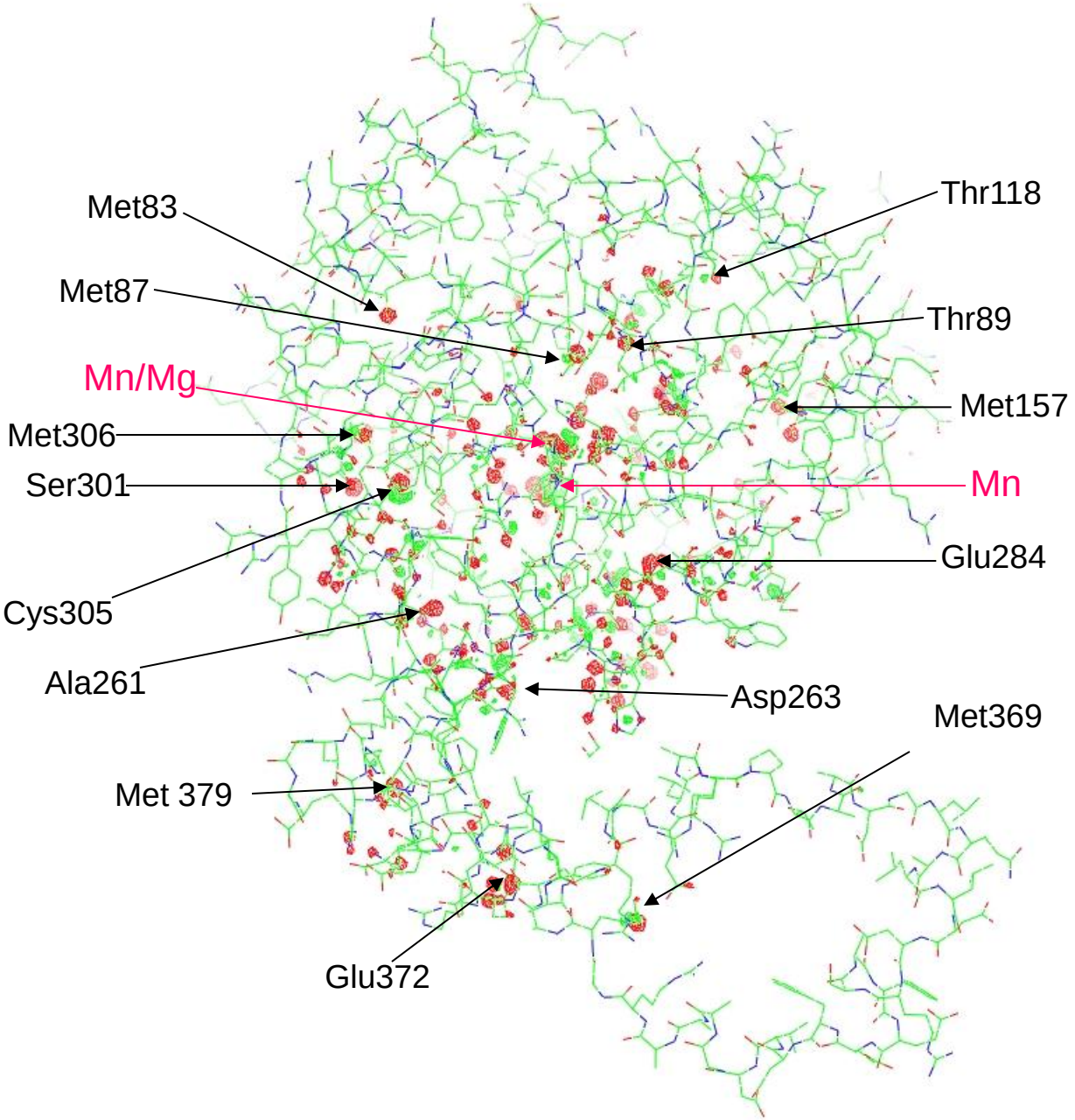
4.92×10^6 Gy

Fo_i-Fo_n for structural data set 1 and 7



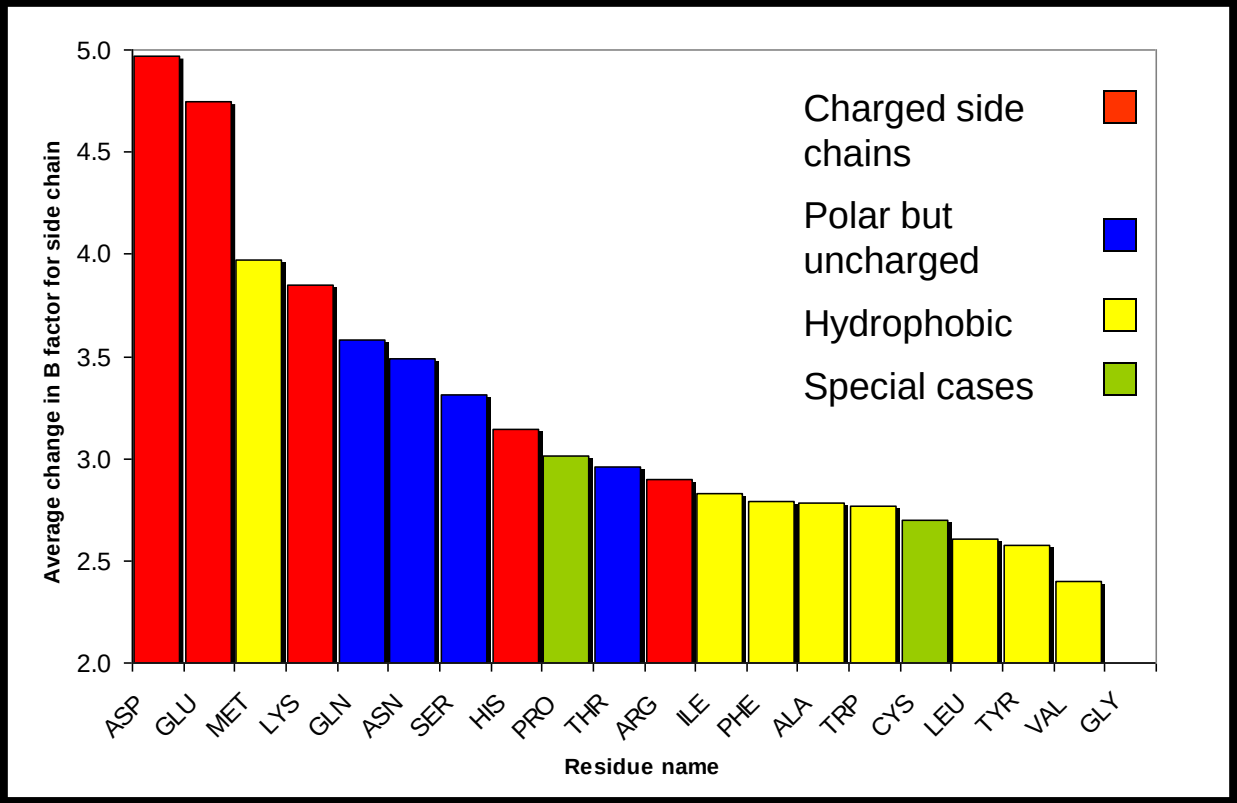
5.74×10^6 Gy

Fo_i-Fo_n for structural data set 1 and 7



Structural refinement

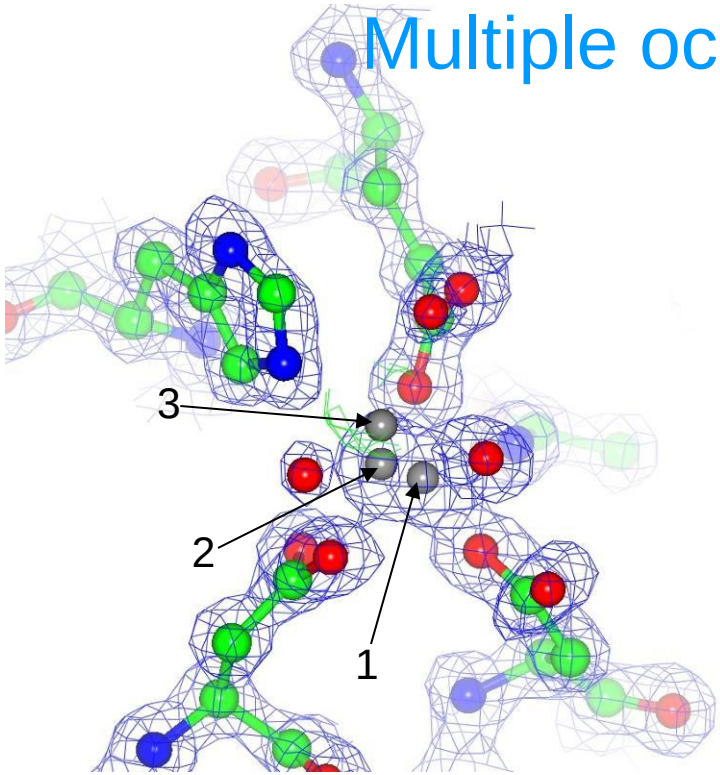
Form the first to the last data set there was no significant difference in atomic positions for the amino acid residues. For all the atoms except for the metal atoms there was no significant difference in occupancy (refined independently for each atom in Phenix).

