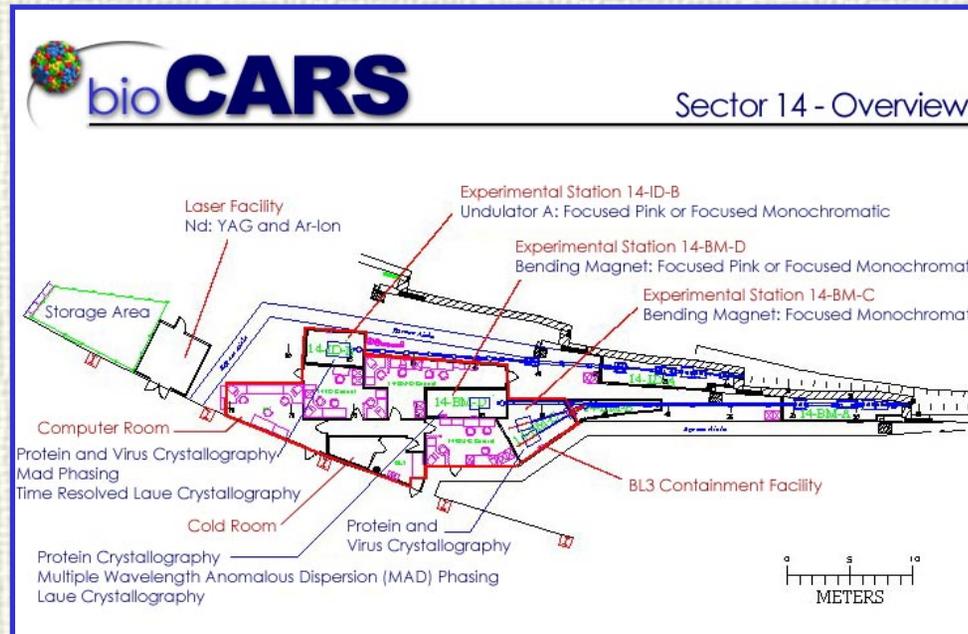


Time-resolved Macromolecular Crystallography

Vukica Srajer

The University of Chicago
BioCARS, Sector 14

Advanced Photon Source, Argonne National Laboratory



How to Capture Structural Intermediates?

Extend the lifetime of intermediates: physical or chemical trapping

- Low temperature
- Trigger-freeze – trap by freezing
- pH change or other solvent modification
- Chemical modification

Kinetic crystallography: Standard monochromatic oscillation technique

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-resolved crystallography: Laue diffraction
pink X-ray beam
stationary crystal

Time-resolved Experiments

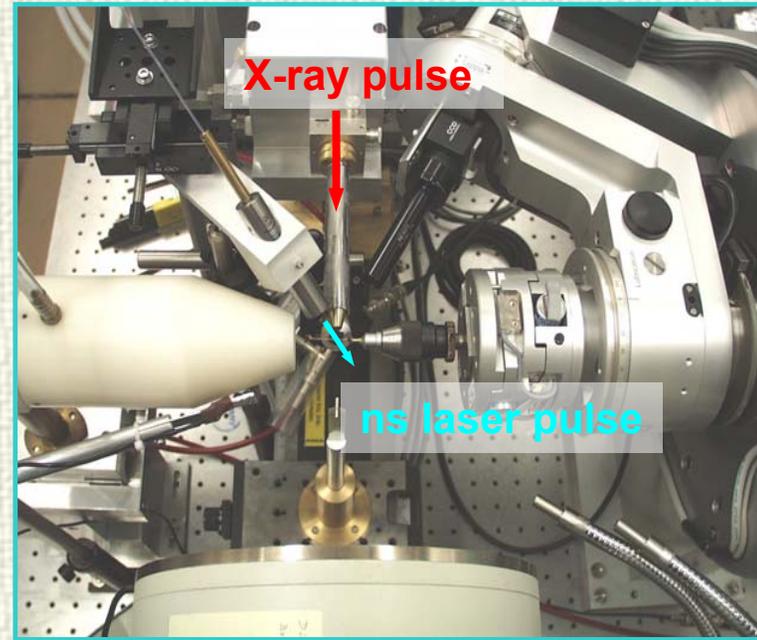
Pump-probe

Pump: laser pulses (100fs-10ns), μ s flash lamps

Probe: 100ps X-ray pulse or longer pulse trains

Data collection strategy

- **Slow variable:** crystal angular setting
- Fast variable:** pump-probe delay time, Δt
- ➔ For each crystal orientation collect:
no laser, Δt_1 , Δt_2 , Δt_3 ... Laue frames



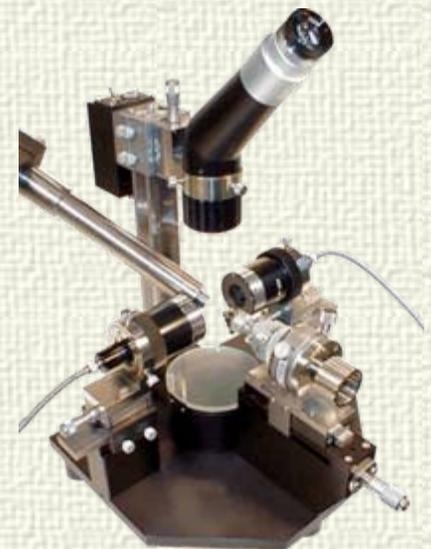
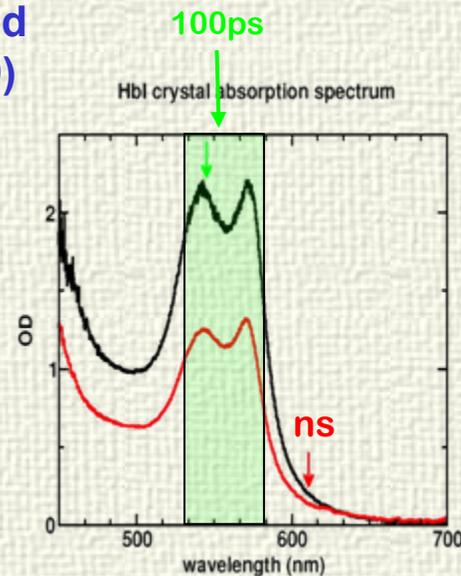
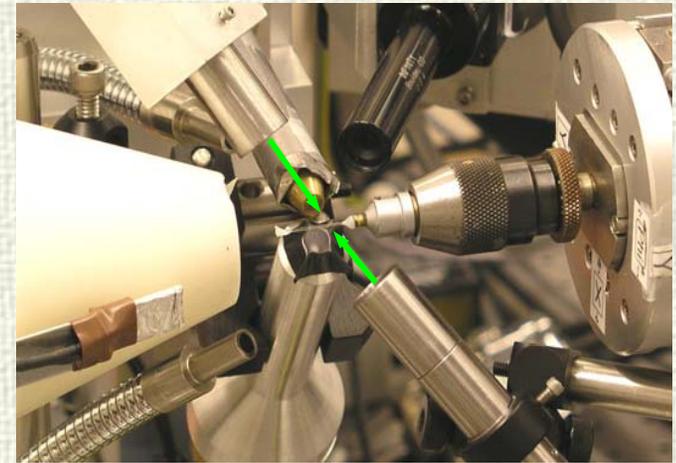
Typically 10-100 pump-probe cycles per image needed (for improved S/N)

➔ pump-probe cycles repeated prior to the detector readout

- **Repetition rate:** sample (lifetimes of intermediates)
heat dissipation (laser-induced heating)
1-3 Hz typical
- 40-60 images per data set
2-3° angular increment with undulator sources (few % bandwidth)

Reaction Photo-initiation in Crystals

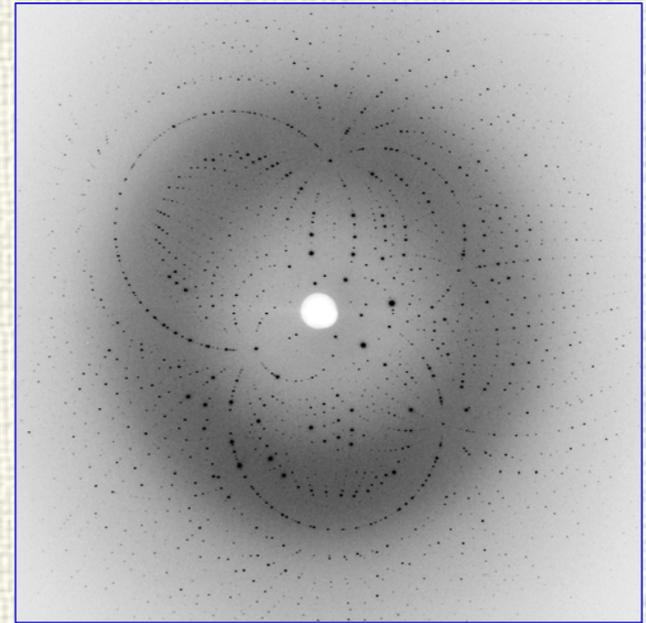
- Short laser pulses: 100fs-10ns duration
- Large number of molecules in crystals: $10^{13} - 10^{14}$
 - ▶ >10-100 $\mu\text{J}/\text{pulse}$
 - ▶ 100ps studies: 40 μJ / 100-200 μm beam diameter
 - ▶ ns studies: 1-2mJ / 600-800 μm beam diameter
- Uniform and efficient photo-initiation
 - ▶ tunable wavelength
 - ▶ double-sided illumination (BioCARS)
 - ▶ optically thin crystals (OD < 1) or
 - ▶ probing by X-rays only laser-illuminated surface layer of the crystal (ESRF ID09)
- Avoiding crystal damage
 - ▶ maximum laser pulse energies?
 - ▶ 100fs pulses typically stretched to 10-100ps to avoid sample damage
- Extent of photo-initiation?
 - ▶ monitoring absorption changes by a micro-spectrophotometer



Laue and Time-resolved Data Analysis

Laue data processing

- **Mature stage:**
 - problems of wavelength normalization, spatial and harmonic spot overlap resolved in mid 1990-ies
 - automation ongoing – minimal user input and intervention needed
 - ➔ online data processing
- **Very good software packages:**
 - **TReX** (Schotte & Anfinrud)
 - **Precognition** (Renz Research Inc)
 - **LaueView** (Ren and Moffat, J. Appl. Cryst. 28: 461,1995)
 - **PrOW** (Bourgeois, Acta Cryst. D55: 1733, 1999)
 - **Daresbury Laboratory Laue Software Suite**
(Campbell J.W., J. Appl. Cryst. 28:228, 1994; Arzt et al., J Appl. Cryst. 32: 554, 1999)
 - **Leap**
(Wakatsuki, S. Data Collection and Processing, Sawyer, N. W. Isaacs & S. Bailey, Eds., Daresbury Laboratory, Warrington, UK, p.71, 1993)



Quality of Laue data and electron density maps derived from Laue data are comparable to monochromatic data.

Difference electron density maps

Laue structure factor amplitudes: $h k l$ $|F(t)|$ $\sigma_{F(t)}$

→ time-dependent difference electron density maps $\Delta\rho(t)$: $|F(t)| - |F(I_o)|$, φ_{I_o}

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
- Maps for pure intermediates, $\Delta\rho(I_i)$ (time-independent)
- Structures of intermediates I_i
- Reaction mechanism

Most promising method: Singular Value Decomposition

Ihee et al., PNAS 102, 7145-7150 (2005)

Rajagopal et al., Structure 13, 55-63 (2005)

Rajagopal et al., Acta Cryst. D60, 860-871 (2004)

Schmidt et al., PNAS 101, 4799-4804 (2004)

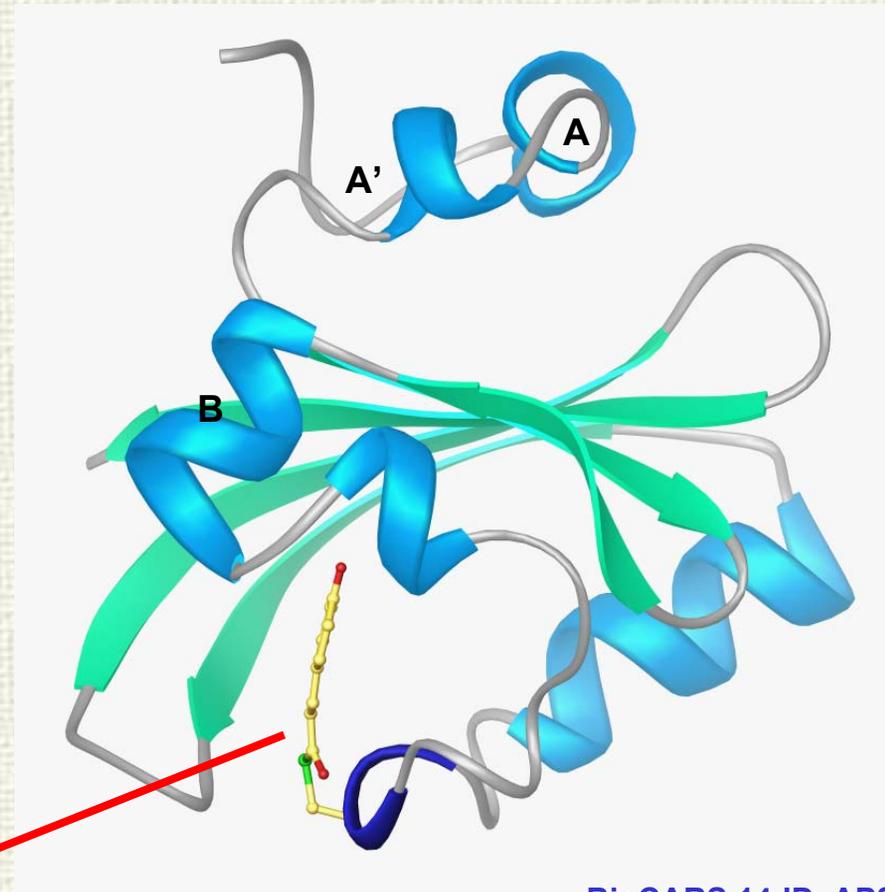
Schmidt et al., Biophys. J. 84, 2112-2129 (2003)

