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Report:

The objective of our Apri1 95 experiment (experiment number LS177) was to determine the structure of short-lived (ns-μs) intermediates resulting from ligand photodissociation of carbonmonoxy myoglobin(MbCO) . Several experimental improvements were made compared to earlier attempts in1994, in order to achieve and maximize the extent of ligand photolysis in crystals and to observe the resulting structural changes; in-situ optical monitoring of crystal photolysis was implemented to assure that photolysis occurred during the actual X-ray data collection; the coupling of the laser light to the optical fiber was improved and enabled us to increase the laser pulse energy delivered to the crystals ; and finally, signal averaging and a higher storage ring current of 15mA were used in single bunch mode in order to improve the signal-to-noise ratio.

Two complete data sets were collected in the single bunch mode on both MbCO and its photoproduct (Mb*), with a 4ns delay between the laser and X-ray pulses for the Mb* data collection. Complete data sets were also collected at time delays of 500ns, 7μs, 50μs, 350μs and 1.9ms using multibunch mode at 150mA. Delay times were accurately measured using an avalanche diode to capture both the laser and X-ray pulses. All data sets covered 180° in crystal angular setting in increments of 4°, to record complete and redundant data for the monoclinic $P2_1$ space group of MbCO crystals. Typical crystal size was between 0.35x0.25x0.06 mm^3 and 0.50x0.35x0.08 mm^3. In the single bunch mode three X-ray exposures were collected for each angular setting of the crystal to improve the data quality by signal averaging. Crystals were photolyzed using 10ns laser pulses at 635nm from a Nd:YAG pumped dye laser. The wavelength of 635nm was selected since the crystal absorption at 635nm is ≤ 0.20D. This ensures uniform photolysis in the longitudinal direction and minimizes thermal gradients. The laser beam with 13mJ pulse energy was focused to a 0.75mm diameter. Only a small part of this energy, about 0.5-lmJ, was actually absorbed by the crystals due to their small size and low absorption cross section at 635nm. This absorbed energy corresponds to about 5 photons absorbed per pulse per molecule and to a photolysis rate of $10^9 s^{-1}$. Only -45% of molecules were photolyzed as estimated from the optical density change. A further increase in laser pulse energy, however, resulted in an irreversible crystal damage.

The X-ray diffraction data was reduced independently in Chicago, using the LaueView software[1] and in Grenoble, using the Daresbury Laue/CCP4[2,3] packages. The two protocols yielded similar structural results. Data were 81 to 85% complete to 2Å, with an R-factor of ~ 10% on I and an overall redundancy of ~5. The resulting time-dependent difference Fourier maps (Mb* -MbCO) show clearly several significant features. The loss of the CO ligand is detected as a prominent negative feature at 4ns(-9.8σ), along the Fe-CO bond. The magnitude of this feature decays with time with the same rate as the optical signal due to CO rebinding. From the integrated electron content of this feature at 4ns we estimate ~40% initial photolysis, in good agreement with ~45% estimated from optical measurements. Another significant and important feature is present (+3 .9σ) along the Fe-NE2(His93) bond and indicates the iron displacement from the heme plane upon ligand photolysis. From the integrated electron content of this feature and its time course, which also parallels the ligand rebinding, we conclude that iron displacement is complete within 4ns. Rearrangements of the residues surrounding the heme are also evident. Most notably, the displacement of the important distal heme pocket residue His64 is evident at 4ns and 500ns and agrees with the differences between the static deoxy and MbCO structures from the Protein Data Bank[4,5]. Residues along the E and F helices, spanning Lys62 to Va168 and Leu89 to His97, also show signs of rearrangements in agreement with the static deoxy and MbCO structures. Some of these tertiary structural changes seem to be fast and occur in parallel with heme relaxation (≤ 4ns), while others are slower and continue to evolve over at least several μs. The only plausible docking site of the CO molecule is located in the heme pocket, in the vicinity of the water molecule site in deoxy Mb[4] and the site of the photodissociated CO molecule in low temperature MbCO photoproduct[6,7]. The electron density at this site is observed only at 4ns, in agreement with the time-resolved IR measurements that determined the lifetime of CO in the heme pocket docking site to be a few hundreds of ns[8].

These ns time-resolved results on photolysis of MbCO show that ns macromolecular crystallography is indeed feasible. Careful experimental design, Laue data acquisition and reduction strategies result in clearly interpretable difference Fourier maps and reveal valuable information on protein dynamics even from these relatively weak diffraction patterns. The time resolution in this experiment is dictated by the ns duration of the laser pulses and can be extended to utilize the full 150ps resolution of the X-ray source by using shorter laser pulses.

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