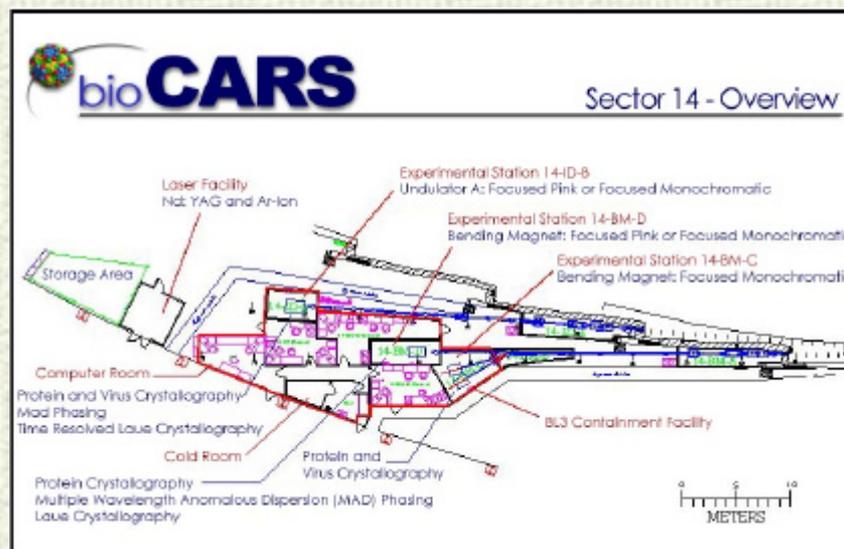


Watching Proteins Function with Time-resolved X-ray Crystallography

Status Report from 14-ID

Vukica Srajer

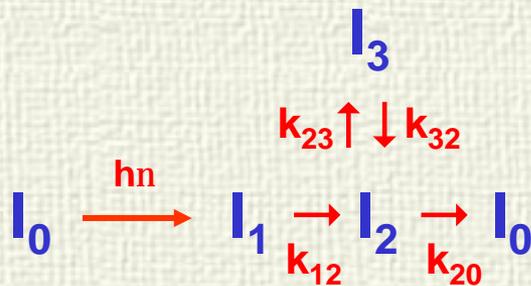
BioCARS, University of Chicago
Sector 14, Advanced Photon Source, Argonne National Laboratory



Time-resolved Crystallography

Why? Static structures of many biological macromolecules are available, but the detailed mechanism by which they function often remains elusive. ➔ **Need to capture molecules in action.**

Ultimate goal: Determine structures of intermediates and reaction mechanism

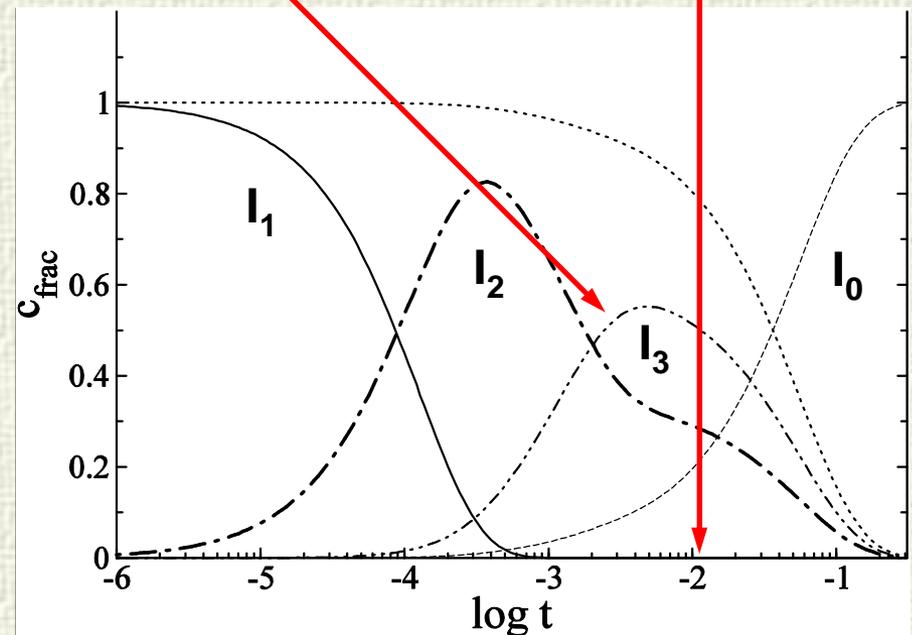


Concentrations of intermediates:

$$C_i(t) = C_{1i} \exp(-K_1 t) + C_{2i} \exp(-K_2 t) + C_{3i} \exp(-K_3 t)$$

Detectable accumulation?

Mixture of states at most time delays



How to Capture Structural Intermediates?

Extend the lifetime of intermediates: physical or chemical trapping

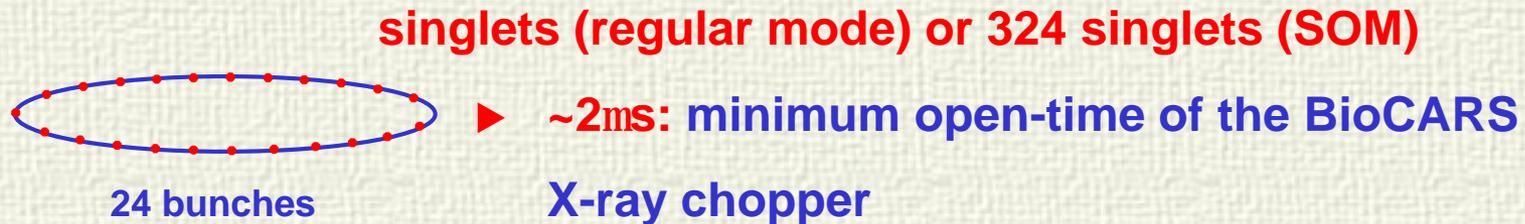
- Low temperature
- Trapping by freezing
- pH change or other solvent modification
- Chemical modification

or

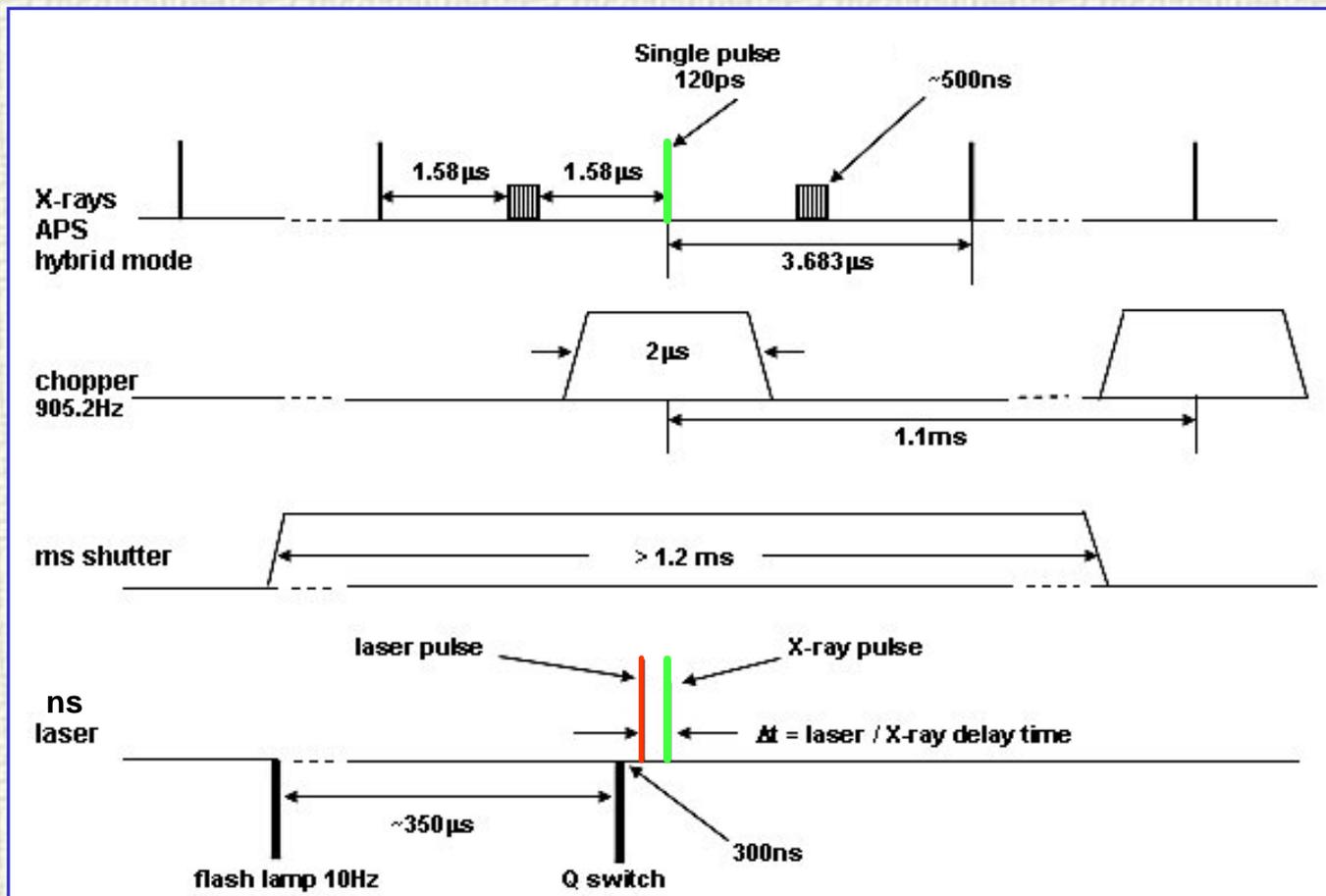
Real-time snap-shots of evolving structural changes: no trapping