Cluster Analysis

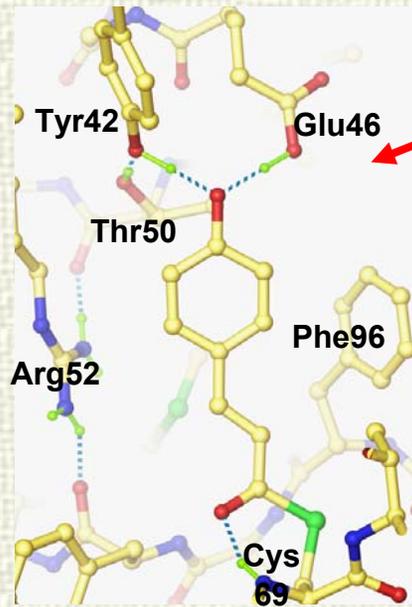
Rajagopal et al., J Struct Biol. 147, 211-22 (2004)

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



light-induced
Trans to *Cis*
isomerization

BioCARS 14-ID, APS

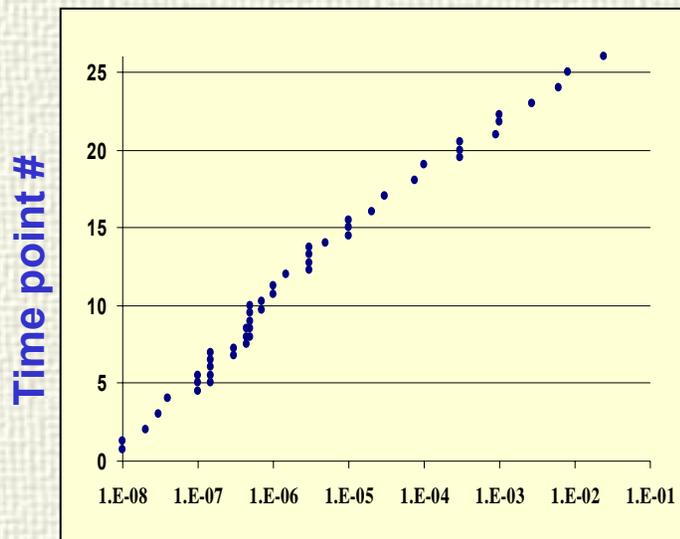
Spencer Anderson, Sudarshan Rajagopal,
Harry Ihee, Marius Schmidt, Keith Moffat
University of Chicago

Vukica Srajer, Reinhard Pahl, BioCARS

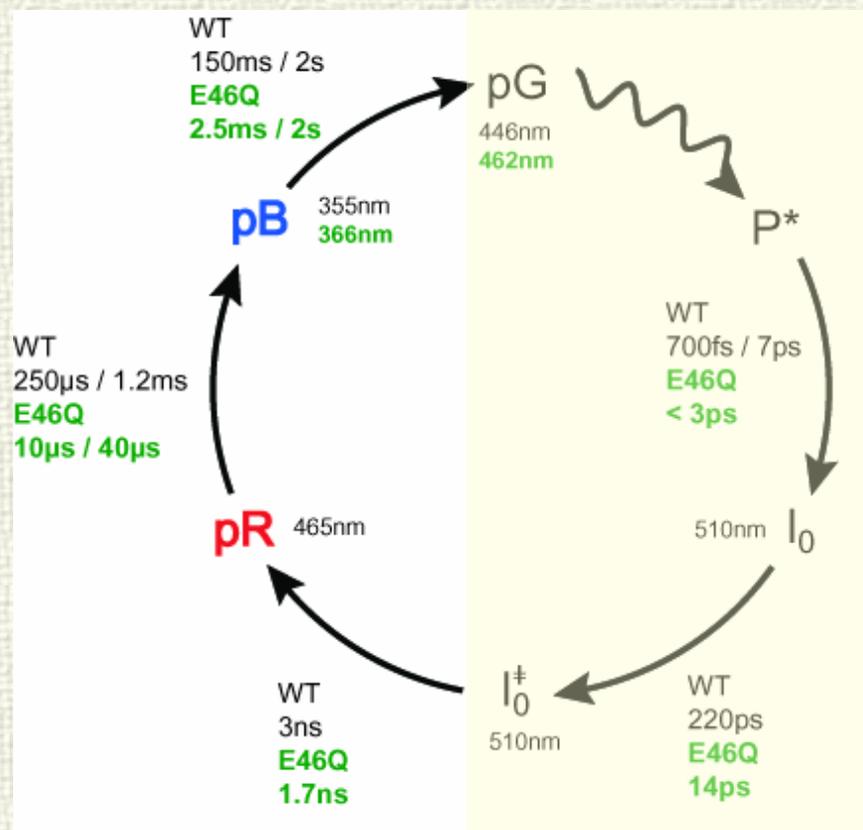
Ihee et al. PNAS 102, 7145 (2005)
Rajagopal et al., Structure 13, 55 (2005)
Anderson et al., Structure 12, 1039 (2004)
Rajagopal et al., Acta Cryst. D60, 860 (2004)

Studies of intermediates PYP E46Q mutant

- 54 Laue data sets collected using 25 crystals, at 30 time delays, from 10ns to 100ms

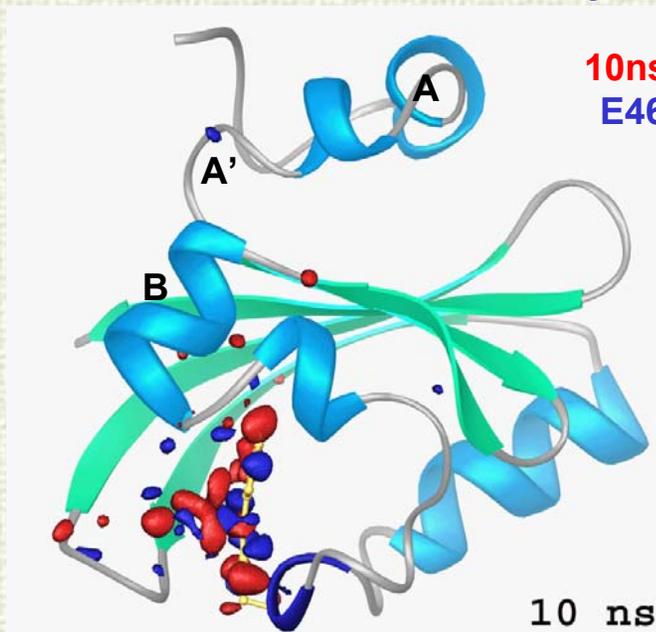
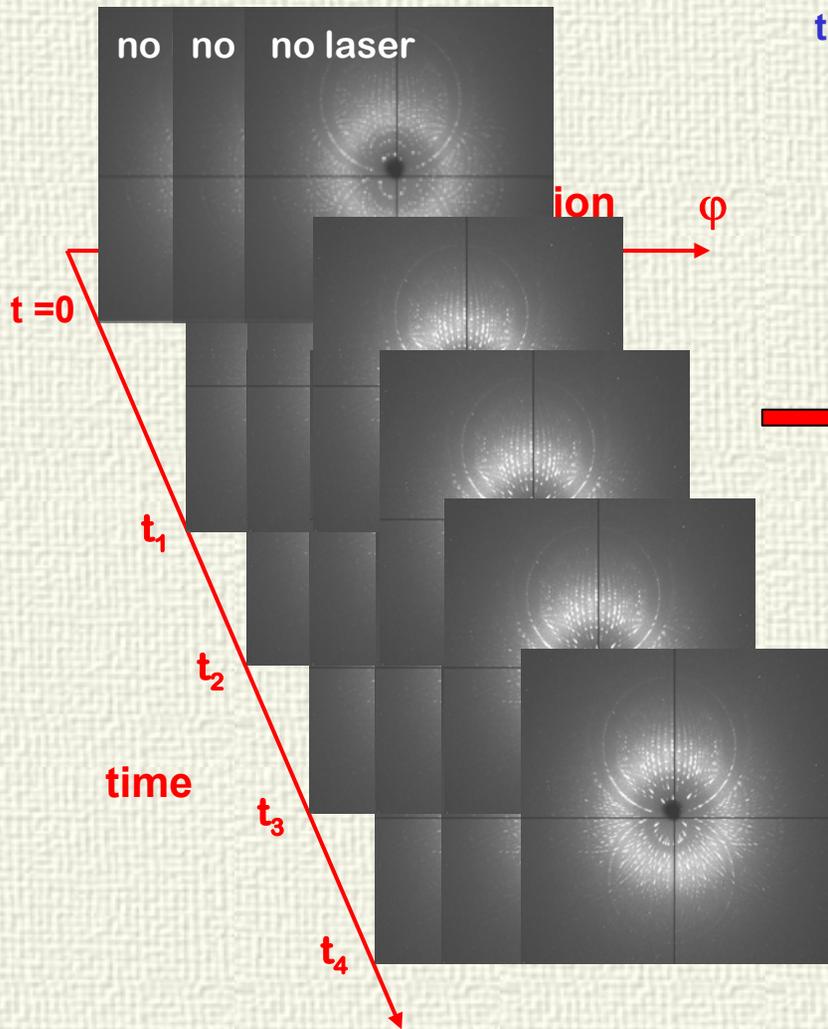


Time delay (s)



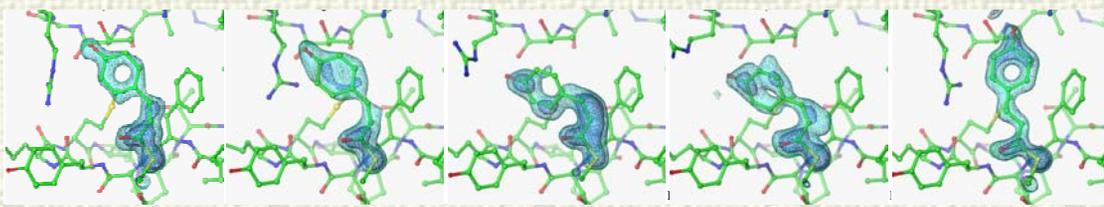
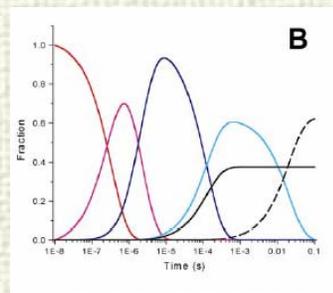
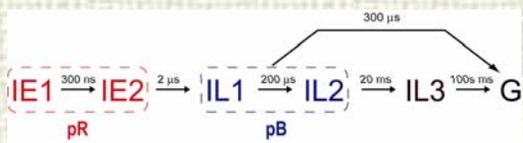
Rajagopal et al., Structure 13, 55 (2005)
Anderson et al., Structure 12,1039 (2004)

time-dependent difference electron density maps, $\Delta\rho(t)$



10ns-100ms
E46Q PYP

SVD/post-SVD analysis



300ns 2μs 200-300μs 20ms >100ms t

X-ray Source Requirements for Time-resolved Macromolecular Crystallography

- **Pulse duration: structural changes to be probed span sub-ps to sec and min**
 - 100ps available presently at synchrotron sources
 - longer pulse trains quite suitable for slow reactions
 - sub-100ps desirable to probe very fast structural changes:
 - short-lived intermediates
 - fast protein relaxation
 - rapid ligand migration
- **Caution: same as in ps time-resolved spectroscopy, effects of laser-induced heating need to be taken into account**

Example: it takes 1-2ps for vibrationally excited heme in myoglobin to cool, based on ps TR Resonance Raman studies
Kitagawa et al., Biopolymers 76, 207, 2002

- **X-ray energy: few % bandwidth at 12-15keV**
 - softer X-rays increase radiation damage
 - harder X-rays diffract less strongly and are detected less efficiently
 - undulators better sources than wigglers:
 - high peak intensity
 - low polychromatic background
 - reduced spatial and harmonic overlap
 - data processing software can handle wavelength normalization in the presence of sharp spectral features
(Srajer et al., J. Sync. Rad 7: 236, 2000)
 - better data as judged by R_{merge} , completeness, map quality
(Bourgeois et al., Acta Cryst. D 56: 973, 2000)

- **X-ray flux: $> 10^{10}$ photons/pulse needed for single pulse image acquisition**
 - at present, at TR PX beamlines (APS 14-ID, ESRF ID09, PF-AR NW14)
>10-100 single X-ray pulses needed
 - the aim of the 14-ID upgrade: single pulse acquisition in hybrid and 24-bunch mode (dual, in-line undulators; KB mirror pair – focused beam size 80 (h) X 50 (v) μm^2)
 - single-pulse acquisition will allow study of fast, irreversible processes
 - higher pump laser pulse energies can be used (crystal motion not a problem)

■ X-ray focal spot:

- in principle should be able to match the sample size
- investigating small crystals requires small beam but at full flux (short exposures)
- small vertical beam size needed for isolating single X-ray pulses by a chopper
- low energy fs-ps laser pulses require illumination of smaller crystal volume for efficient photo-initiation – need a small probe X-ray beam

< 50-100 μm focal spot

■ Storage ring mode and beamtime availability:

