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# Time-resolved optical and x-ray studies of P<sub>2</sub>, MbCO crystals

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## Overview

The principal goal of this run was to develop experimental protocols suitable for picosecond time-resolved macromolecular crystallography. Once established, we aim to move beyond the “still” camera of conventional static crystallography and study the structural evolution of various proteins as they execute their designed function. This new knowledge will help to reveal *how* proteins function at an atomic level of detail. To pursue this vision, two major issues have to be addressed. First the signal-to-noise ratio of diffraction images must be **maximized** to reveal the weak, high angle spots that contain the high resolution data required to generate a functional interpretation. Second, to attain ~ 100 ps time resolution, a limit dictated by the electron bunch duration, the synchronization of x-ray and optical pulses must be precise down to ~ 20 ps. Both of these areas were addressed and our results are summarized below.

## Experimental Results and Discussion

### Sample photolysis

If a photolysis pulse were to trigger a reaction in every protein molecule within a crystal, its time-resolved x-ray diffraction pattern would correspond to the structure at a particular point in time along the reaction pathway (resolvable to ~100 ps at the ESRF). When the photolysis is incomplete, the diffraction image corresponds to a linear combination of photolyzed and unphotolyzed images. Because the information of interest resides in the difference between photolyzed, and unphotolyzed images, the signal-to-noise ratio for the difference depends on the fraction photolyzed. Indeed, in the shot-noise limit, attaining a S/N of 1 with 10% photolysis requires 181 x-ray photons whereas a S/N of 1 with 100% photolysis requires only 1 x-ray photon<sup>1</sup>. Consequently, to acquire high resolution (high S/N) time-resolved diffraction images, it is crucial to develop experimental protocols that maximize the level of photolysis. To that end, we developed a femtosecond microspectrophotometer that measures both static and transient absorbance spectra of protein crystals. From these spectra, the fraction photolyzed can be determined. By systematically varying the excitation pulse characteristics, the optimum conditions for attaining high levels of photolysis can be determined.

### Femtosecond microspectrophotometer

The femtosecond microspectrophotometer is based upon a single-filament continuum that is generated by focussing a high peak power optical pulse (~ 100 fs; 800 nm; <1 μJ) in a thin crystal of sapphire. The divergent continuum is collimated by an Ealing all-reflective objective,

and is focused into the protein crystal with a second objective. A third objective recollimates the continuum and an achromat lens focuses the continuum into a fiber optic which delivers the continuum to an imaging spectrometer equipped with a CCD detector. A portion of the continuum is sampled before the crystal and is delivered through a second fiber to the same imaging spectrograph. The all-reflective Ealing objectives are aligned with the continuum off center so that it passes through the objective without obstruction (by directing the beam through one of the three sectors). The sample and reference spectra are imaged onto the CCD which is binned into two tracks that we read out after each laser flash. The continuum pulses are generated at a 900 Hz rate and a millisecond Uniblitz shutter selects a single pulse for the spectroscopic measurement. To select a single pulse, the continuum beam had to be positioned close to the edge of the aperture which is the last to open and the first to close. This could be arranged with the continuum beam, whose collimated dimension was less than 1 mm. The photolysis pulse, on the other hand, is much bigger in diameter and when placing the shutter as close to the crystal as was possible, the beam dimensions were too large to permit single pulse selection. The best we could do was transmit two photolysis pulses for every continuum probe pulse<sup>2</sup>. The photolysis pulse is selected with a second Uniblitz shutter. To acquire the time-resolved spectra, a sequence of four images were acquired in rapid succession (as fast as the computer and CCD could run): sample/reference without photolysis; sample/reference with photolysis; photolysis only; background (blocking both sample/reference and photolysis pulses). From these images, the scattered photolysis light could be subtracted and the photolysis-induced change in the absorbance of the sample could be calculated. The equilibrium spectrum required a background measurement without a sample, which was obtained by translating the capillary out of the beam.

### P<sub>2</sub>, MbCO Absorbance Spectrum

This figure shows a series of spectra from a P<sub>2</sub>, MbCO crystal that is approximately 300 microns along the long axis (I believe it was crystal #12, but I'm unable to confirm this). The polarisation of the continuum was adjusted to find the minimum absorbance axis of the crystal. The spectral series corresponds to the “unpumped” spectra acquired over the period of time over which transient absorbance spectra were accumulated (a period of a few hours). While there appears to be a modest degradation of the spectral amplitude, the spectra do not reveal features that could be identified as met-Mb or deoxy-Mb, so the modest reduction in amplitude could be due to drift of the alignment over a period of hours. The peak absorbance is around 0.7 in optical density units at 540 nm, and about 0.3 at the photolysis wavelength of 575 nm. These optical densities are less than the values listed by Vukica Strajer for the same crystal. I can't come up with any explanation for how the spectra in this figure could be scaled incorrectly. It may be that Strajer's microspectrophotometer is in error. The continuum beam is quite clean and can lead to more accurate spectroscopic measurements than an incoherent source that is focused with large numerical aperture optics. The photolysis pulse was oriented at about 45 degrees relative to the crystal normal, similar to the geometry used in the x-ray goniometer. I believe we used circularly polarized photolysis light and focused the photolysis beam to about 200 microns to duplicate the conditions on the x-ray goniometer. Friedrich and/or Simone may be able to confirm this. Because the absorbance along the other crystal axis (orthogonal to the probed axis) is several times larger (I don't have crystal spectra to quantify this) the transmission of circularly