- Probe fast structural changes at ambient temperature
- Requires
 - ▶ rapid reaction initiation (short laser pulses)
 - ▶ rapid data collection (short X-ray pulses, Laue technique)

Time-resolution: APS Operating Modes

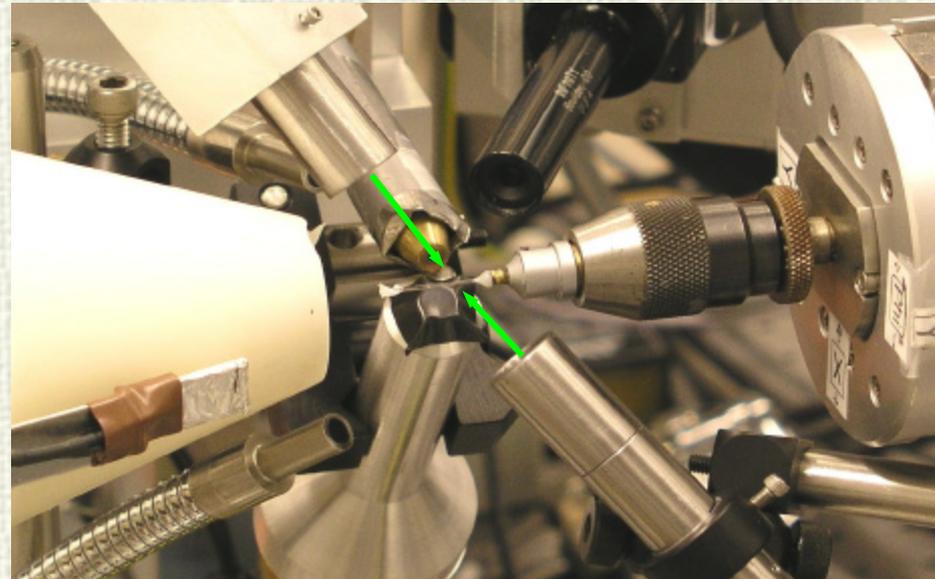


BioCARS Timing Scheme



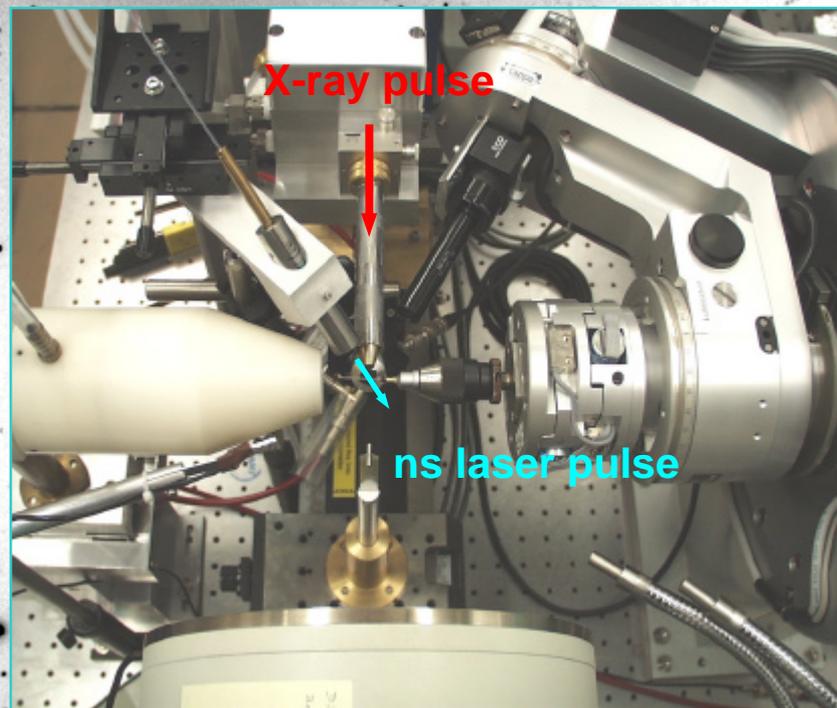
Reaction Photo-initiation in Crystals

- large number of molecules in crystals:
 $10^{13} - 10^{14}$
 - ▶ 10-100 mJ/pulse
- uniform and efficient photo-initiation
 - ▶ tunable wavelength
 - ▶ thin crystals (OD < 0.2)
- avoiding crystal damage
 - ▶ maximum laser pulse energies?
- extent of photo-initiation?
 - ▶ monitoring absorption changes



Ns Time-resolved Experiments at BioCARS

- Pump: 4-7ns laser pulse at appropriate wavelength
- Probe: 120ps, 500ns or 2ms X-ray pulse
- Laser–X-ray pulse delay times: 1ns to seconds
- Best data collection strategy
 - Slow variable: crystal angular setting
 - Fast variable: time delay
- ➔ For each crystal orientation collect a no laser frame and a series of frames at different time delays
- Merge data from several crystals
- 10-100 pump-probe cycles per image
 - ➔ pump-probe cycles repeated prior to detector readout
- 40-60 images per data set (2-3° angular increment for Undulator A)
- ~1-5 sec between laser pulses
- 0.5-10h elapsed time per data set



Time-resolved Data Analysis

Challenge:

Time-dependent series of difference electron density maps, $\Delta\rho(t)$, each possibly a mixture of states



- Number of intermediates
- Time-independent maps for pure intermediates
- Structures of intermediates
- Reaction mechanism

Most promising method: Singular Value Decomposition (SVD)

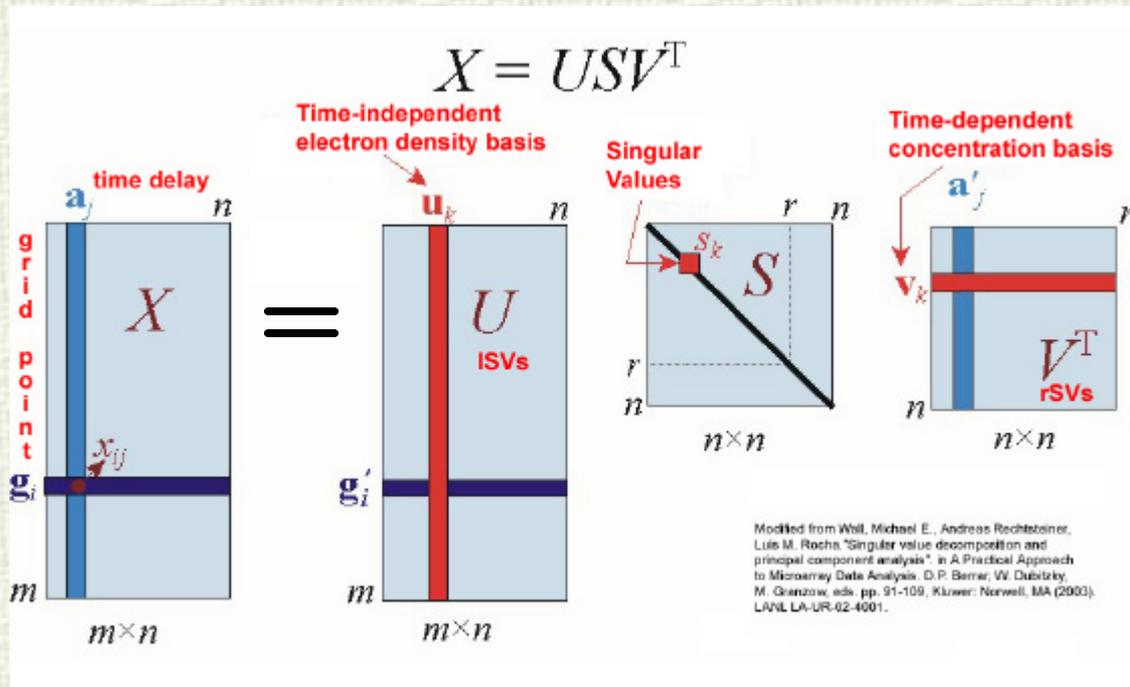
Rajagopal, S., Schmidt, M., Anderson, S., Ihee, H., and Moffat, K. Analysis of Experimental Time-Resolved Crystallographic Data by Singular Value Decomposition. *Acta Cryst. D* 60, 860-871 (2004).