- need to isolate a single X-ray pulse in pink mode:
 - current BioCARS fast chopper can isolate single pulse only in the APS hybrid mode
 - upgraded chopper will be isolating single pulses in the 24 bunch mode
- technically challenging experiments – unlike standard PX data collection require significant beamtime ➔ standard rather than special operating mode

Upgraded BioCARS fast chopper

Geometry

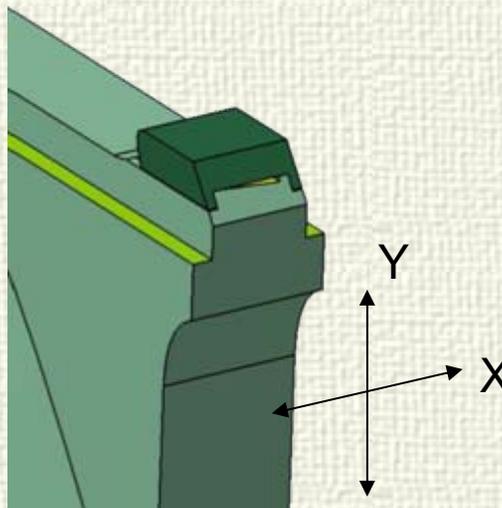
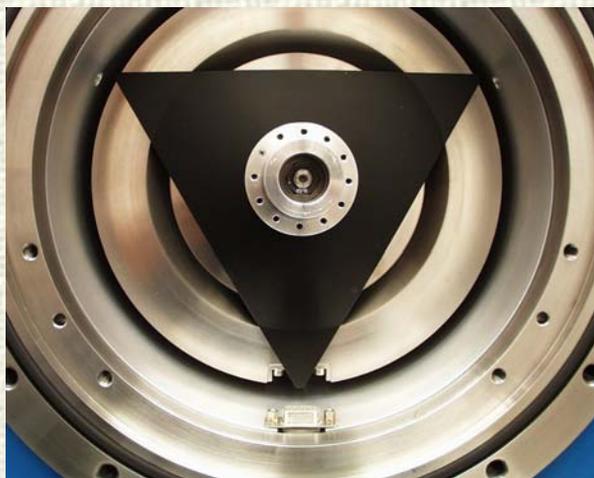
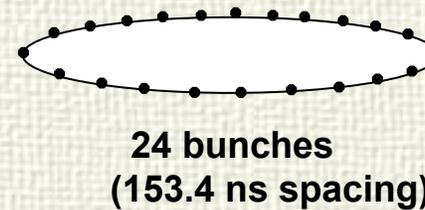
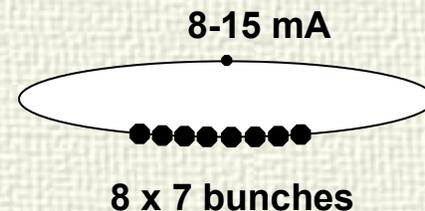
TIMETAL (Ti6Al4V)
triangle, 166 mm side
multiple aperture size
vacuum ($< 10^{-3}$ Torr)

Rep. Rate

60,320 rpm (994.7 Hz)
T = 1.005 ms

Pulse Width

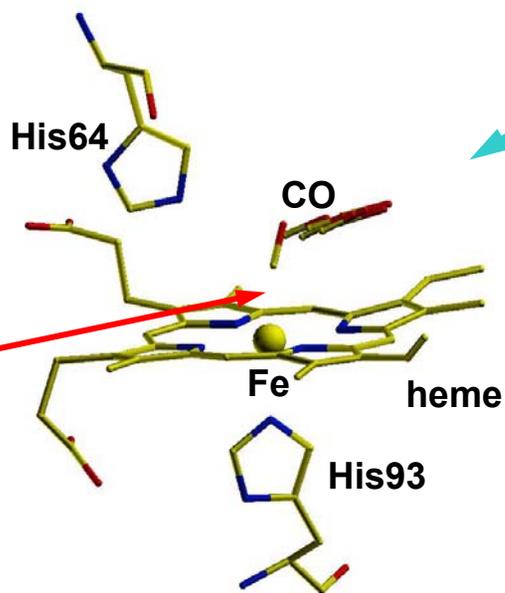
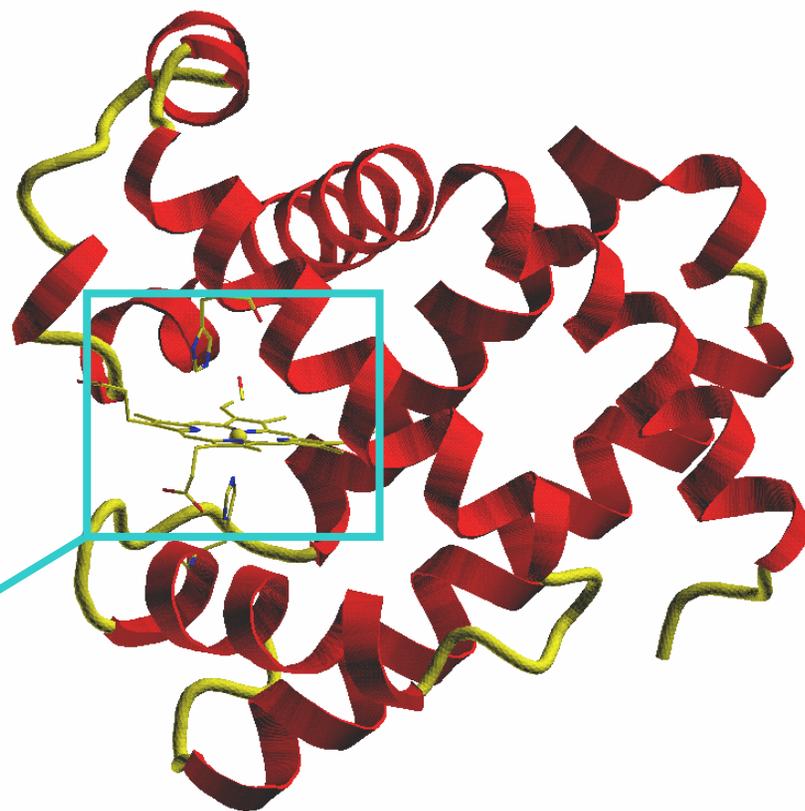
$t_{\text{open}} = 190 - 420$ ns, $0.46 - 11.5$ μs , $> \text{ms}$



P. Anfinrud
F. Schotte
R. Pahl
B. Lindenau

Myoglobin

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



laser pulse
breaks Fe-CO
bond

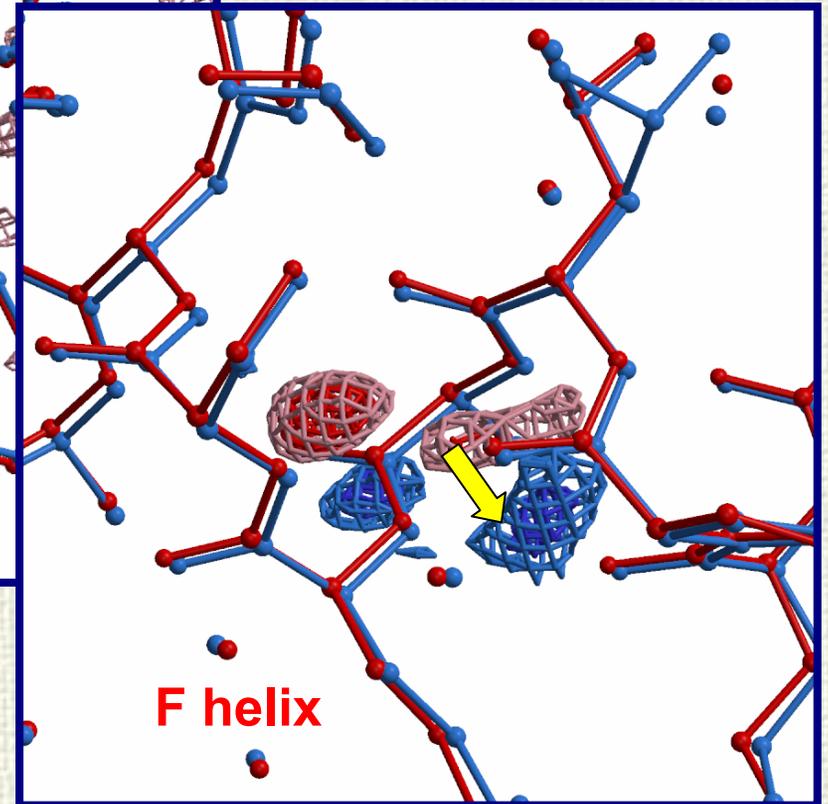
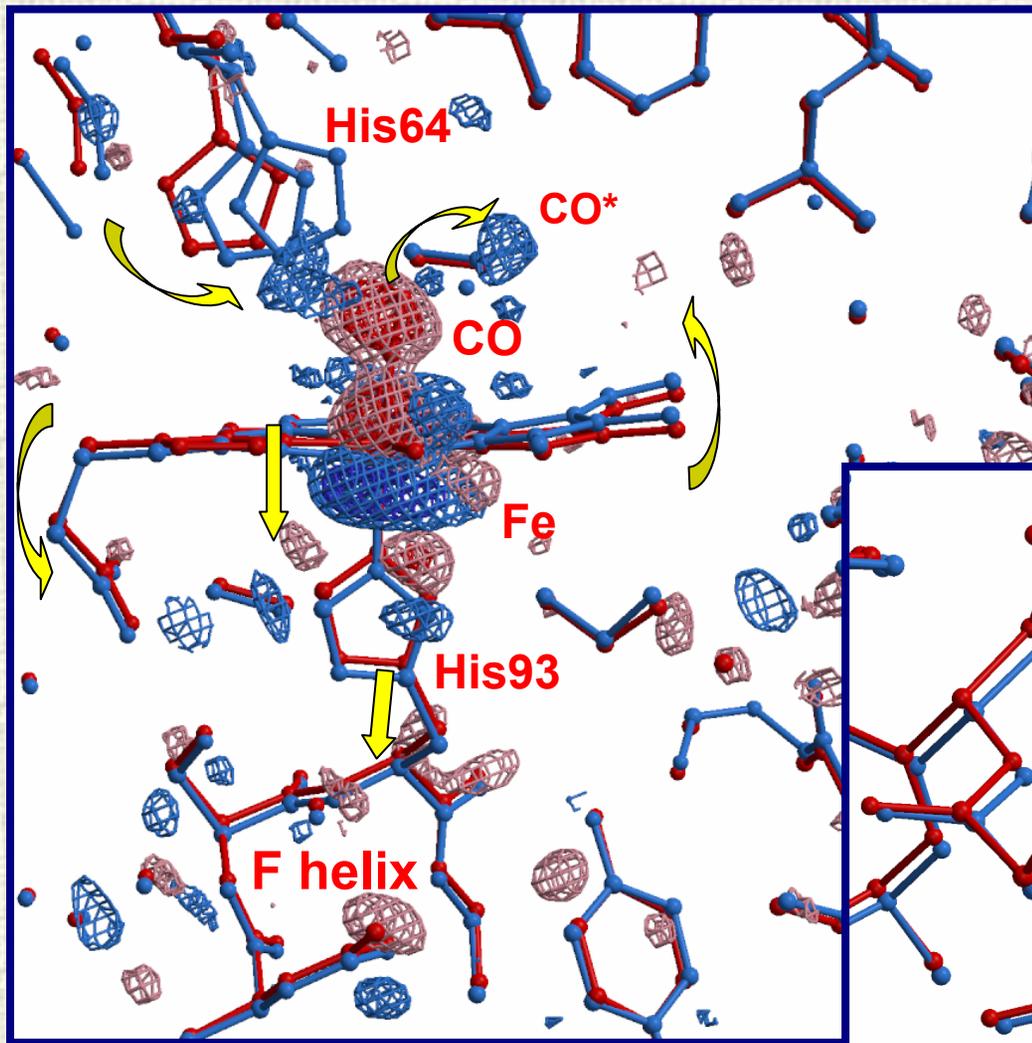
ID09, ESRF

Srajer / Moffat, University of Chicago
Michael Wulff, ESRF
Shin-ichi Adachi, PF-AR/KEK

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

Changes at 1ns: red → blue

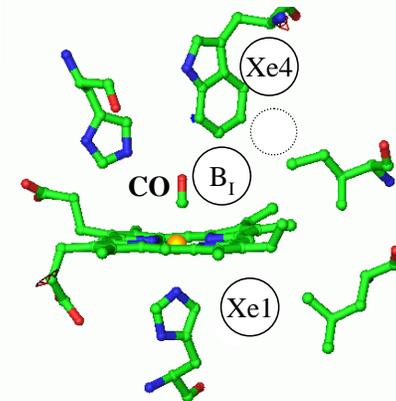
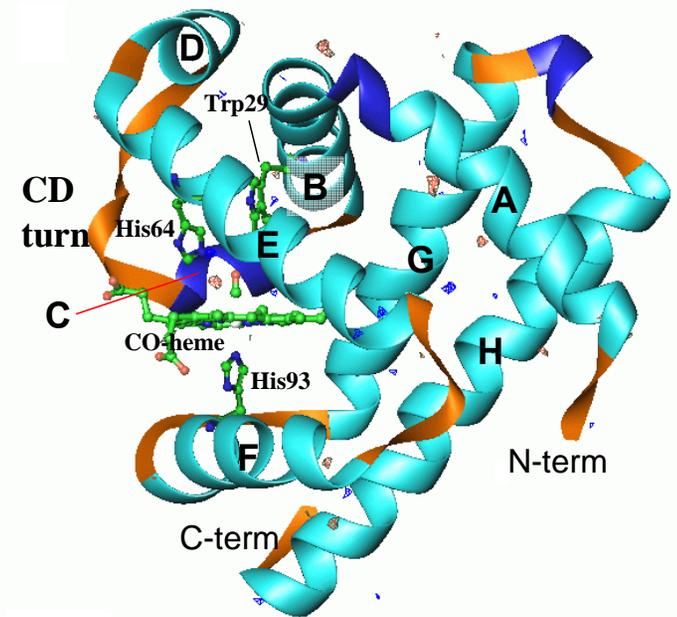
- Fe-CO bond breaks
- Fe moves out of the heme plane
- Heme buckles
- CO* at site B (~2.5Å away)
- His64 swings in
- Heme rotates
- F-helix moves ! Fast global changes.