<sup>1</sup>  $S = fN$  where  $S$  is the signal arising from the photolyzed fraction,  $f$  is fraction photolyzed, and  $N$  is the total number of photons detected.  $\sigma_s^2 = N + (f-2)N$  where  $\sigma_s^2$  is the variance of  $S$ . Signal-to-noise ratio is  $S/\sigma_s$ .

<sup>2</sup> A faster shutter is available from Uniblitz; it might be worthwhile acquiring one

polarized light would be less than 50%. A 200x200–50 micron MbCO crystal contains  $5.9 \times 10^{13}$  hemes and 10  $\mu\text{J}$  of 580 nm light contains  $2.9 \times 10^{11}$  photons. Consequently, if half of the photons were absorbed, the fraction photolyzed would be expected to be  $\leq 0.5 \cdot (2.9/5.9) = 0.25$ . For the sake of comparison, I have plotted the solution spectrum of MbCO on the same figure. I have also plotted the continuum spectrum. One can see a strong correlation between the continuum spectrum (log plot) and the systematic oscillations in the red (600 - 700 nm) region of the MbCO spectrum. This error in the MbCO spectrum is evidently due to incomplete subtraction of the background, which must be done to recover the static spectrum. The continuum is strong from 500-600 nm where the features are most prominent, so the spectra recovered over this window should be quite accurate.

#### Transient Absorbance Spectra of MbCO

Static solution spectra of MbCO and Mb are plotted along with their difference spectrum (Mb-MbCO). The negative-going difference is largest at 538 nm where the “bleach” is  $(-0.256/0.764) = -0.33$  times the peak absorbance at the same wavelength (numbers came from solution spectra). The minimum of the transient absorbance spectrum at 0 fs delay is 539.5 nm and at 10 ps it is 53X run. Because both transient “bleach” features are centered near that for the solution phase difference spectrum, we should be able to get a reasonable estimate for the fraction photolyzed by measuring the magnitude of the transient bleach at 538 nm and comparing this number to the peak absorbance of the static spectrum at the same wavelength. For example, at 10 ps, the magnitude of the bleach is -0.041 and the peak absorbance is 0.778 (average of spectra shown in other figure), so the fraction photolyzed is  $(-0.041/0.778)/(-0.33) = 0.16$ . This level of photolysis (16%) was achieved with approximately 10  $\mu\text{J}$  of pulse energy at 575 nm, of which slightly more than half was absorbed (given an optical density of about 0.33 at 575 nm). We need to improve the photolysis substantially. If we set > 50% photolysis as our goal, we require more than 30  $\mu\text{J}$  in our photolysis pulse (delivered to the crystal, not generated by the OPA).

The transient absorption spectrum at 0 fs does not reveal a deep bleach, so there is no “magic” wavelength for photolyzing MbCO (as there is with bR). Ideally, one would like to photolyze MbCO at a wavelength where the excited state and/or photoproduct absorbance is small compared to the ground state absorbance. Upon photoexcitation, an excited state absorbance appears which is red-shifted relative to the Soret band (published by J.-L. Martin) and this absorbance reduces the magnitude of the bleach seen in the Q-band region. Furthermore, the photoproduct (the ground state of Mb) is initially “hot” and this causes the red edge of the Soret band to extend into the Q-band region, also reducing the magnitude of the bleach. About 75% of the bleach is attained within 1 ps and the rest of the bleach develops on the time scale of thermal cooling, which I measured before to be 6.2 ps (time constant). Consequently, the most effective photolysis of MbCO would probably require a pulse of -10 ps duration that is tuned to the wavelength where  $A_{Mb}/A_{MbCO}$  is minimized. According to the solution spectra, this occurs at 475 nm where  $A_{Mb}/A_{MbCO} = 0.64$ . (The ratio becomes 0.66 at 538 nm.)