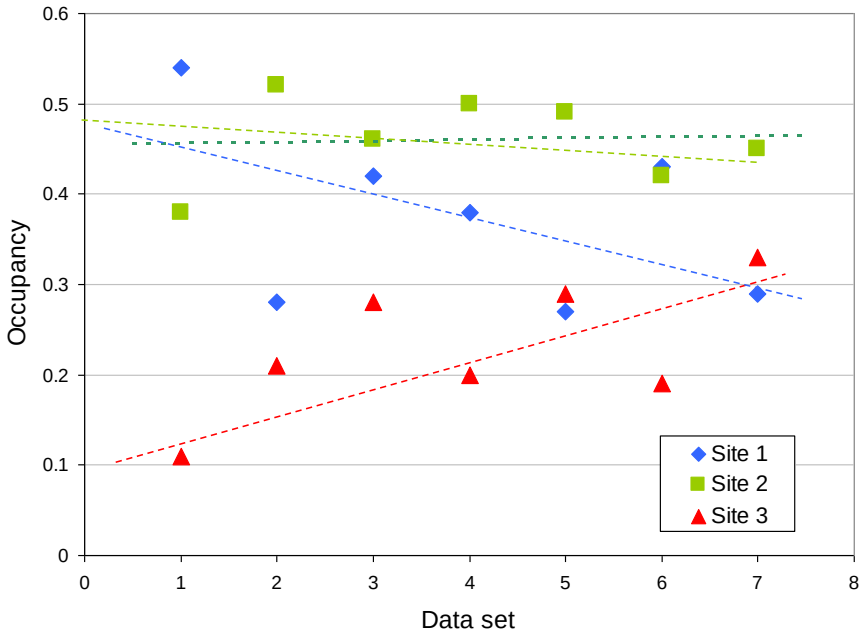


Residues B_{factor} determined by baverage from the CCP4 suite.

However, there was an increase in average and specific atomic B_{factor} for all the residues. This average side chain difference is shown above for the first and last data sets.

Multiple occupancy of metal site 2





With dose:

Site 1 decreases in occupancy
Site 2 remains constant
Site 3 increases in occupancy

For the second metal site the occupancy for Mn and Mg remain constant over the data sets.

Summary

In the X-ray data we see:

- A decrease in reflection intensity at high resolution.
- A linear decrease in signal-to-noise as a function of dose.
- An increase in cell parameters as a function of dose.
- No increase in mosaicity (so small we cannot measure it).

In the structural results we see:

- No significant change in amino acid overall occupancy or position as a function of dose.
- A significant increase in the B_{factor} as a function of dose
- B_{factor} increasing predominantly in the order of charged side chains, polar but uncharged then hydrophobic residues. MET is a special case containing sulfur, a large X-ray target.
- An occupancy change (and some positional change) for one of the metal sites but not the other.

Are there a different physical processes for radiation effects on amino acid residues and metal sites?

Beware

- The radiation data is from a single crystal.
- Until the experiments have been reproduced it is not statistically valid.
- There are still features to take care of in the refinement.

- The best lesson from this work – if your crystal diffracts to beyond 1Å … move the detector back, tell no one and ignore it!

Where we are heading now

- Computational modeling of the structures from the individual data sets.
- Reproduction of the experiment with this and other macromolecues.
- Molecular biology to try and increase or decrease the response to study both the target and environment.
- Other stuff!

Conclusion

- There is evidence that X-ray dose causes shifts and changes in occupancy of metal sites.
- The evidence is limited and will require more experiments before it is statistically validated.
- Shifts in occupancy and position in the active site can easily be interpreted as having biological mechanistic significance rather than a physical artifact due to data collection.

Acknowledgements

Thanks to SSRL for help and beamtime, and the John R. Oishei Foundation for funding support. Thanks to the organizers and hosts for this series of workshops.