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

Ns Protein Relaxation Study: L29W Myoglobin Mutant

- Leu 29 → Trp 29:
ligand rebinding 200 times slower than in wt
- examine protein relaxation:
can we follow it to the full extent
before the CO ligand rebinds?



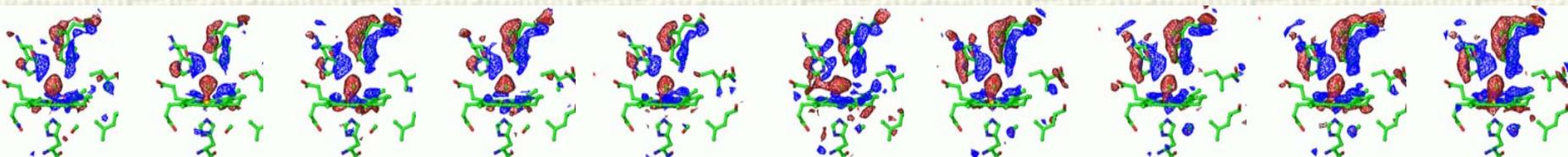
BioCARS, 14-ID, APS
Marius Schmidt, Technische Universität München
Vukica Srajer, Reinhard Pahl, BioCARS

Schmidt *et al*, PNAS 102, 11704 (2005)

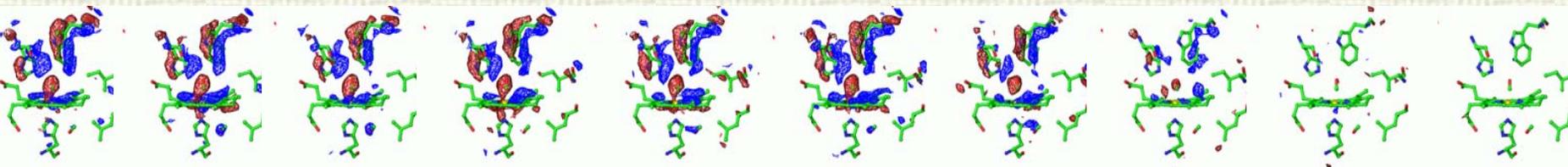
L29W difference electron density movie, $\Delta\rho(t)$: 20 pump-probe time delays

← 100ps X-ray pulse → ← 500ns →

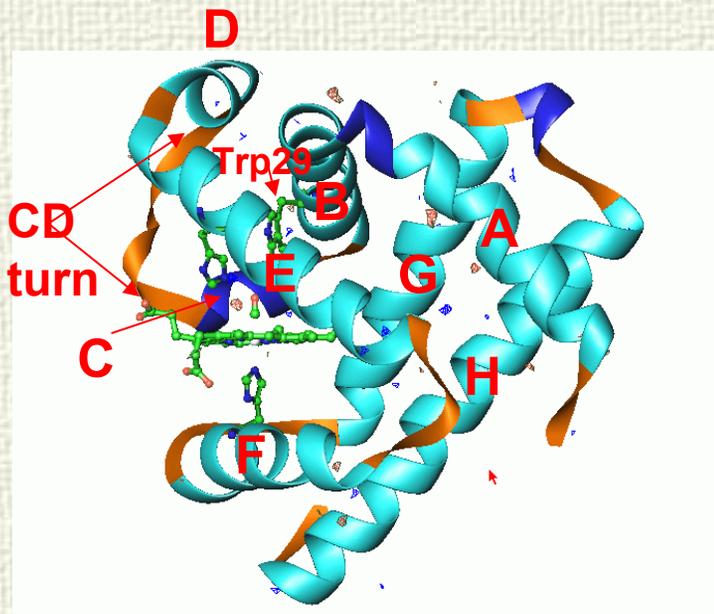
1 ns 4 ns 10 ns 35 ns 100 ns 290 ns 800 ns 2 μ s 5 μ s 10 μ s



24 μ s 50 μ s 110 μ s 250 μ s 650 μ s 1.3 ms 6 ms 35 ms 210 ms 4 s



← 6 μ s →

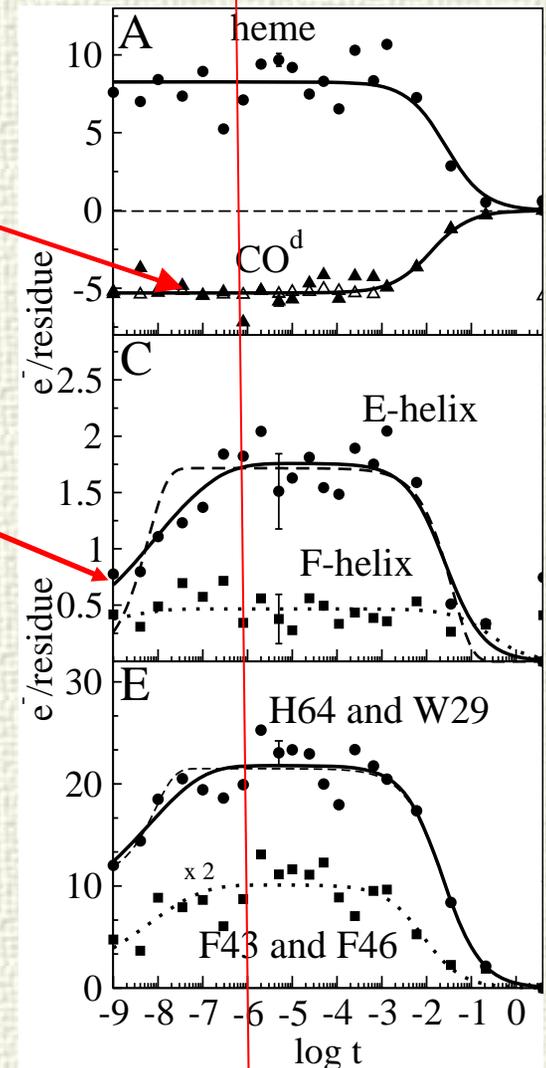


CO rebinding

partial E-helix relaxation by 1ns

Protein relaxation

- plot integrated $|\Delta\rho(t)|$: includes + and - signal
- heme relaxation: $<1\text{ns}$
- prominent relaxation for distal helices E and B
- relaxation of helices starts early
 - ➔ sub-ns data needed
- stretched exponential
- relaxation complete within $1\mu\text{s}$

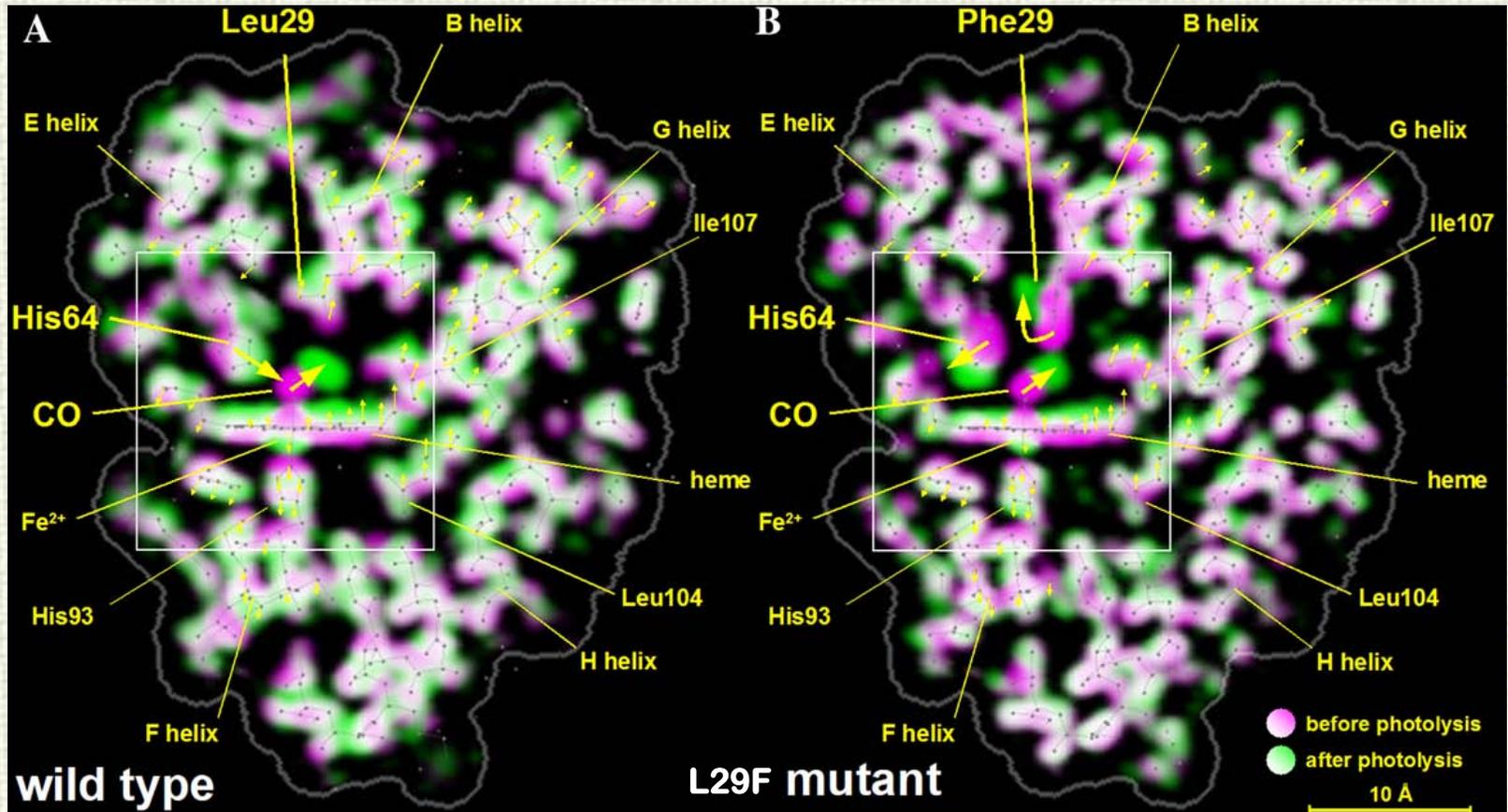


$1\mu\text{s}$

Sub-ns data: 100ps Mb studies

significant global protein relaxation by 100ps

➔ sub-100ps data needed

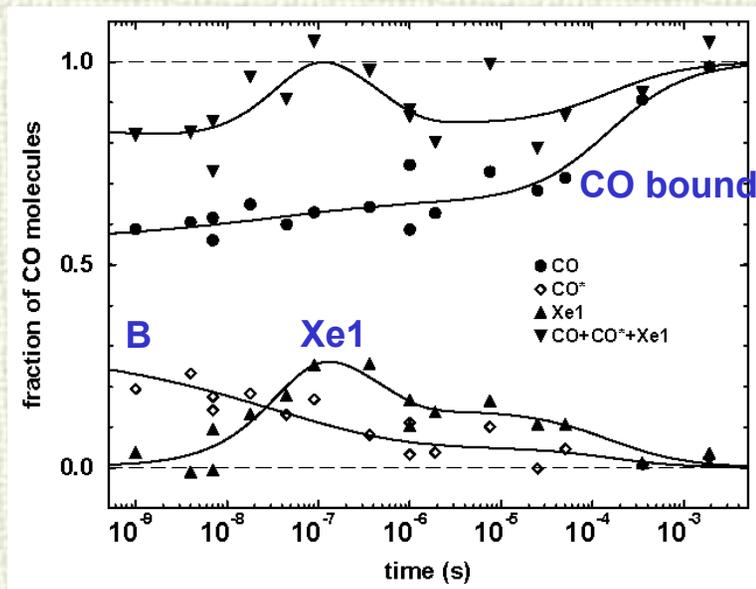
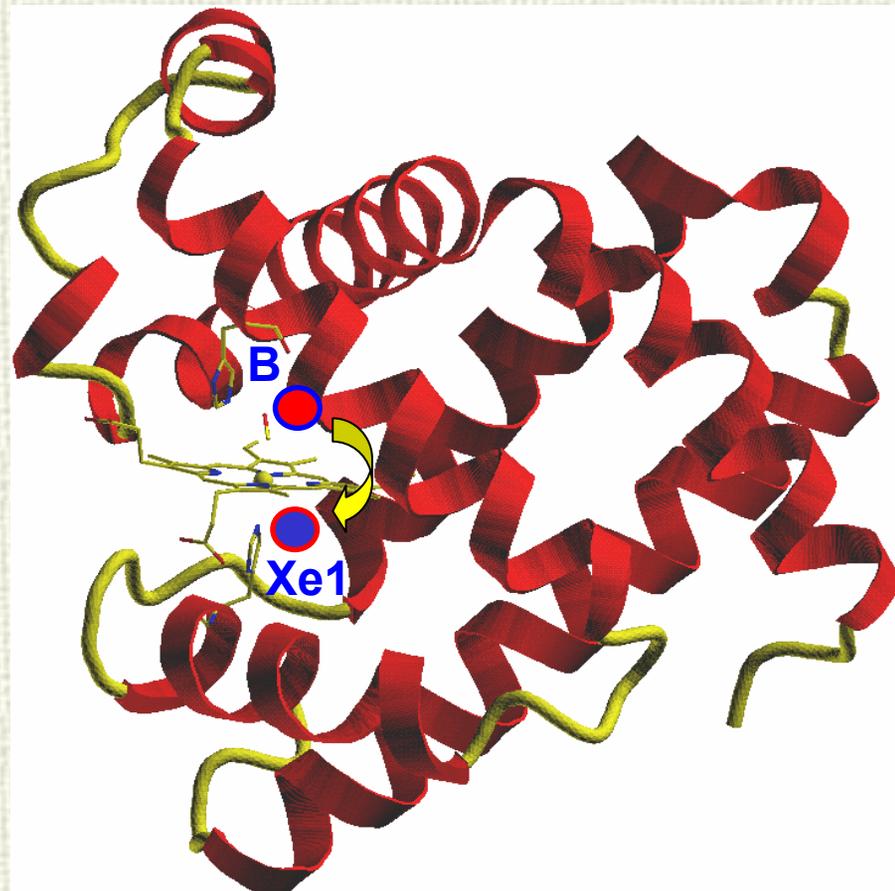