The best way to photolyze MbCO with your laser system is to tune the Mira to 950 nm. The amplified fundamental would be frequency doubled to generate 475 nm photolysis pulses. This would require a different optics set in the Mira and the regenerative amplifier and might also require swapping a few multi-layer dielectric mirrors along the path to and from the regenerative amplifier (some optics may be optimized for 800 nm but not 950 nm). To operate at 950 nm, the

Mira might have to be purged with  $N_2$  to eliminate absorption by water vapor. A Coherent representative could advise us on whether or not a purge is needed at that wavelength. The pulse energy that could be generated at 950 nm should be several hundred microjoules, and with efficient doubling, we would have more than enough energy to photolyze a sample of MbCO. Another benefit of working at 450 nm is the reduced optical density of the crystal, which would permit more uniform photolysis through the crystal. Moreover, the absorbance of Mb is 0.64 times that absorbance of MbCO at 475 nm, so multi-photon absorption by a single heme would be minimized.

#### One vs. two-photon photolysis

One photon photolysis is often plagued by a strong absorbance of the photolysis pulse which leads to shallow penetration of the light and nonuniform photolysis within the crystal. If the sample could be photolyzed through a 2-photon absorbance, many of these problems would go away. In general, any chromophore should exhibit a reasonably strong 2-photon allowed transition at some wavelength. If the one photon cross section for the ground state and the photoexcited state is negligible at the optimal wavelength for 2-photon absorption, the photolysis pulse would “bathe” the entire volume of the crystal and 2-photon absorption events could occur randomly (and uniformly) within the crystal volume. If the crystal would tolerate the high peak power required for efficient 2-photon absorption, it might prove to be a viable means for achieving photolysis.

To test this idea, we toned the fs laser system to 844 nm where the MbCO and Mb absorbance is negligible. 844 nm was chosen because two photons would have the same energy as 422 nm, the peak of the MbCO Soret band. Unfortunately, we failed to see any significant photolysis. There was one case where we thought we found a real signal, but we couldn't reproduce it with other MbCO crystals. It remains a possibility that no (negligible) signal was seen due to incorrect pump-probe pulse timing. However, I suspect 844 nm is not an optimal wavelength for 2-photon absorbance I am unaware of anyone that has attempted 2-photon photolysis of hemes. It is probably worth trying it in a laboratory setting where it can be done carefully. I'm interested enough to perhaps give this a try in my lab at the NIH once my lasers are set up and functional. If this idea works with MbCO, it may work with many systems. For example, PYP would be a great candidate to try this, given its extremely strong optical density.

Given this negative result, we went on to characterize how much energy could be focussed onto the crystal before nonlinear absorption and sample damage occurred. When 60  $\mu\text{J}$ , 80 fs (120 fs autocorrelation) pulses were focussed down to 280 microns (which for a 2-photon process would generate a 200 micron spot), bubbles slowly formed within the mother liquor surrounding the crystal. At 520  $\mu\text{J}$ , the crystal shattered. At intermediate pulse energies a plume of water (mist) was generated which spattered the opposite side of the capillary. Evidently, the maximum pulse energy must be less than 60  $\mu\text{J}$ . If the 2-photon idea is to work, it would have to have a very strong 2-photon cross section. In my own lab, we have excited bR with a 2 photon process and saw a sizable signal. I seem to recall about 5% photolysis with perhaps <10  $\mu\text{J}$  in a 200-300 micron spot. Because the photolysis scales as the power squared, 4 times the energy would generate 16 times the photolysis, so this approach seems viable

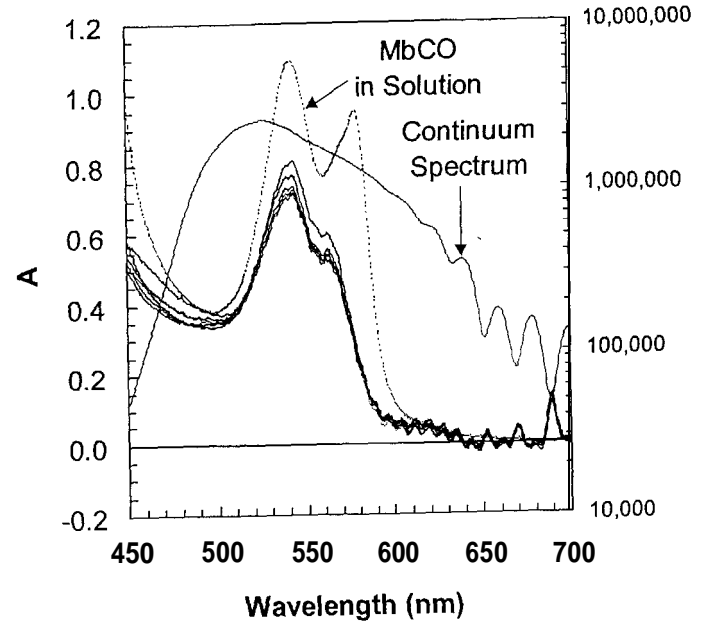
**Signal-to-Noise Ratio of Diffraction Images: Undulator (U26) vs. Wiggler (W70) Radiation**

Diffraction images were obtained with both U26 and W70 radiation using the same crystal at the same orientation. Friedrich knows the order in which they were collected. The images correspond to a 200 ps time delay after 16% photolysis (because the determination of photolysis is approximate- compare 16% with the 25% estimate from pulse energy, optical density, size of the crystal, and focus size - I rounded it to 20%). When the brightness of the image was set so that the background new the edges of the images was about the same, spots clearly seen in the U26 image are lost in the background noise of the W70 image. It is clear that the U26 offers much greater potential for obtaining high quality diffraction images.