Schmidt, M., Pahl, R., Srajer, V., Anderson, S., Ren, Z., Ihee, H., Rajagopal, S. and Moffat, K. Protein kinetics: Structures of intermediates and reaction mechanism from time-resolved x-ray data. *PNAS* 101 (14) 4799-4804 (2004).

Schmidt, M., Rajagopal, S., Ren, Z., and Moffat, K. Application of singular value decomposition to the analysis of time-resolved macromolecular x-ray data. *Biophys. J.* 84, 2112-2129 (2003)

SVD

- Method of **global analysis** commonly used in spectroscopy to analyze time-dependent optical spectra
- Separates **space and time variables** by decomposing data matrix X into **time-independent Dr maps** (left singular vectors U , **ISV**) and their **temporal variations** (right singular vectors V , **rSV**)



Attractive Features of SVD-based analysis of Time-resolved Crystallographic Data

1. **Major time courses, rSVs, are identified in an automated and global way** from $D_r(r,t)$ maps, with no assumptions about the reaction mechanism.
2. **Relaxation rates, common to all rSVs, are determined from rSVs.**

Number of intermediates in the reaction:

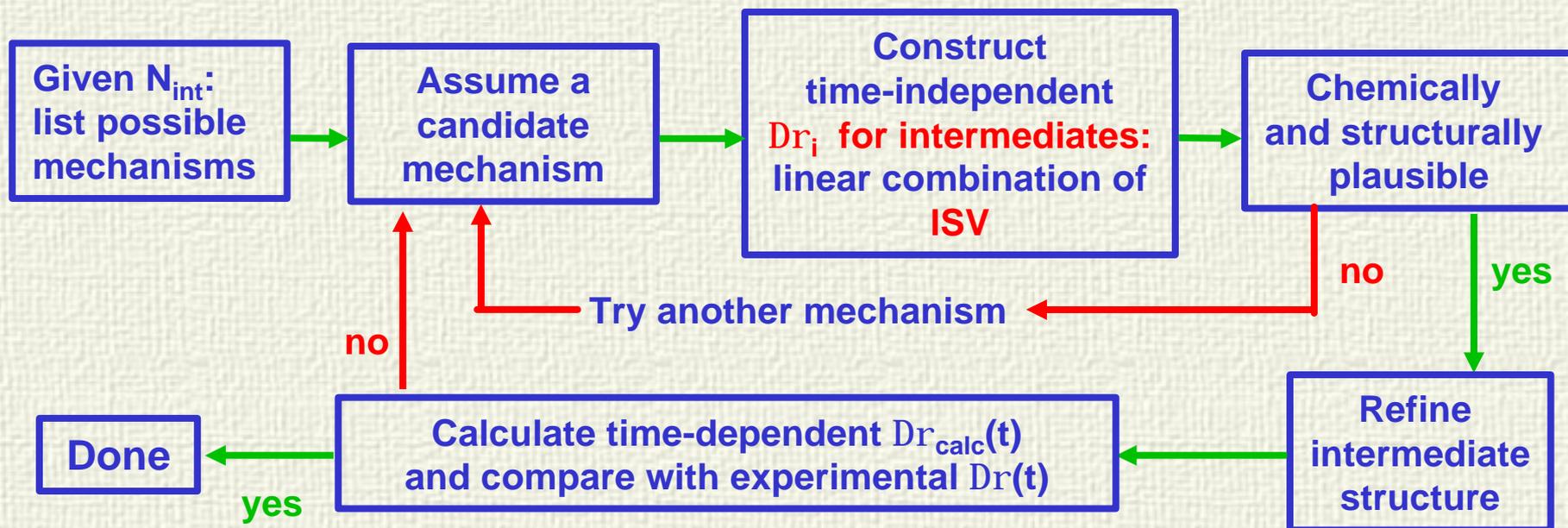
$$N_{\text{int}} \approx N_{\text{relax}}$$

3. **Signal is typically contained in the first few ISV only.** The input series of maps can be approximated by S/N improved maps $D_r'(r,t)$, reconstituted from the first N' singular vectors: **effective noise filter.**
4. **Significant ISV are used to construct** the time-independent difference electron density maps for reaction **intermediates.**



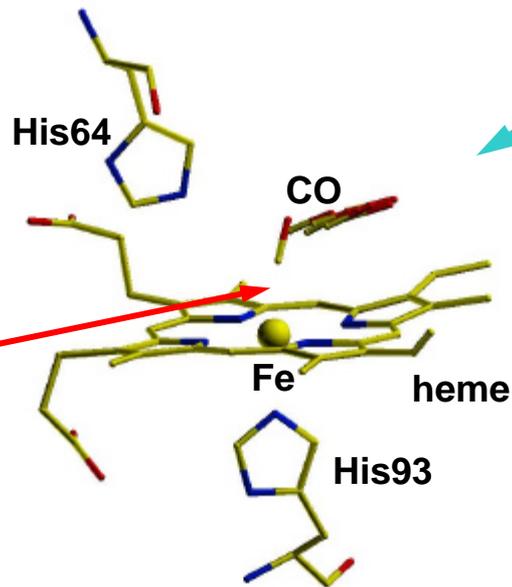
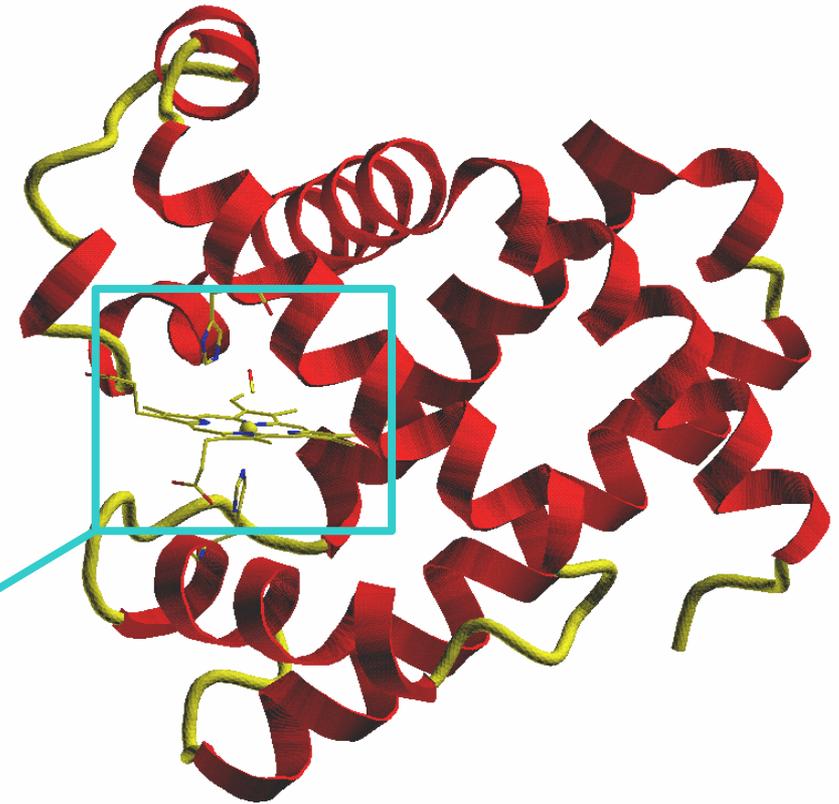
SVD-based Analysis: Reaction Mechanism and Structures of Intermediates