Schotte et al, Science 300, 2003

Schotte et al., JSB 147, 2004

Ligand Migration Pathways

- Photo-dissociated CO in wt Mb: two docking sites
- B: max populated at 1ns
depopulates in ~70ns
- Xe1: populates in ~30ns
depopulates in ~10 μ s



- CO docking sites with < 20% occupancy are detectable

Srajer *et al.*, *Biochemistry* 40, 13802 (2001)

All ns and 100ps Mb studies:

L29F Phe29; $\Delta t_{\min/\max}$ 100ps/ 3 μ s
(Schotte et al., JSB 147, 2004)

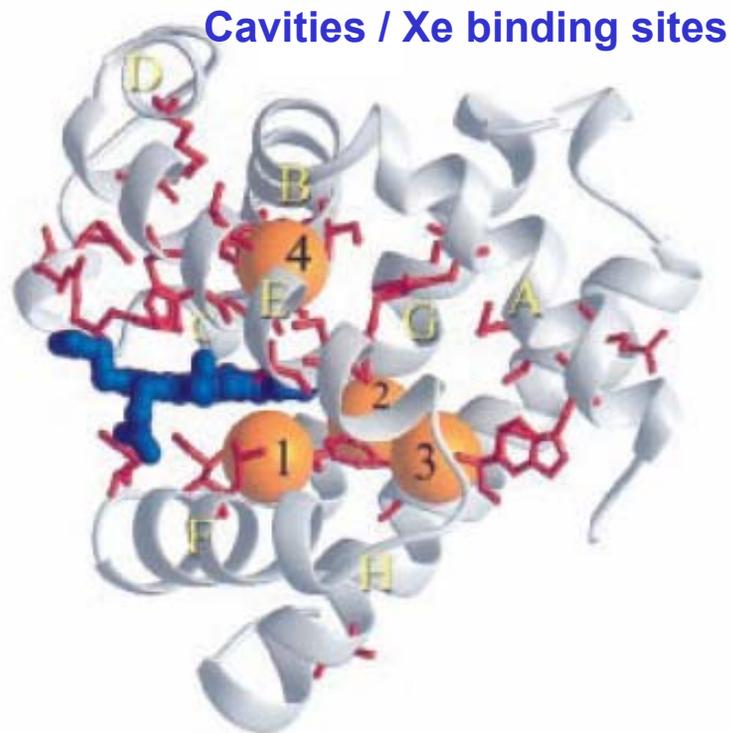
- B: 100ps
- Xe4: <1ns
- Xe1: 30ns / > 3 μ s
- Xe2: ~30ns (?)

WT Leu 29; $\Delta t_{\min/\max}$ 1ns/ 2ms
(Srajer et al, Biochem 40, 2001)

- B: 1 ns / 7 ns
- Xe1: 30 ns / 10 μ s
- Xe4: -
- Xe2: -

L29W Trp29; $\Delta t_{\min/\max}$ 1ns/ 200ms
(Schmidt et al, PNAS 102, 2005)

- Xe1: 500 ns / ~1-2 ms
- B: -
- Xe2: -
- Xe4: -



YQR-Mb Tyr 29; $\Delta t_{\min/\max}$ 100ps/ 3ms
(Bourgeois et al., PNAS 103, 2006)

- Xe4: 100ps / 30ns
- Xe1: 30 ns / > 3 μ s
- B: -
- Xe2: -

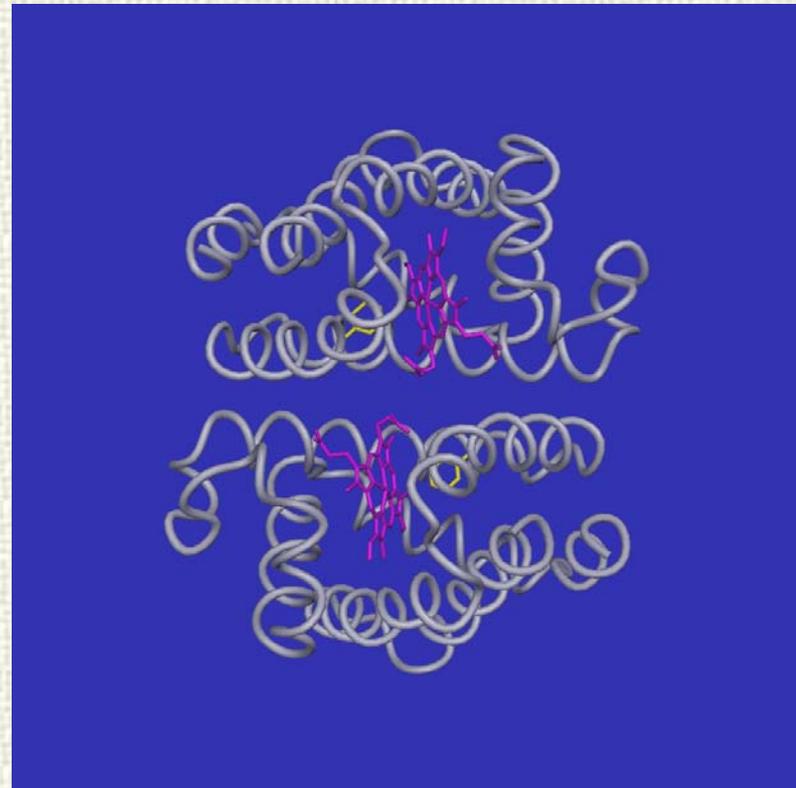
**Fast ligand migration to
remote Xe1 site (8Å)!**

Dimeric Hemoglobin Hbl

(from clam *Scapharca Inaequalis*)

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

Knapp et al., *PNAS* 103, 7649 (2006)

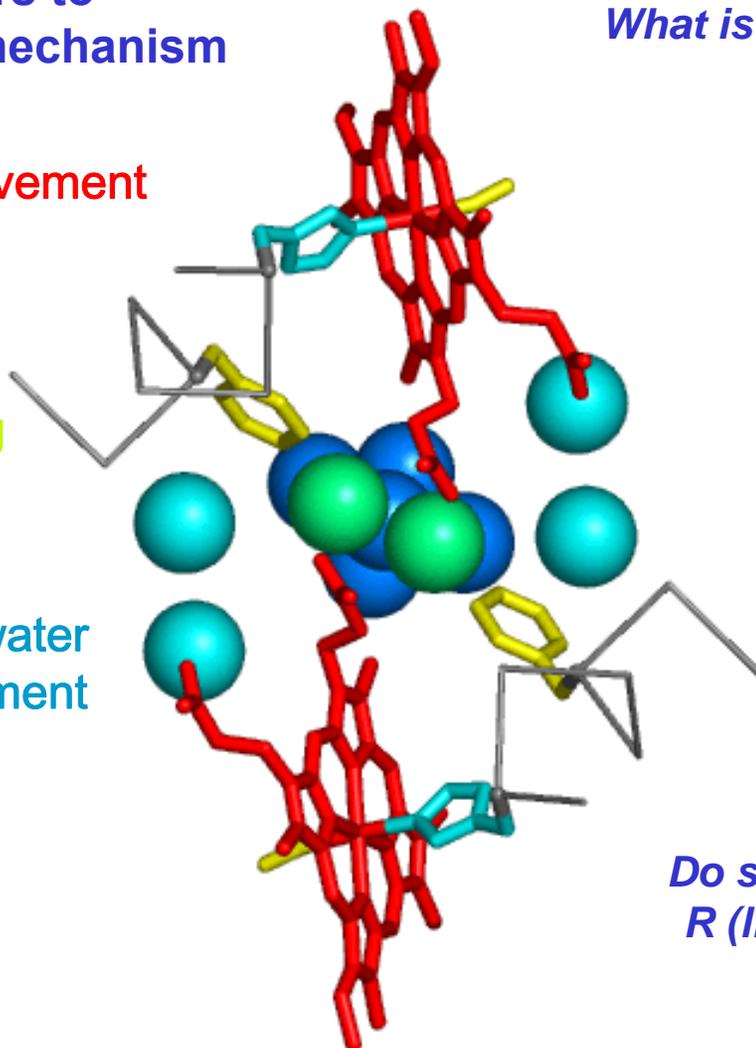
Key contributors to cooperativity mechanism

What is the cascade of structural events?

Heme movement

F4 Phe flipping

Interface water rearrangement



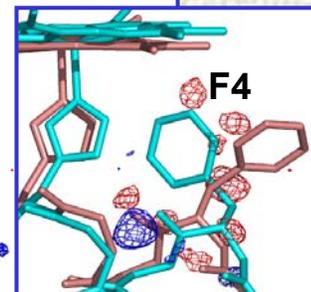
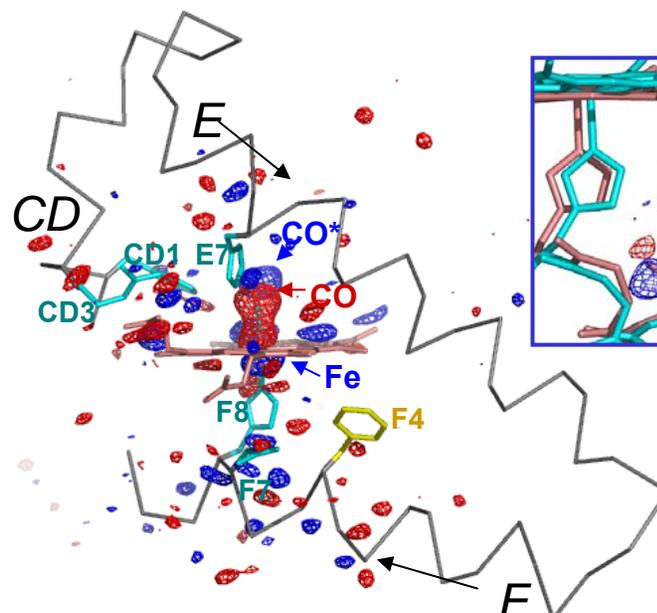
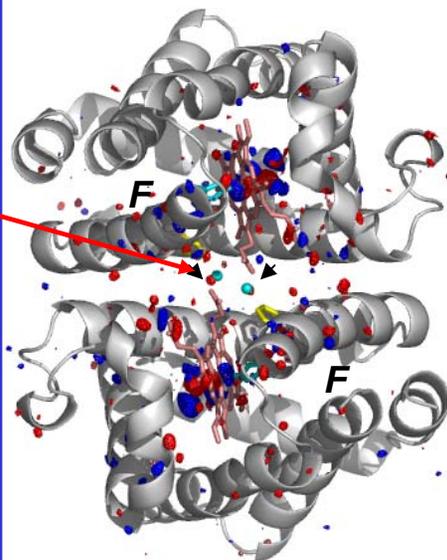
Are these transitions concerted or sequential?

What is the triggering event?

Do structural intermediates facilitate R (ligands bound) to T (no ligands) transition?

Two water molecules displaced!