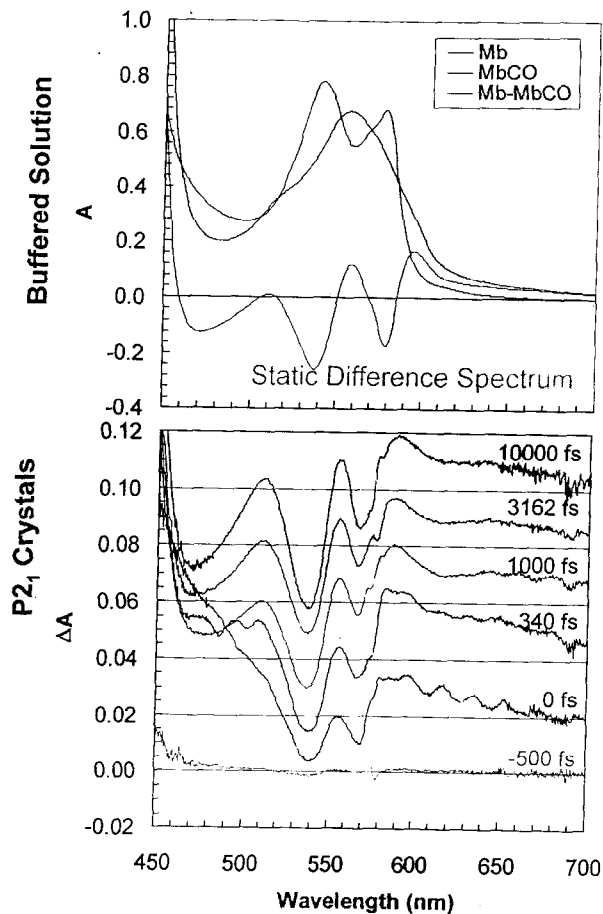
**Determination of x-ray and optical pulse time delay**

A convent means to measure the time delay between the x-ray and photolysis pulse was developed. Using an avalanche photodetector that is responsive to both x-rays and light pulses and a LeCroy digital oscilloscope, we found that we could signal average the traces and reproduce time-difference measurements to a precision of less than 40 ps.

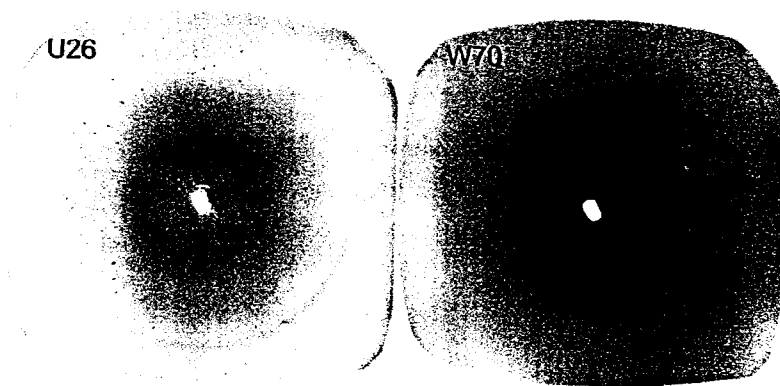
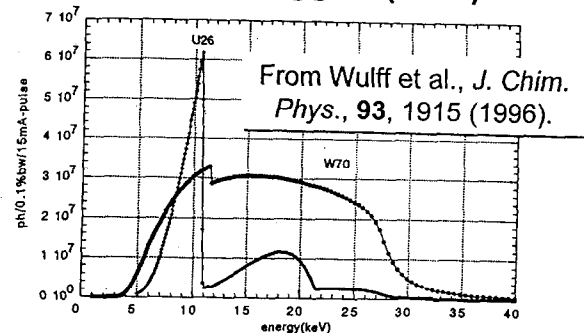
**P<sub>21</sub> MbCO Absorbance Spectrum**



## Transient Absorbance Spectra of Mb\*CO



## Signal-to-Noise Ratio of Diffraction Images: Undulator (U26) vs. Wiggler (W70) Radiation



X-ray diffraction of P<sub>21</sub> MbCO measured 200 ps after photolysis (~20%); accumulated 64 shots (ESRF ID09, Feb. 22, 1998)