- SVD analysis:
 - number of intermediates N_{int}
 - relaxation times
- Post-SVD processing: analyze possible reaction mechanisms



Myoglobin

Challenging case:
structural changes following
ligand photo-dissociation
are small (0.2-0.3Å)

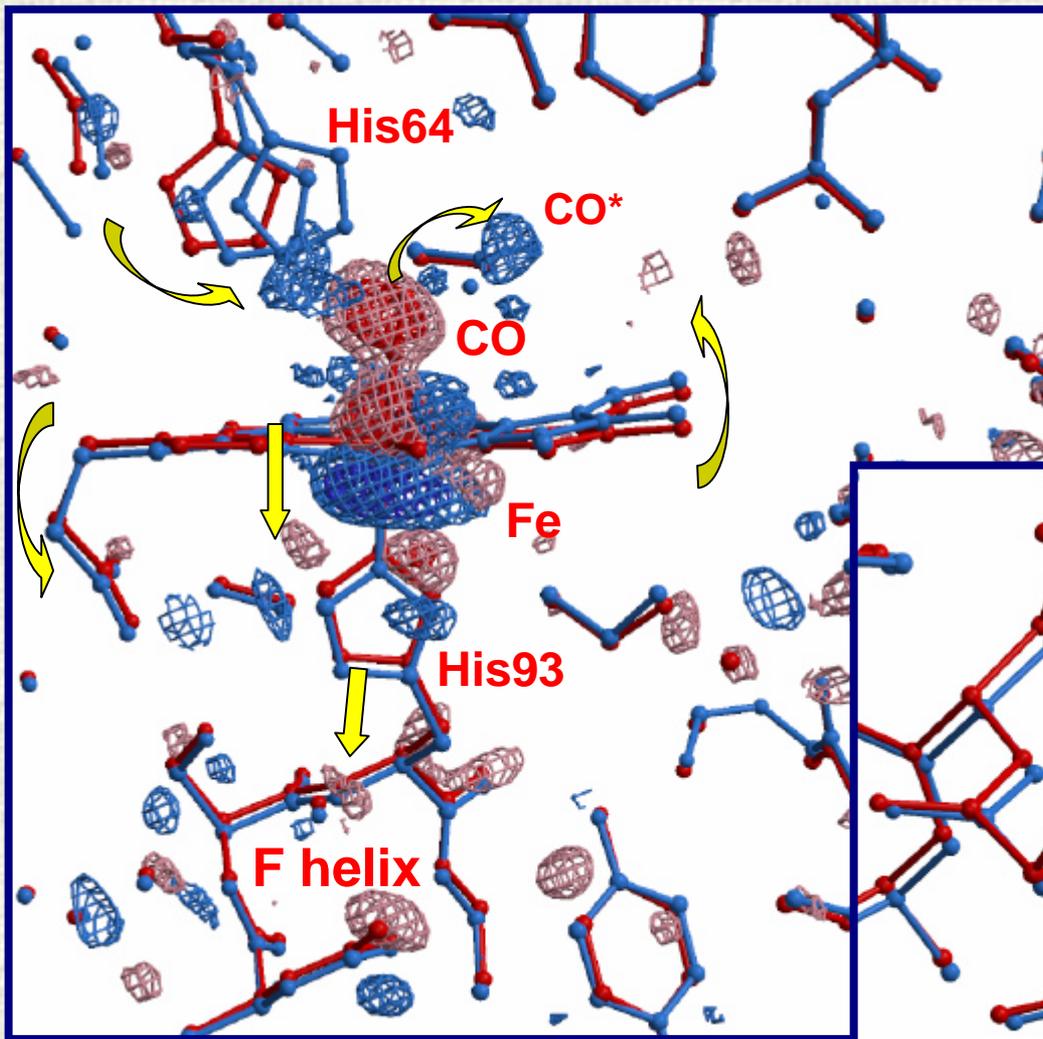


laser pulse
breaks Fe-CO
bond

ID09, ESRF

Keith Moffat, University of Chicago
Michael Wulff, ESRF

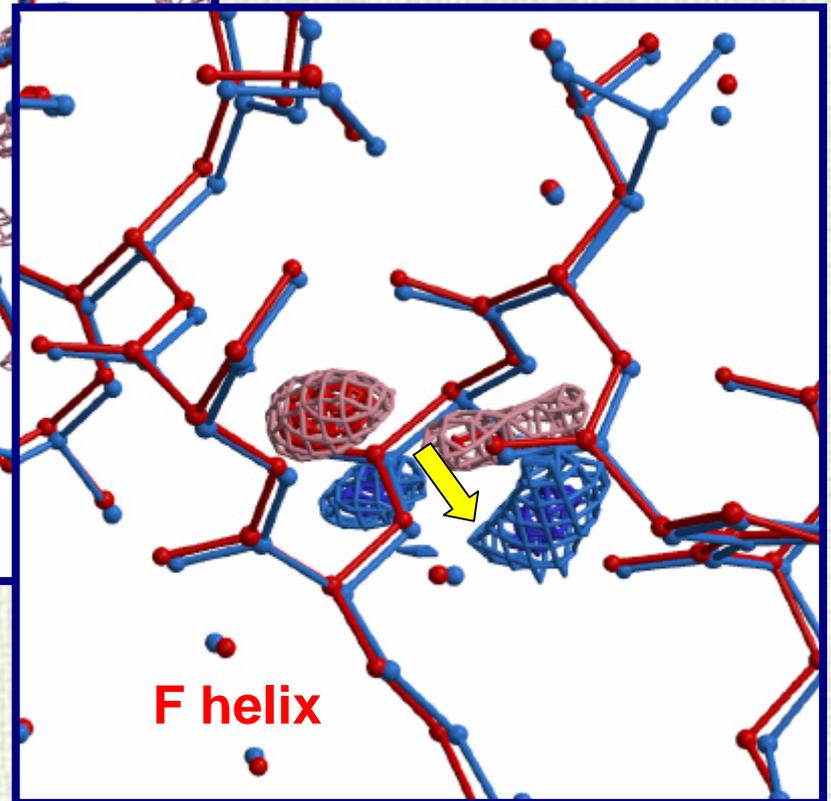
Srajer *et al.*, *Science* 274, 1726 (1996)
Srajer *et al.*, *Biochemistry* 40, 13802 (2001)



Changes: red → blue

Fe-CO bond breaks
 Fe moves out of the heme plane
 Heme buckles

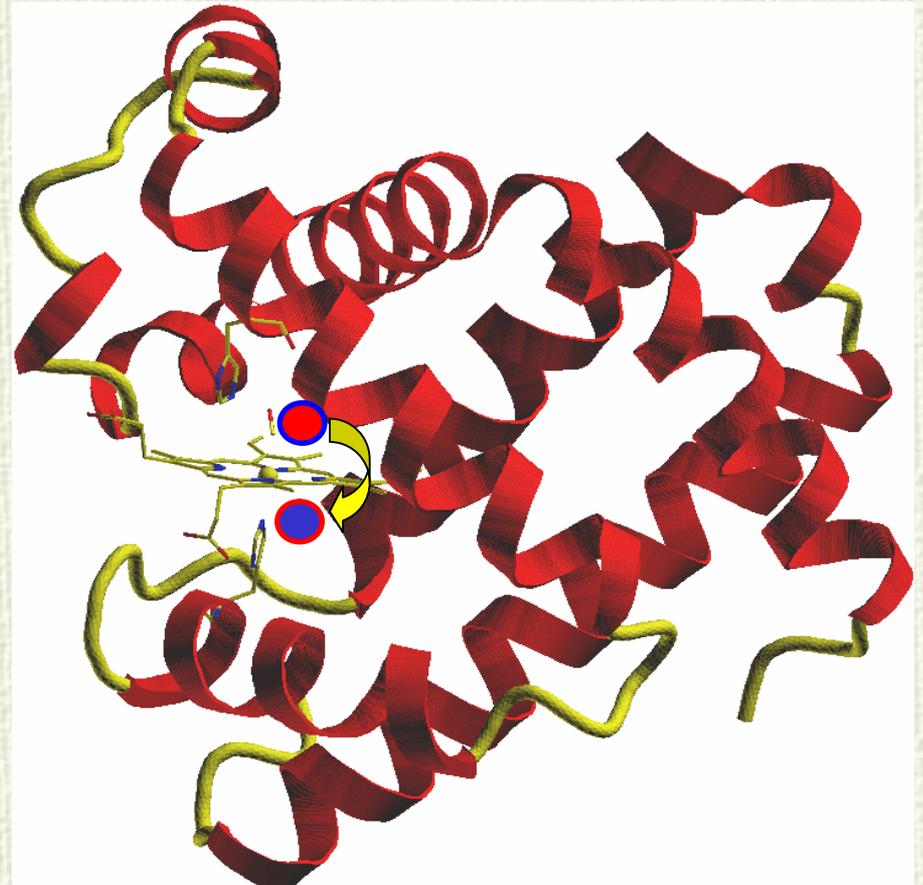
His64 swings in
 Heme rotates
 F-helix moves