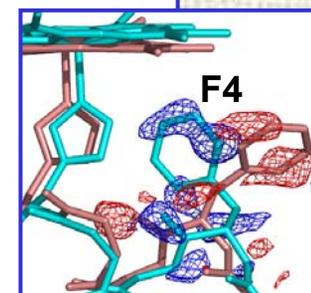
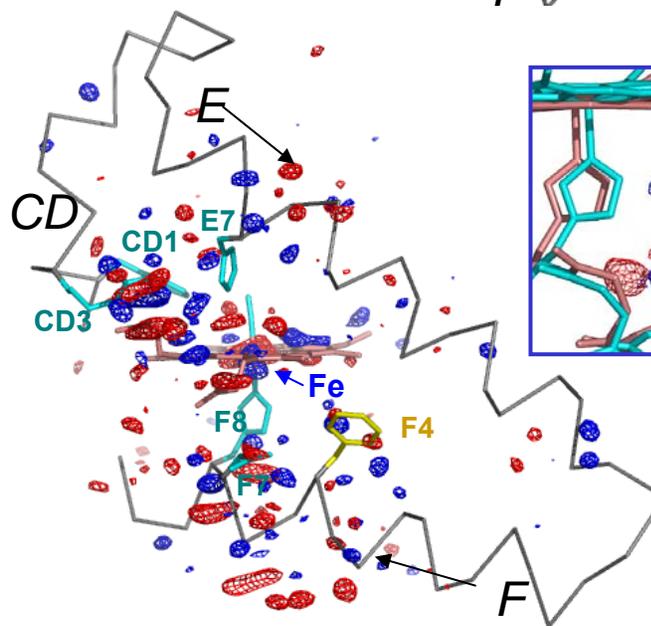
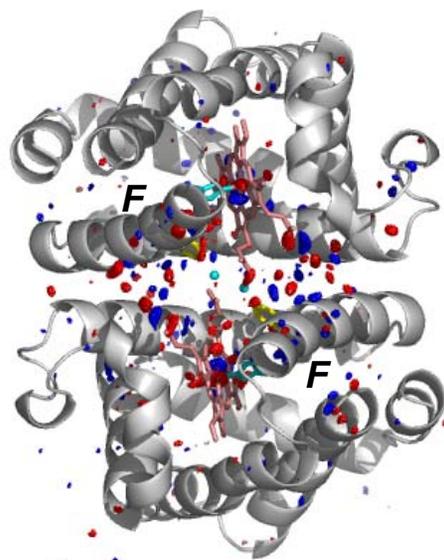
5ns



red: -2.5σ
blue: 2.5σ

F_o(light)-F_o(dark)
red: -3σ
blue: 3σ

60 μ s

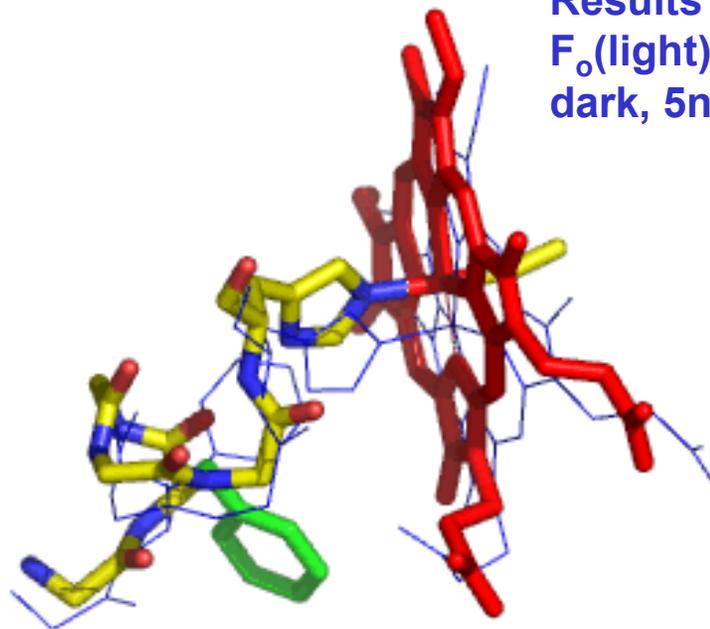


red: -2.5σ
blue: 2.5σ

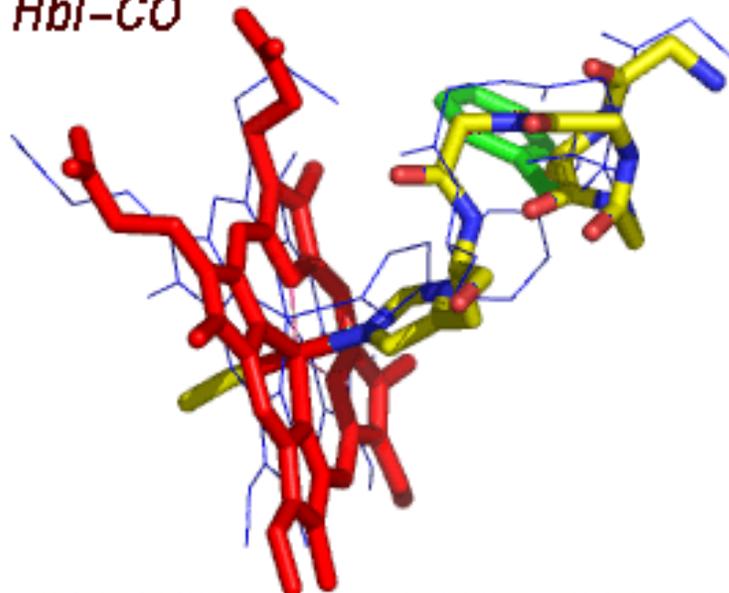
Subunit A

Results of difference Fourier refinement
 $F_o(\text{light}) - F_o(\text{dark})$ coefficients:
dark, 5ns, 200ns, 700ns, 2 μ s, 9 μ s, 80 μ s

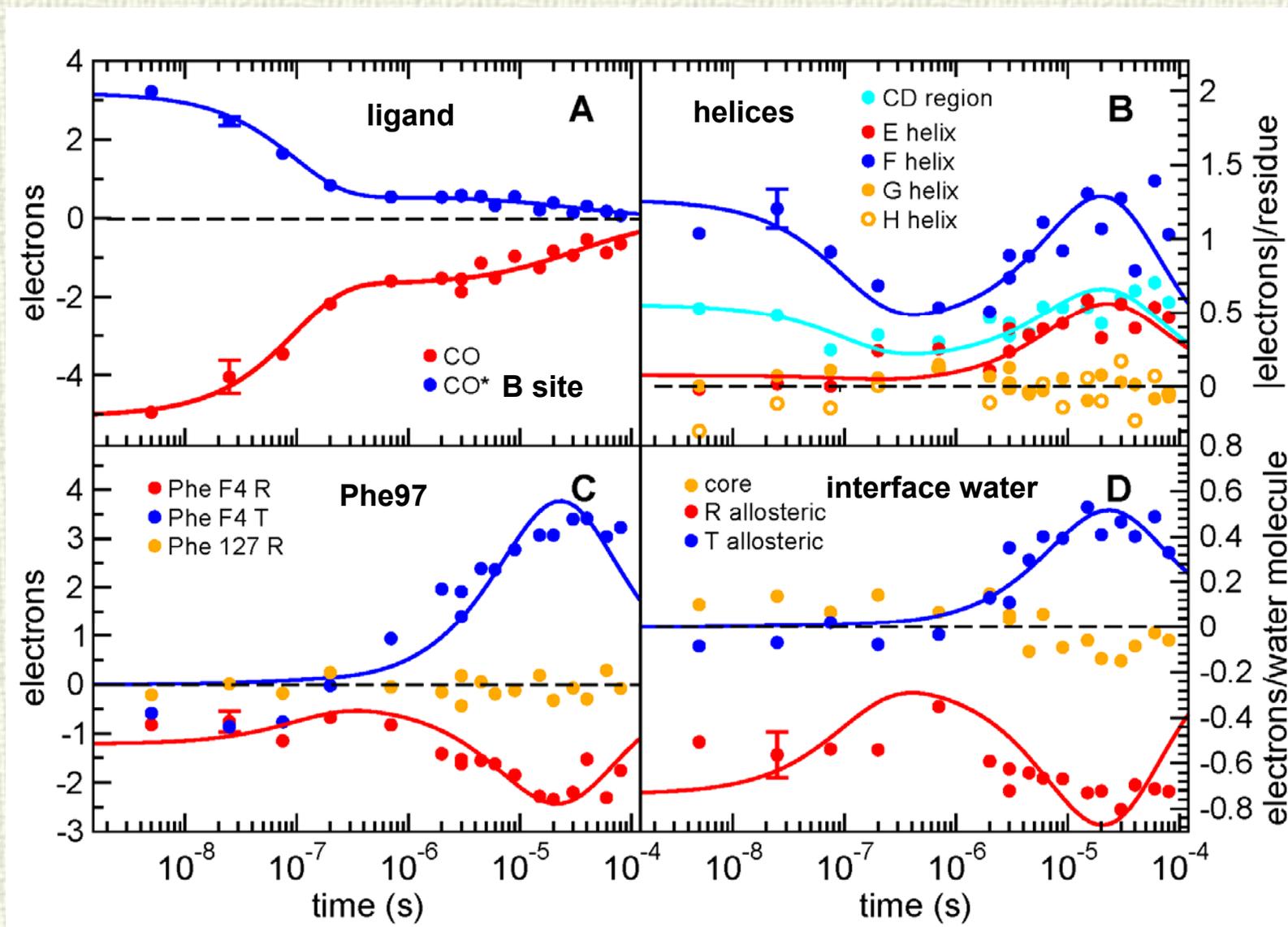
heme transition



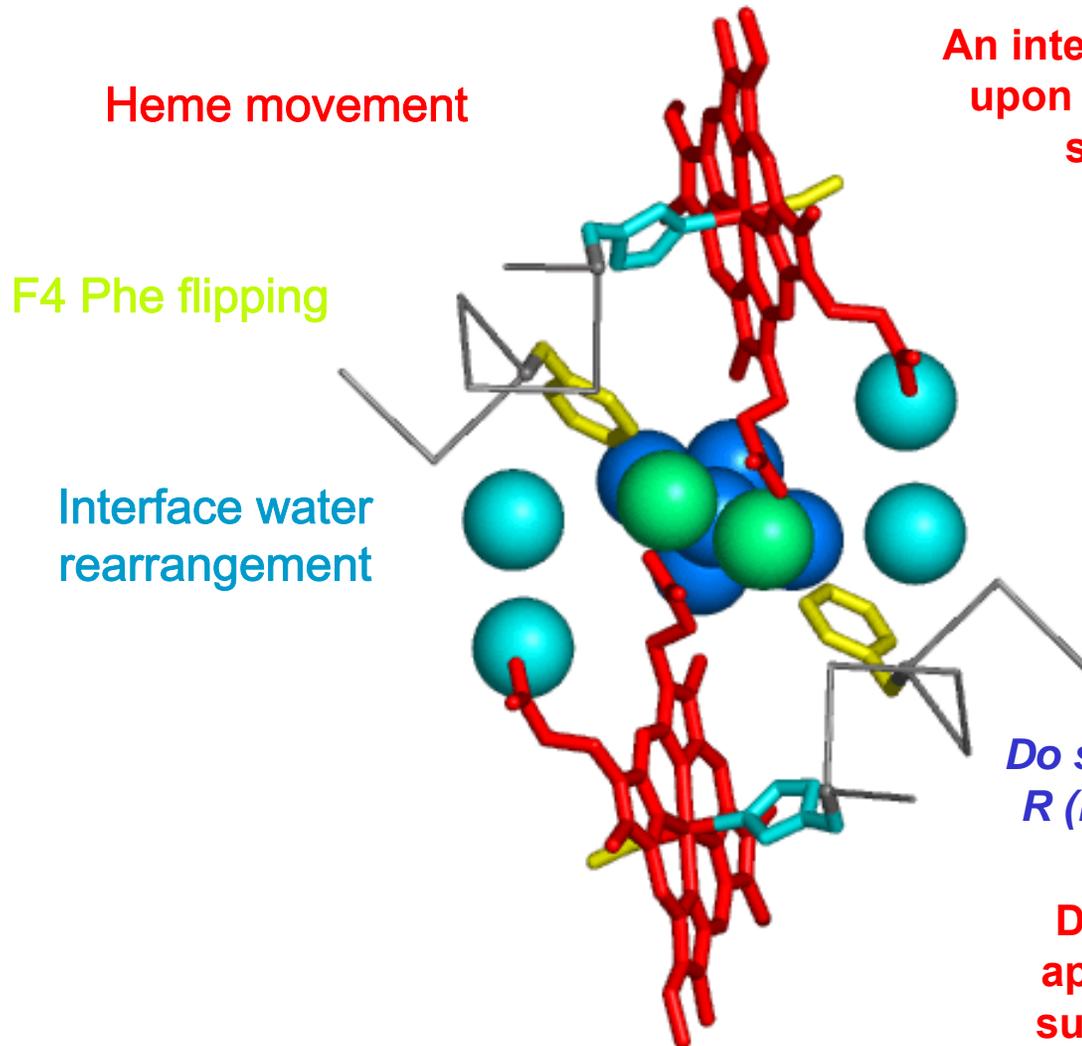
Hbl-CO



Integrated difference $[F_o(\text{light})-F_o(\text{dark})]$ electron density values



Key structural transitions with functional ramifications



What is the cascade of structural events?

An intermediate is formed rapidly (<5ns) upon ligand release, relaxing to T-like structure in μ s time domain.

Are these transitions concerted or sequential?

Key allosteric changes appear to be tightly coupled.

What is the triggering event?

Rapid disordering of water molecules H-bonded to heme propionates.

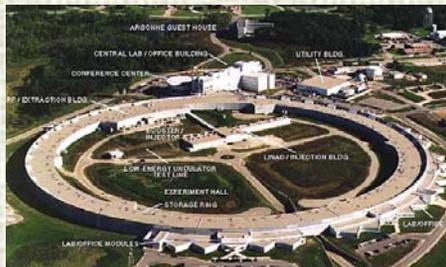
Do structural intermediates facilitate R (ligands bound) to T (no ligands) transition?

Disordering of water molecules appears to lay the foundation for subsequent heme movement and coupled Phe97 flip.