1ns difference electron density map:
 Mb* - MbCO
 ~40% photolysis

Myoglobin – Summary of results

- **Sub-ns structural changes:**
heme buckling, His64 motion,
initial displacement of F and E
helices
- **Photo-dissociated CO pathway:**
two docking sites
 - **distal (half-life of 70ns)**
 - **proximal (half-life of 10ms)**
- **CO docking sites with 20%
occupancy are detectable: ~3 σ**
- **Small structural changes can
be detected: 0.2-0.4Å**



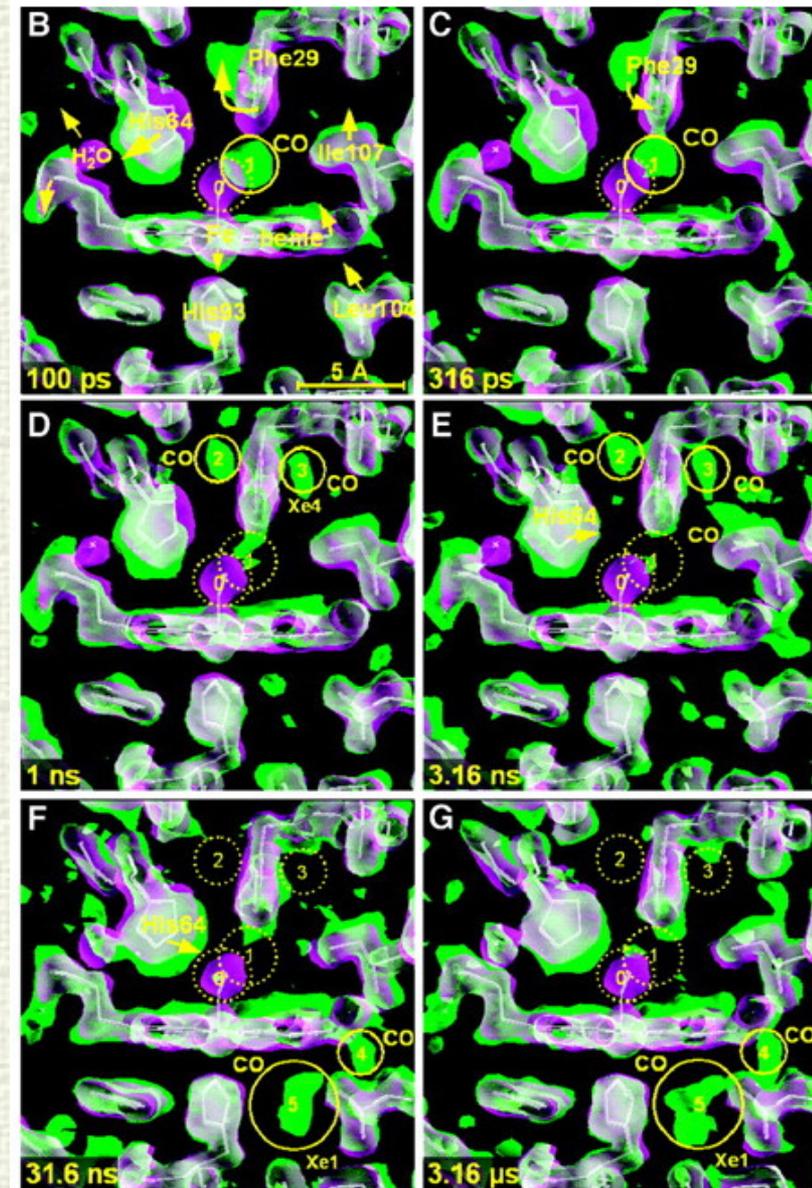
Latest Myoglobin Results

First sub-ns time-resolved experiment
(ID09, ESRF):

F. Schotte et al., *Science* 300, 1944 (2003).
Watching a Protein as it Functions with 150-ps
Time-Resolved X-ray Crystallography

Protein structural dynamics:

D. Bourgeois et al., *PNAS* 100, 8704 (2003).
Complex landscape of protein structural dynamics
unveiled by nanosecond Laue crystallography



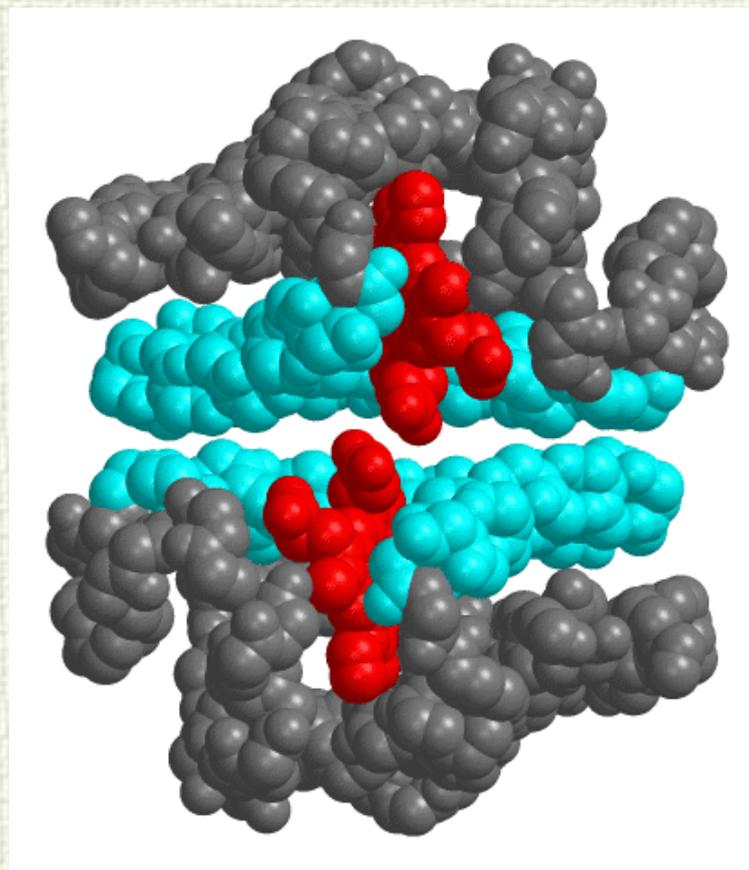
From: Schotte et al., *Science* 300, 1944 (2003)

Dimeric Hemoglobin Hbl

(from clam *Scapharca Inaequalvis*)

Model for studies of cooperative protein behavior by time-resolved crystallography

- Cooperative ligand binding demonstrated in crystals
- Structural transitions involved in ligand binding and dissociation are localized and not too large: crystals survive quaternary change
- Successful Hbl-CO \rightarrow deoxy Hbl \rightarrow Hbl-CO transformation in the crystals
(Knapp J. and Royer W., *Biochemistry* 42, 4640, 2003)
- Crystals diffract to atomic resolution ($\sim 1\text{\AA}$)



BioCARS 14-ID, APS

James Knapp and William Royer
U of Mass Medical School, Worcester, MA

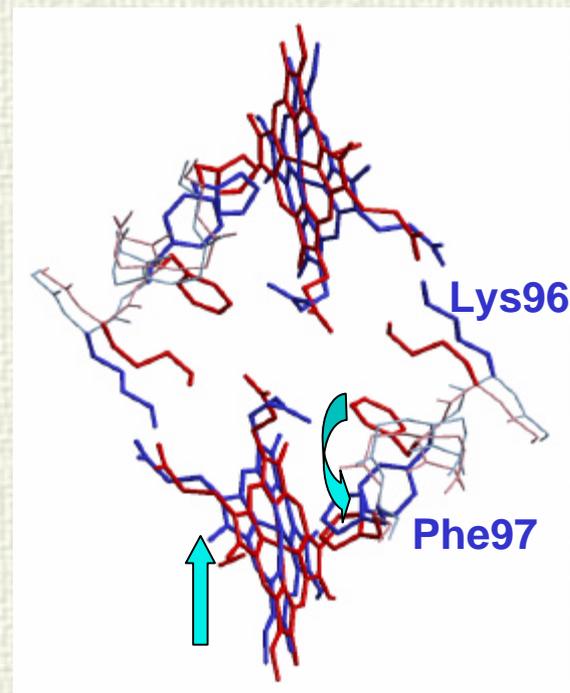
Vukica Srajer, Reinhard Pahl, BioCARS

Cooperativity in Hbl

Three key contributors to the cooperativity mechanism:

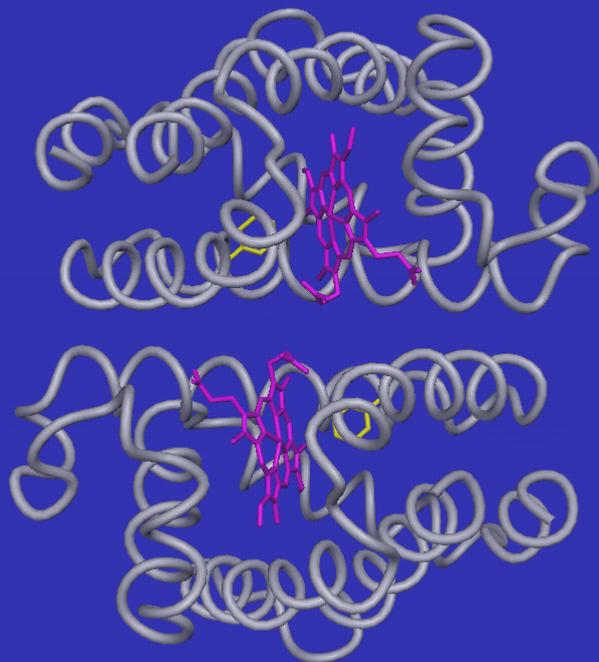
- Phe 97
- hemes
- water molecules at the dimer interface

Hbl-CO (R): red
Deoxy Hbl (T): blue

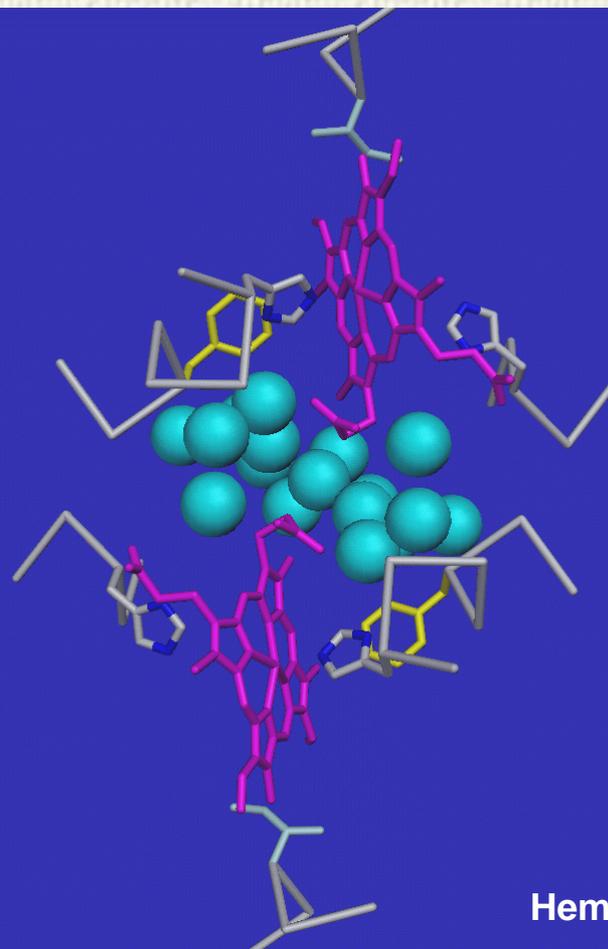


Structural transitions upon ligand release (red → blue):

- Phe 97 moves from the dimer interface to the proximal heme pocket
- the heme group moves toward the interface
- cluster of water molecules at the dimer interface rearranges



Hbl dimer



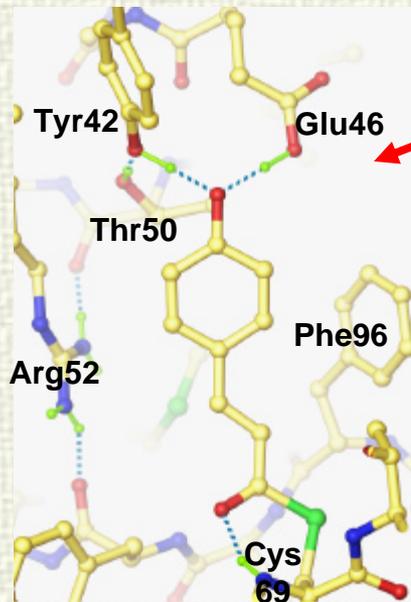
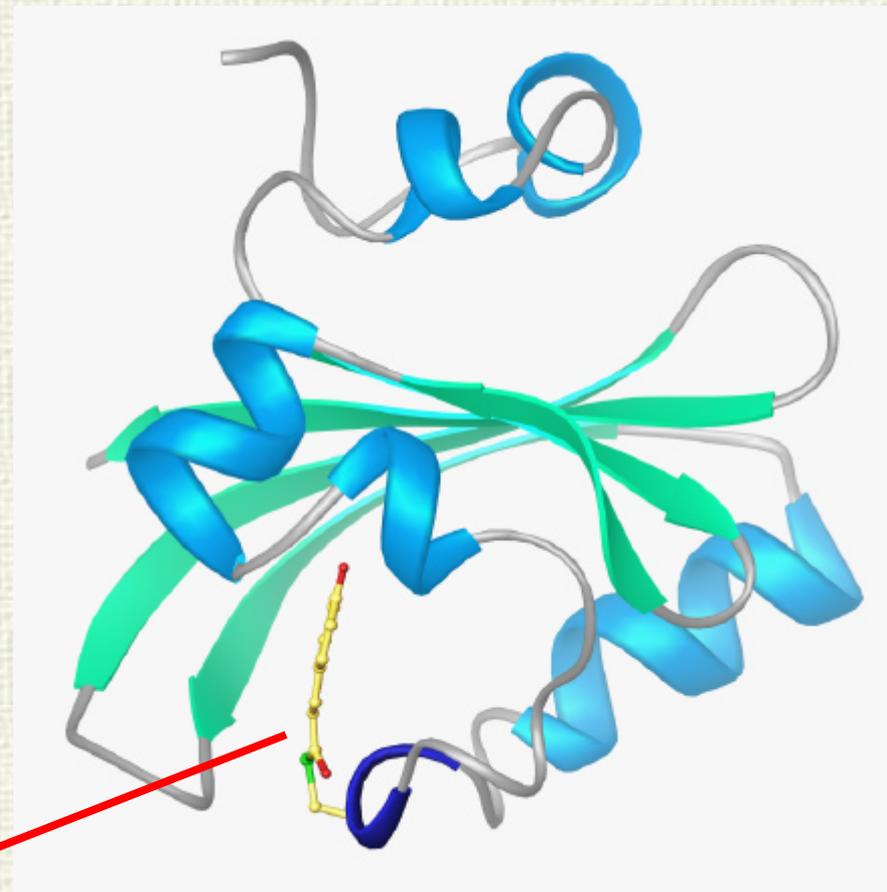
Heme region

Colors: **Hbl-CO heme**
deoxy Hbl heme
Phe 97
interface water molecules

Results of time-resolved experiments will be presented at the workshop.