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 crystals: 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





Acknowledgements



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Spencer Anderson, Sudarshan Rajagopal, Harry Ihee,
Zhong Ren, Wilfried Schildkamp, Claude Pradervand, Tsu-yi Teng

Technische Universität München

Marius Schmidt

University of Massachusetts

James Knap and William Royer

ESRF

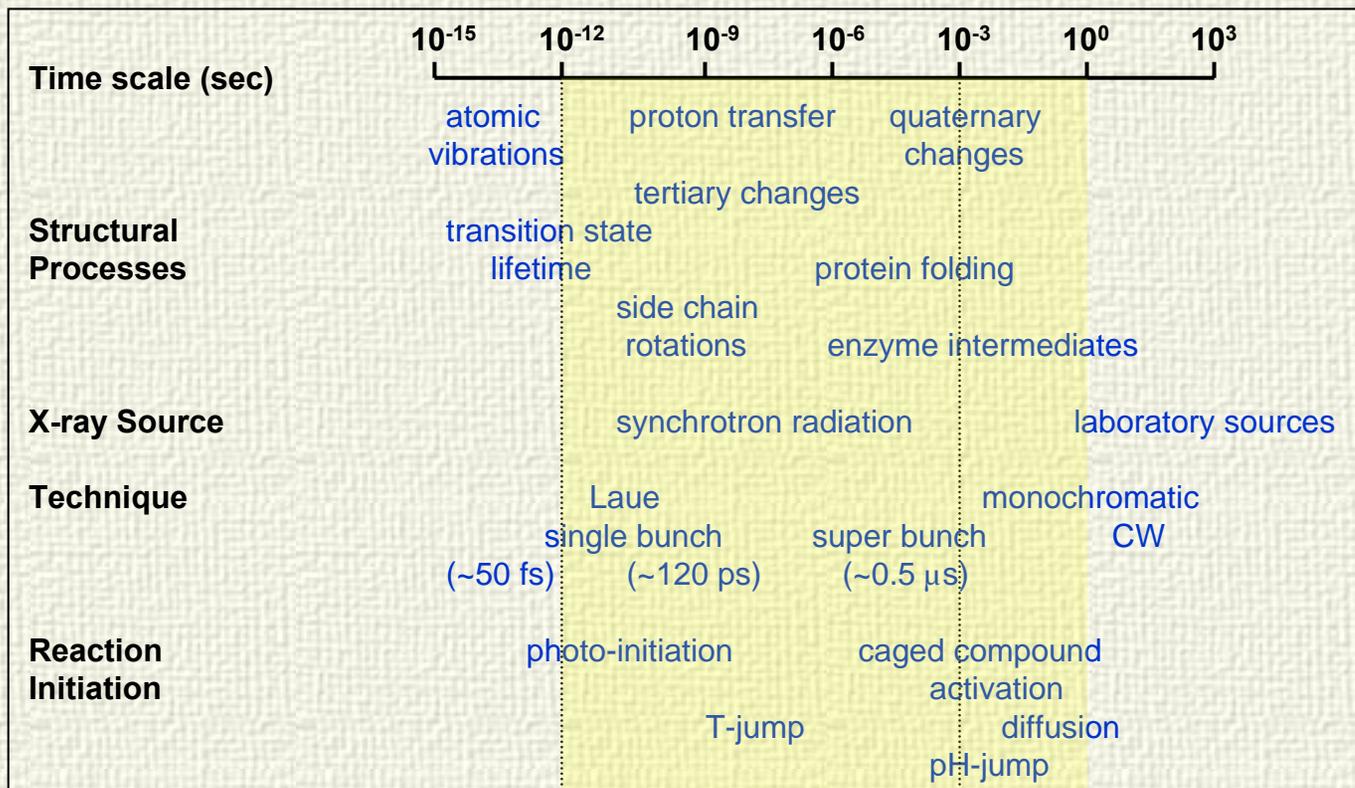
Michael Wulff, Dominique Bourgeois, Thomas Ursby,

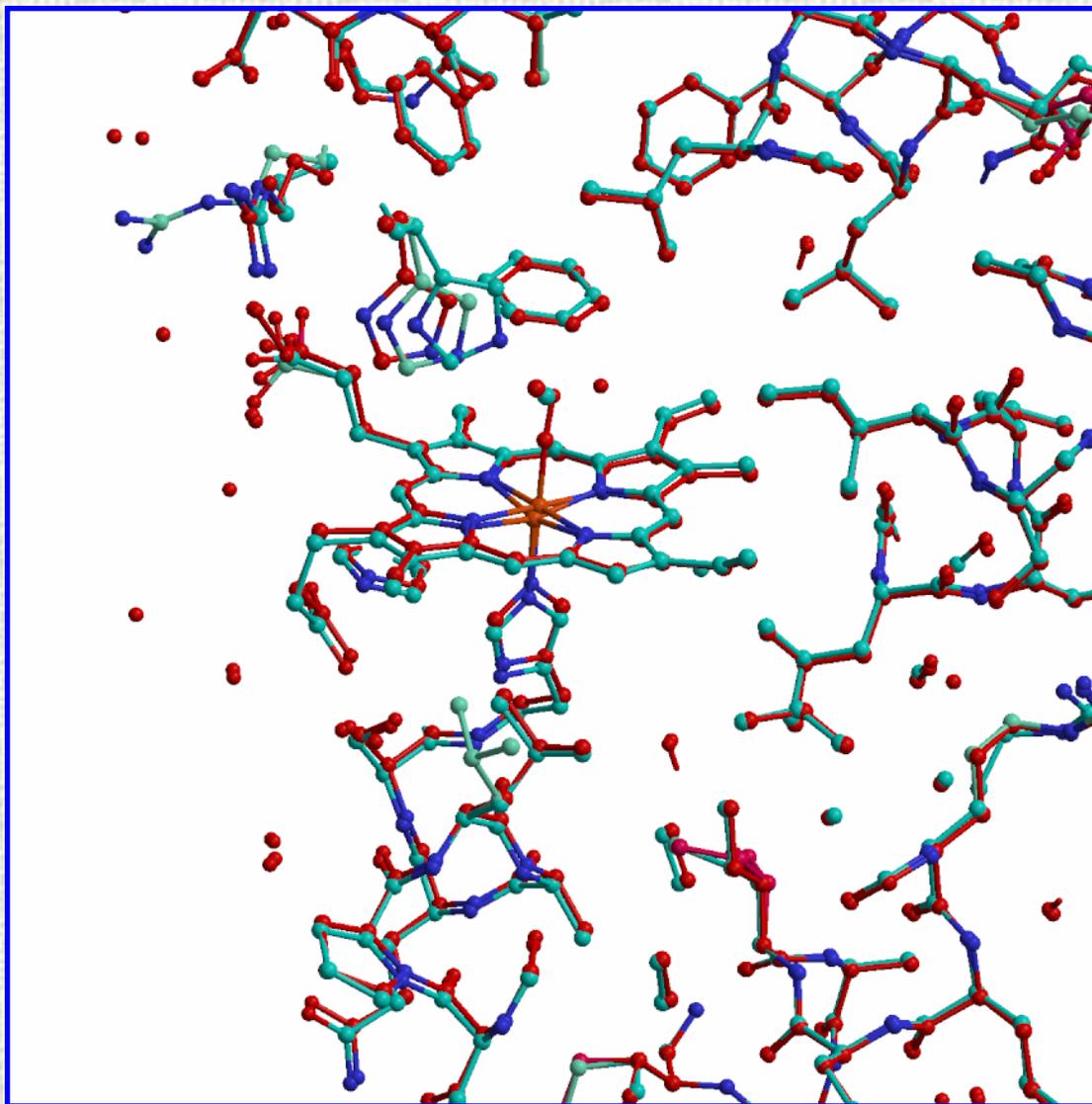
NIH

Friedrich Schotte, Philip Anfinrud

Systems for time-resolved crystallography

- heme proteins
- bacteriorhodopsin
- rhodopsins: visual, sensory, transport
- reaction centers (photosynthesis)
- bacterial and plant photoreceptors:
 - cryptochromes (crys)
 - phototropins (phots)
 - phytochromes (phys)

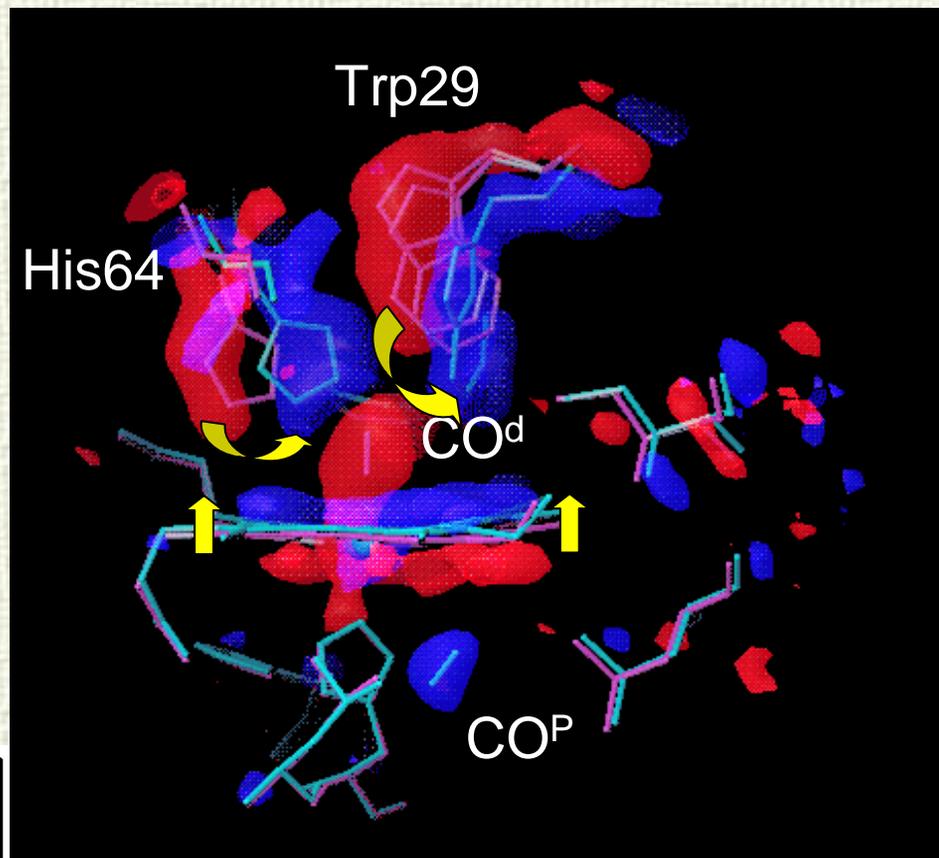
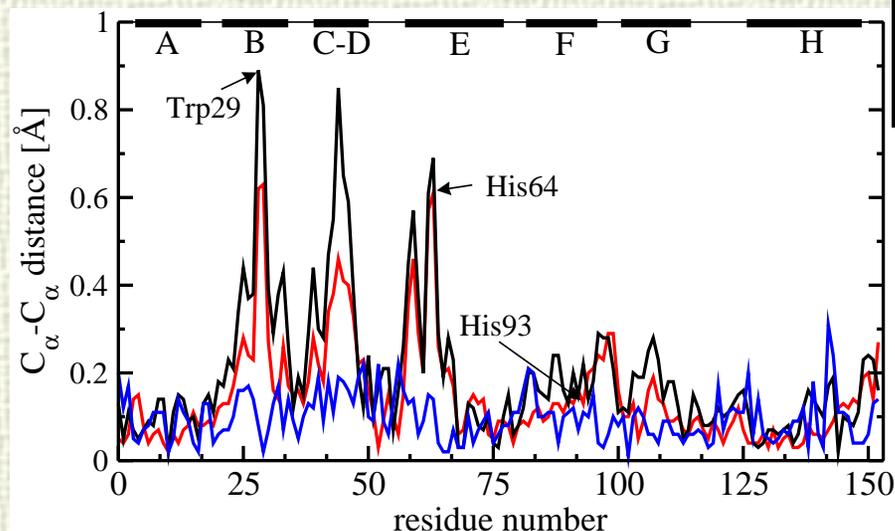




Static structures of **MbCO** and **deoxy Mb** at 1Å resolution (room T)
Kachalova et al. Science 284, 473-476 (1999)

Relaxed photoproduct structure:

- refined from 2-100 μ s data
- virtually identical to deoxy Mb, with CO* captured at the Xe1 site (refined model shown in blue)



- Mb* vs MbCO
- Deoxy vs MbCO
- Deoxy vs Mb*

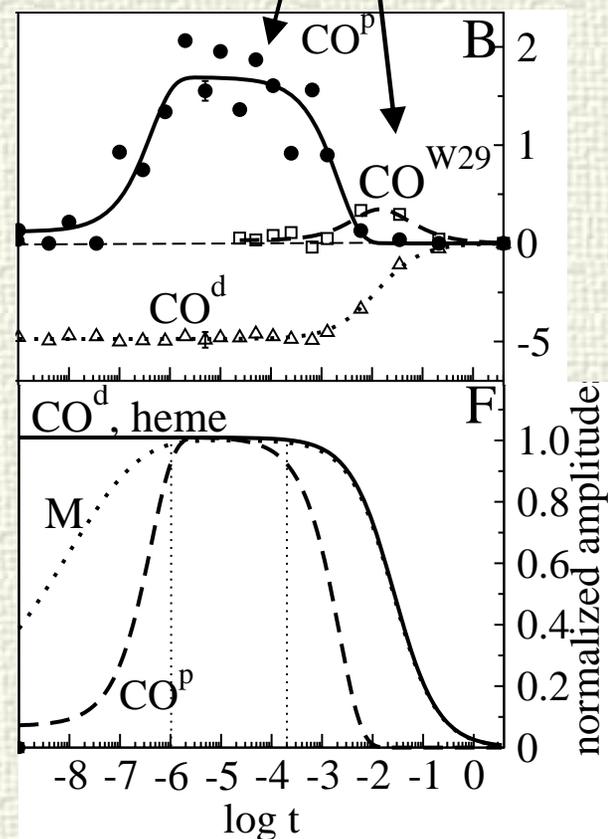
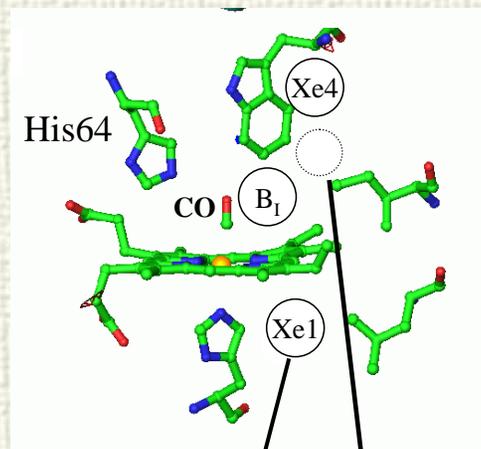
Ligand pathways