Photoactive Yellow Protein

- Blue light photoreceptor from the purple eubacterium *Ectothiorhodospira halophila*
- Involved in negative phototactic response of *E. halophila* to blue light
- PYP exhibits a photocycle: several intermediates spanning time-scales from <ps to seconds



Coumaric Acid
Chromophore

BioCARS 14-ID, APS

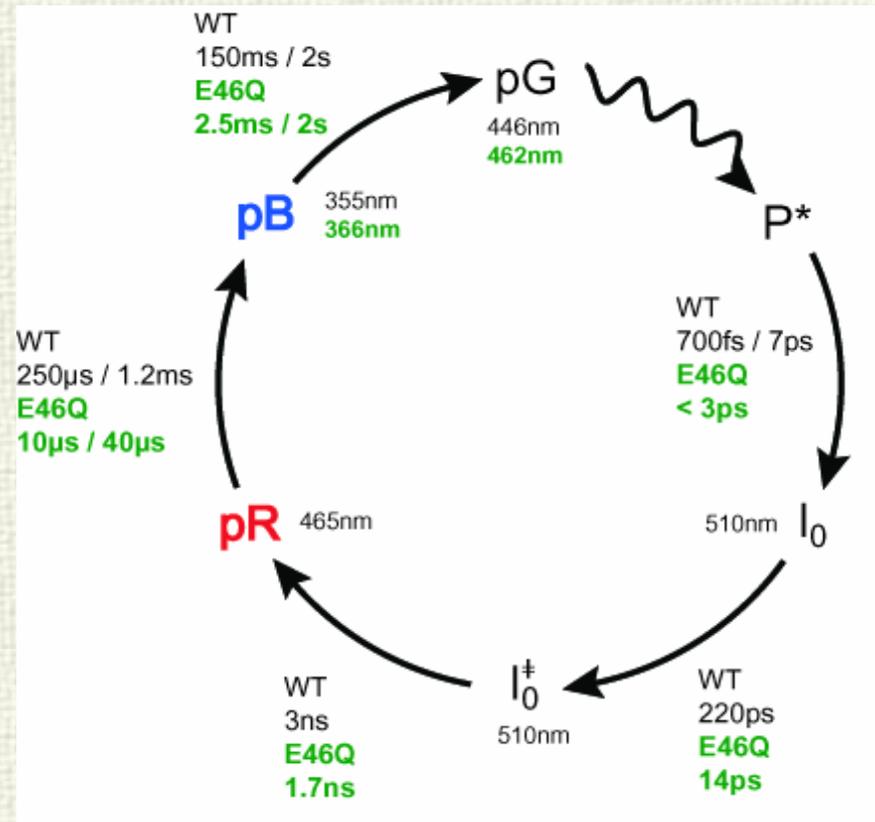
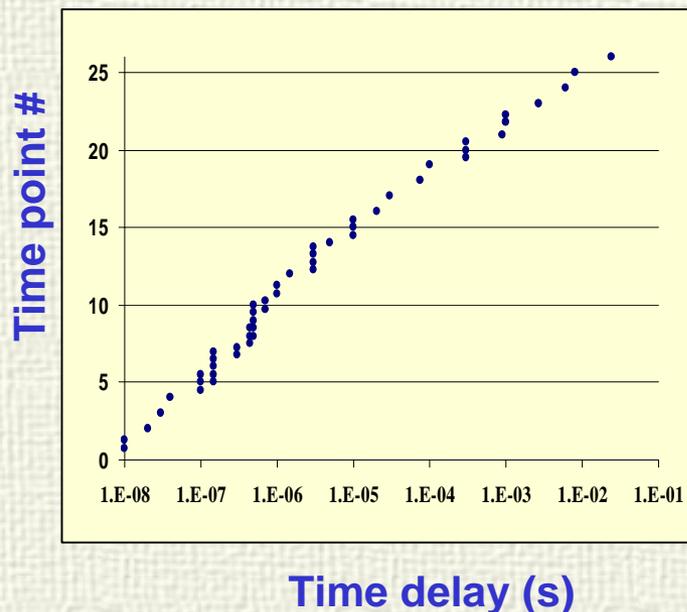
Spencer Anderson, Sudarshan Rajagopal
Keith Moffat
University of Chicago

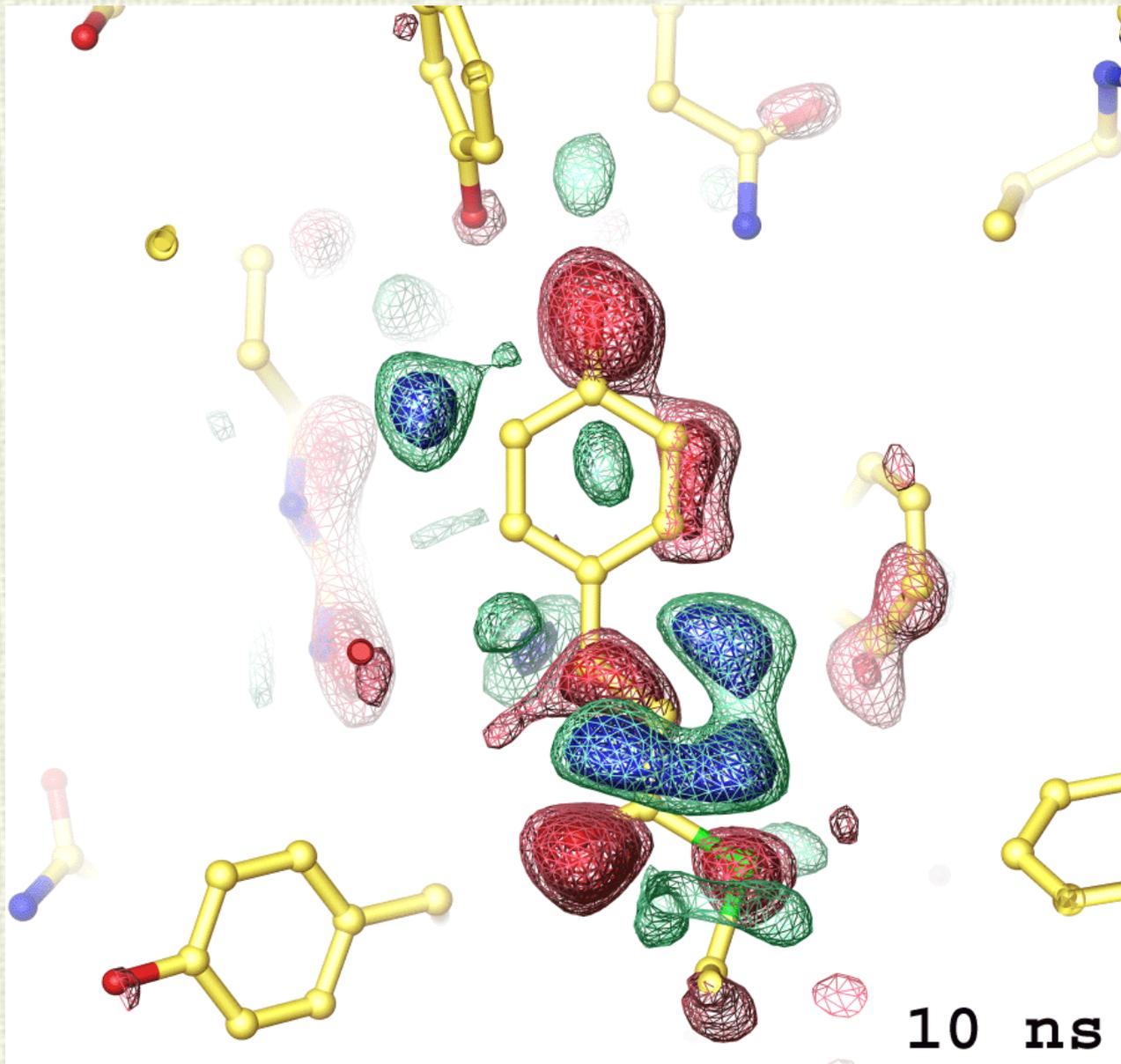
Vukica Srajer, Reinhard Pahl, BioCARS

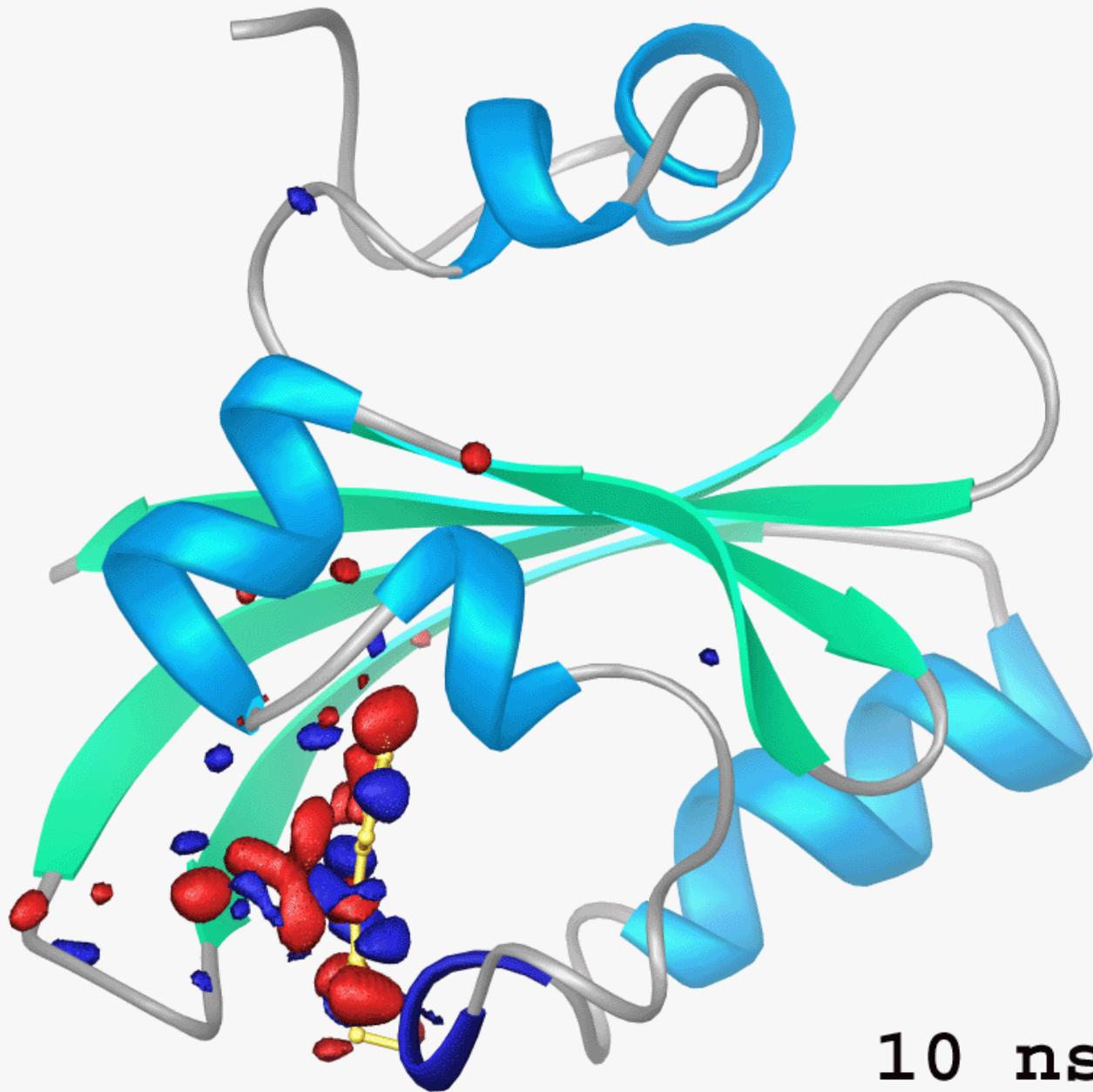
Anderson et al., Structure 12,1039 (2004)
Rajagopal et al., Acta Cryst. D60, 860 (2004)

Studies of intermediates of the PYP E46Q mutant

- Goal: observe structural changes throughout the E46Q photocycle
- 54 Laue data sets were collected using 25 crystals, at 30 different time delays, from 10ns to 100ms







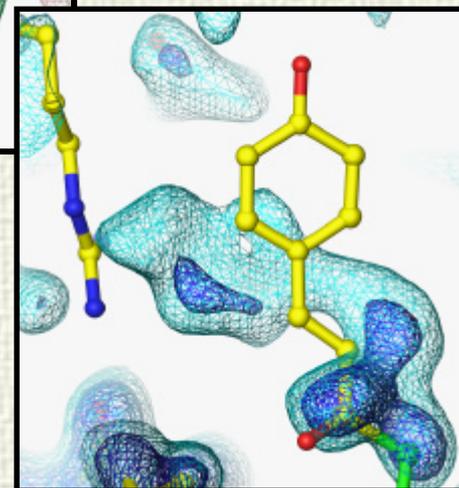
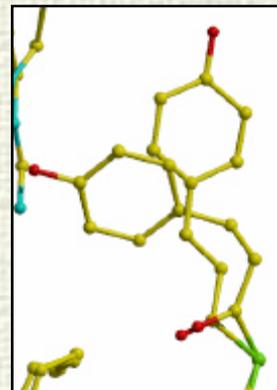
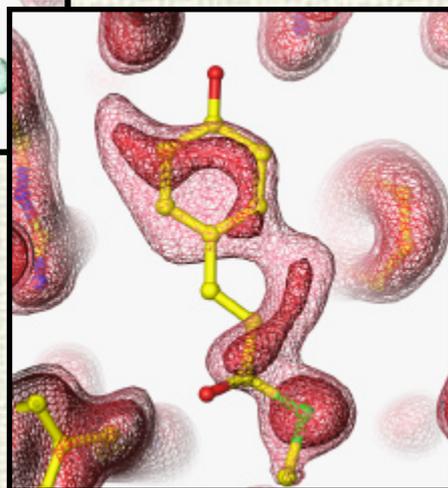
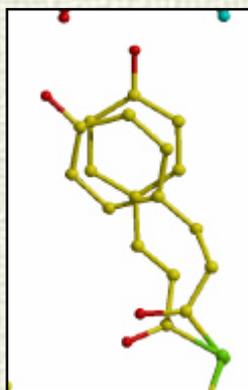
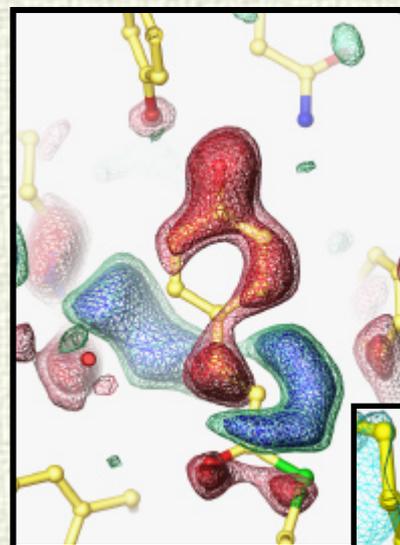
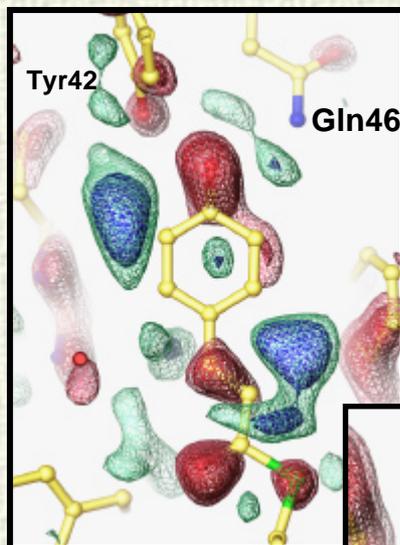
Two major intermediates identified: pR and pB

pR

10ns to 500ns

pB

10 μ s to 2.7ms



$F_{\text{light}} - F_{\text{dark}}$ difference electron density maps (contoured at ± 2.5 and 3.5σ)
(1/Photoactive Occupancy) $\times (F_{\text{LIGHT}}^{\circ} - F_{\text{DARK}}^{\circ}) + F_{\text{DARK}}^{\text{c}}$

Further results will be presented at the Workshop.

Time-resolved Crystallography: Conclusions and Future Outlook

- **Mature phase of the technique: demonstrated ability to detect small structural changes even at relatively low levels of reaction initiation (15-40%)**
- **Development of essential methods for global time-resolved data analysis, such as SVD, is well under way**
- **Challenges:**
 - ▶ **Application of the technique to other systems of biological interest, photosensitive and beyond**
 - ▶ **Reaction initiation: system-specific efforts to determine a suitable reaction initiation method**
 - ▶ **Irreversible processes and smaller crystal: need more intense X-ray sources and faster read-out detectors**
 - ▶ **Further improvements in time resolution: sub-100ps X-ray sources?**
 - ▶ **Combining experimental results from time-resolved crystallography with computational and theoretical approaches to describe reaction pathways completely, including the transition states.**