- no ligand at the distal B or Xe4 sites – blocked by the Trp29 and its relaxation
- Xe1 populates in 500 ns (30 ns in wt)
- CO* stays at Xe1 site for ~1-2 ms (only 10 μ s in wt)!
- Xe1 site depletes before ligand rebinds

➔ no efficient pathway from Xe1 to the solvent
- otherwise depletion of Xe1 site would be similar for wt and L29W

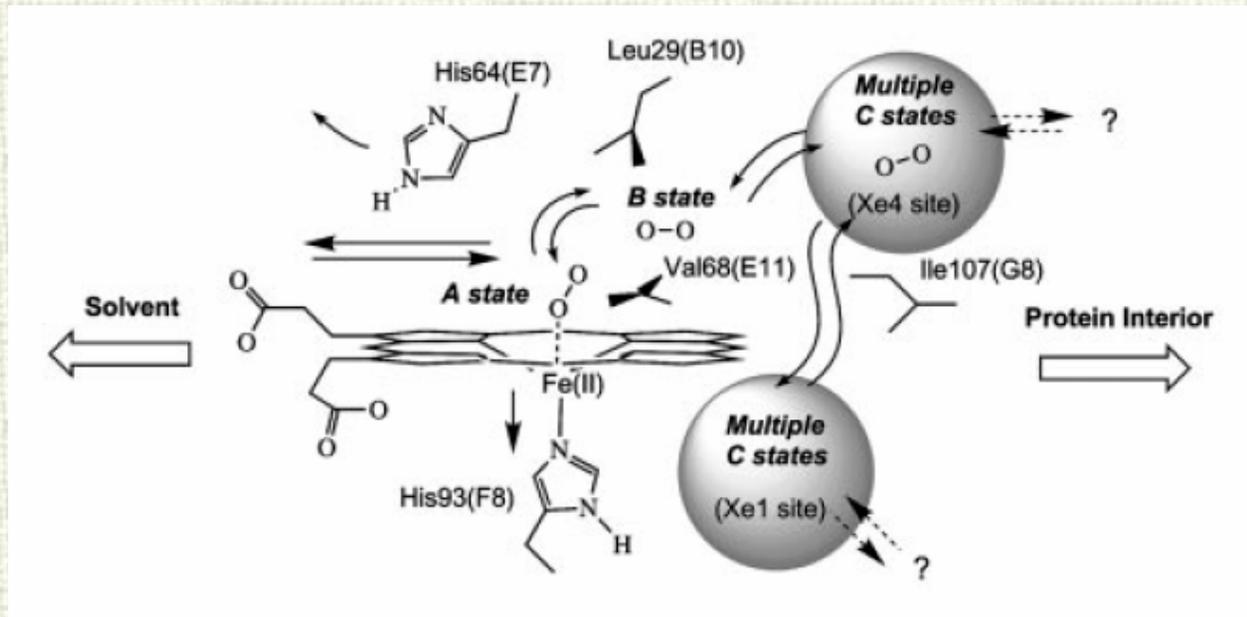
➔ main ligand exit/entrance via His64 gate on the distal side

➔ CO*: Xe1 → distal pocket → rebind/exit via His64 gate



Scott/Gibson proposed model for the role of cavities:

- dead-end states (main exit/entrance via His64)
- but important role in “catching” and “holding” the ligand



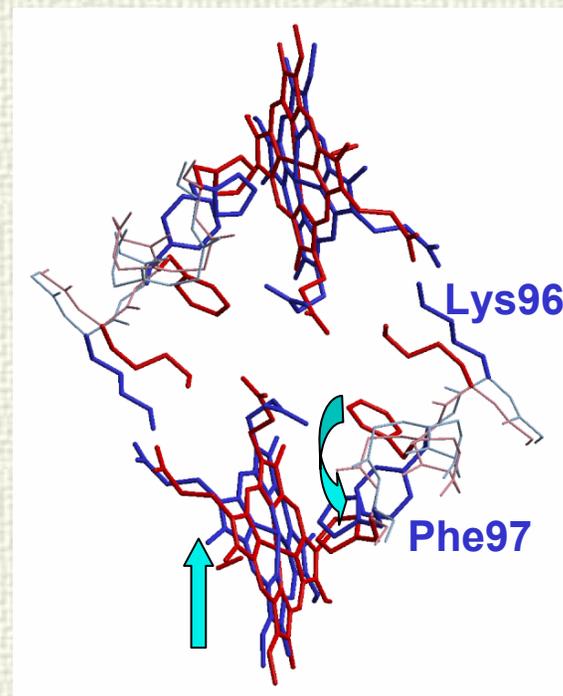
Scott et al., J. Biol. Chem. 276, 5177, 2001

Cooperativity in Hbl

Three key contributors to the cooperativity mechanism:

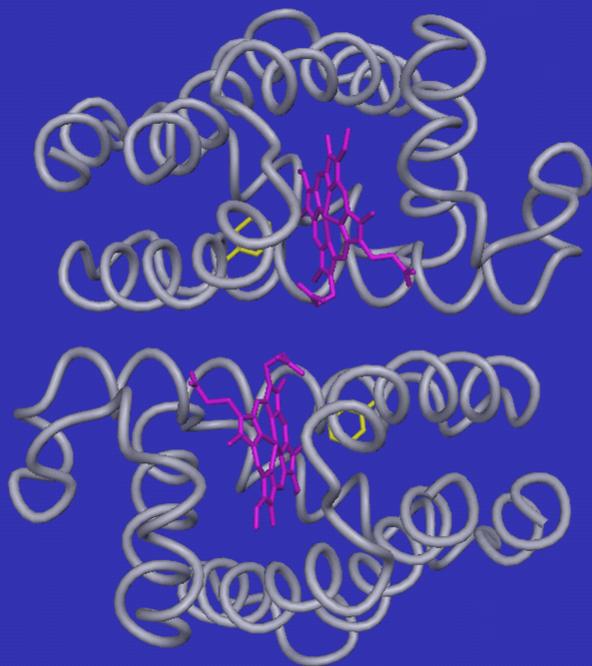
- Phe 97
- hemes
- water molecules at the dimer interface

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

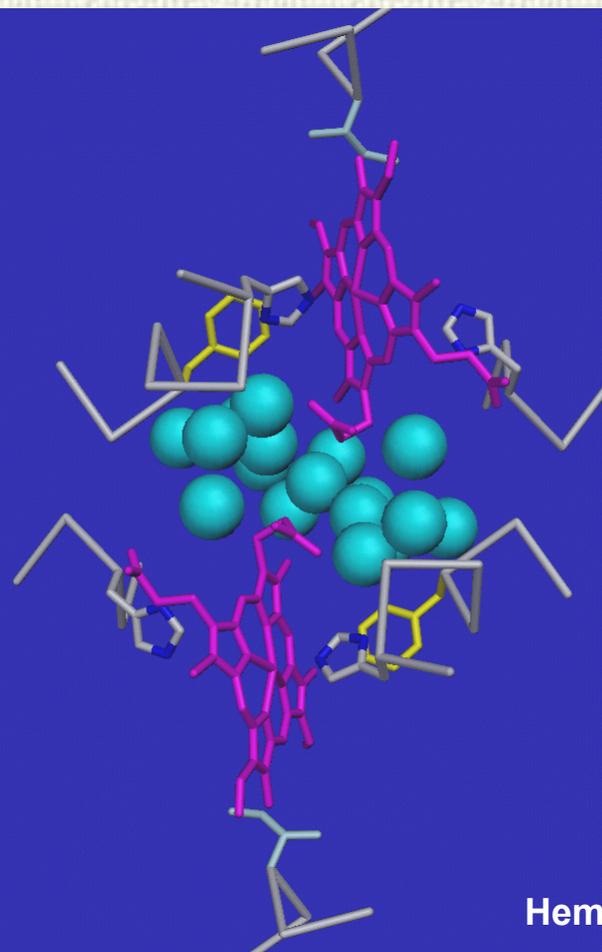


Structural transitions upon ligand release (red → blue):

- 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



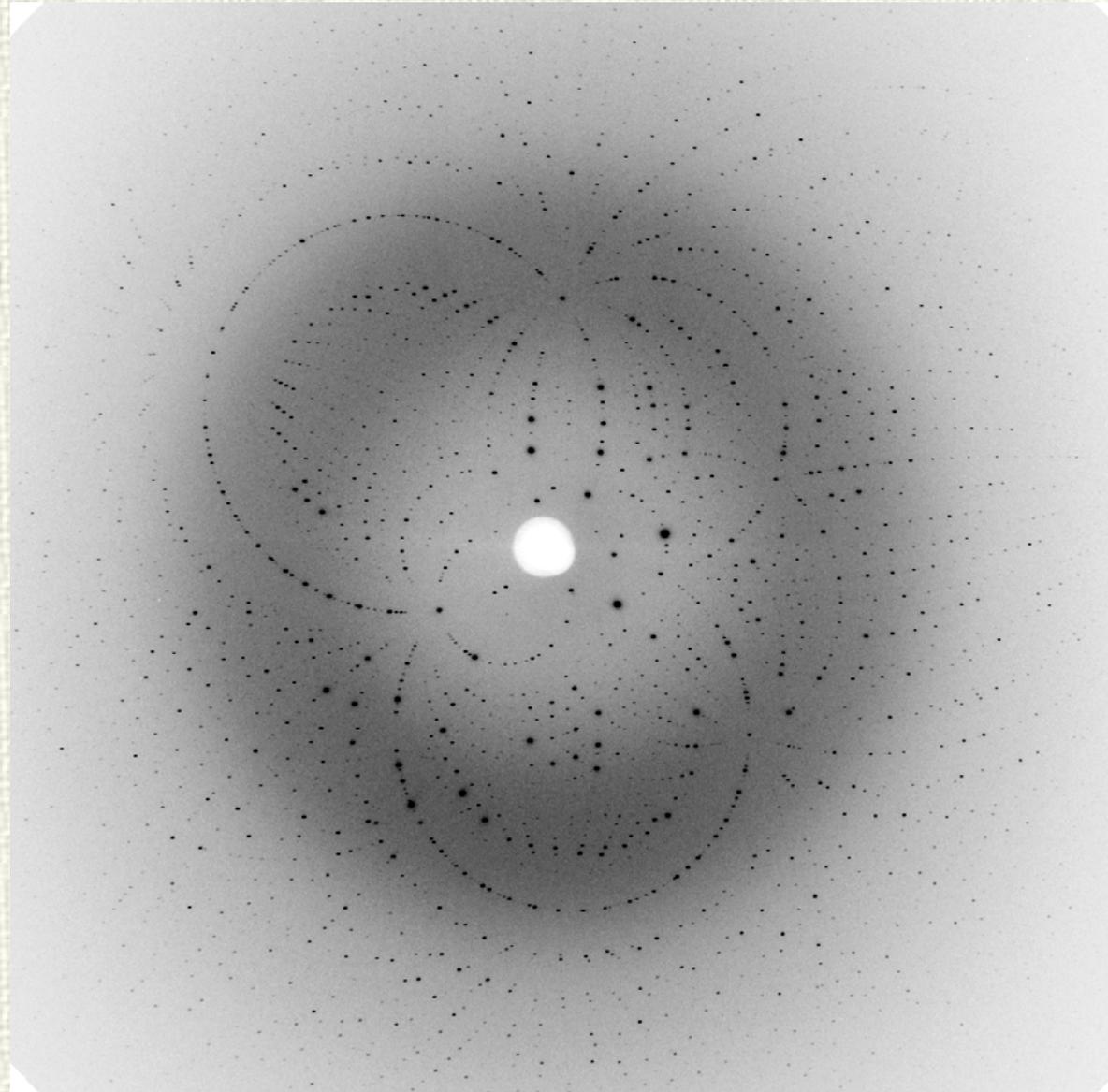
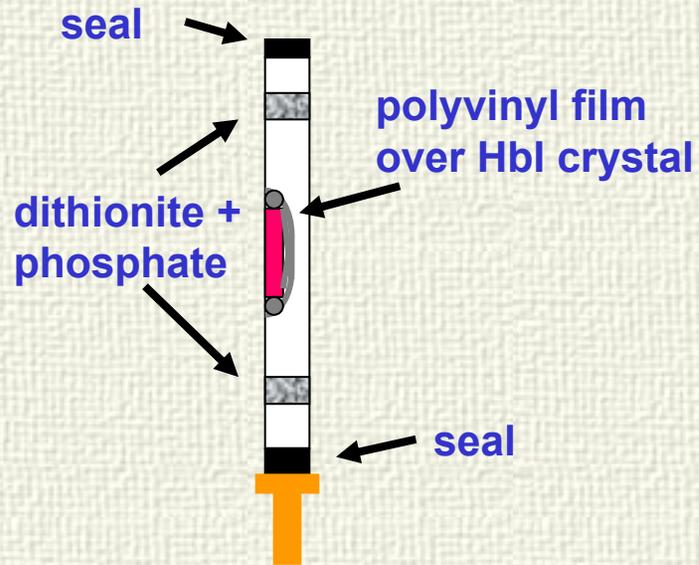
Hbl dimer



Heme region

Colors: **Hbl-CO heme (R)**
deoxy Hbl heme (T)
Phe 97
interface water molecules

Hbl Crystals and Time-resolved Data Collection



Time series

no laser + 16 time delays:
5ns to 80 μ s