Reports

Time-Resolved X-ray Absorption Spectroscopy of Carbon Monoxide–Myoglobin Recombination After Laser Photolysis

Abstract. *Results are presented for the first time-resolved x-ray absorption measurements with a time resolution of 300 microseconds on a dynamically evolving chemical system. By synchronizing a neodymium:yttrium-aluminum-garnet pulsed laser with the bursts of x-rays emitted from the Cornell High Energy Synchrotron Source, it was possible to monitor at room temperature the recombination of carbon monoxide with myoglobin after laser photolysis. Changes in the pre-edge structure and in the position of the iron edge of this protein were detected as a function of time.*

With the development of synchrotron radiation facilities, which can provide high-intensity and tunable x-radiation, x-ray spectroscopy has experienced a renaissance (*1*). X-ray absorption spectroscopy is a powerful tool since it can probe selected atoms in their native environments (*1*). In particular, measurements of the shape and position of K or L x-ray absorption edges and features near these edges can yield information on the oxidation state and local environment of the absorbing atom.

Thus far, only static samples have been successfully studied by x-ray absorption techniques with the use of synchrotron radiation. Storage ring sources, however, have a temporal structure that is very amenable to transient x-ray studies. Because the particles (electrons or positrons) in storage rings are not continuously distributed around the entire ring but are grouped in packets or bunches, the emitted electromagnetic radiation is pulsed. The duration of the pulse is related to the length of the particle bunch, and the repetition rate is dependent upon the number of bunches in the ring and the ring's circumference. The Cornell Electron Storage Ring emits a 160-psec pulse of x-rays every 2.56 μsec. This temporal sequence is ideal for making time-resolved measurements since there is ample delay between x-ray bursts to clear detectors and advance the associated electronics. By synchronizing a laser pulse with the x-ray bursts, we have obtained time-resolved x-ray absorption near-edge spectra of CO recombining with the iron atom in the protein myoglobin (Mb) after laser flash photolysis.

The motivation for using the MbCO system in these initial attempts at time-resolved x-ray absorption spectroscopy was prompted by many factors. First, the energy of the K-absorption edge of the active-site iron atom is readily accessible with the x-rays emitted from the Cornell High Energy Synchrotron Source (CHESS). Second, laser photolysis and recombination of this important chemical system have been extensively studied with other transient techniques (*2*). From such investigations it is known that MbCO undergoes electronic and structural rearrangements after photolysis that span the time regime from picoseconds to milliseconds, so that this model system is an ideal one with which to test the feasibility of using synchrotron sources for such dynamic x-ray measurements. Finally, despite the numerous studies that have been done on this protein, the application of time-resolved x-ray absorption spectroscopy with its sensitivity to both the structural and the chemical environments of the excited atom should yield new insights into the intermediate states important in the photolysis and recombination of CO with Mb.

In order to attempt these measurements, it was necessary to construct a computer interface which synchronized the firing of the neodymium:yttrium-aluminum-garnet (Nd:YAG) laser used for photolysis of the MbCO with the x-ray bursts (*3*). This interface also served to control the data collection process. For a given x-ray energy, data were recorded during selected time intervals after the laser pulses and were stored in separate memory registers in the interface. Fifteen such memory registers or time bins

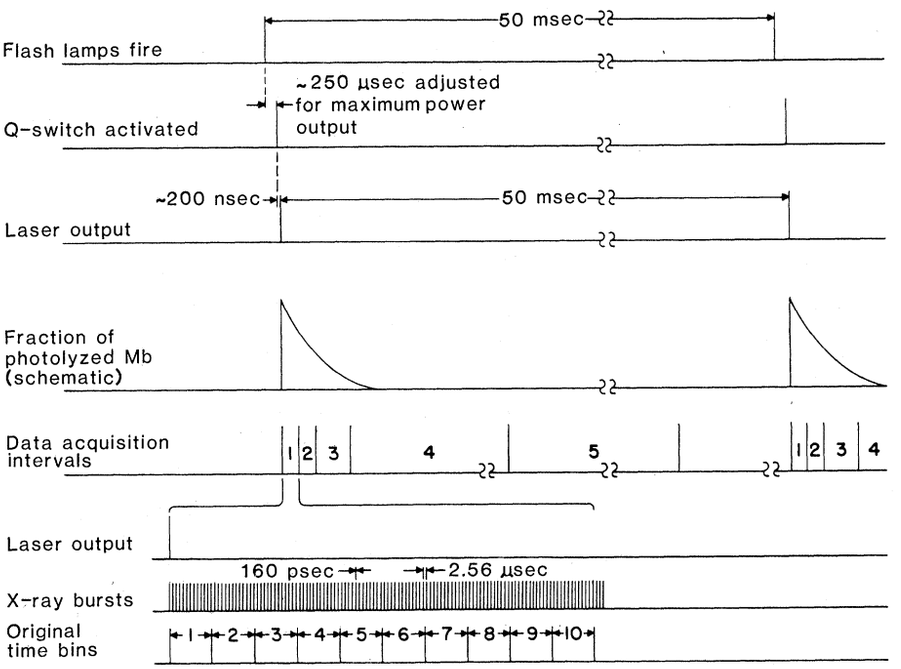

Fig. 1. Timing sequence for the laser firing and data collection. The flash lamps for the Nd:YAG laser fired every 50 msec. This signal was fed into a computer interface where it was delayed by ~ 250 μsec (for maximum laser output power), before triggering the Q-switch which fired the laser 200 nsec later in synchrony with the next x-ray burst. The laser photolyzed ≈ 95 percent of the MbCO, and the fractional recovery of the complex relative to the laser firing is illustrated schematically. Five data acquisition intervals were assigned of the relative durations shown in the figure. We constructed these five data intervals by summing data in 15 individual time bins. The individual time bins corresponding to the first data interval are illustrated at the bottom. Each of these first ten time bins included 12 x-ray bursts from the synchrotron (next to last line). Data interval 2 was the sum of only two time bins, but each bin was collected data from 50 bursts. Data intervals 3, 4, and 5 consisted of one time bin each with 250, 8000, and 8000 assigned x-ray bursts, respectively.

were available. The duration of each time bin was user-selectable through software and specified in terms of the repetition frequency of the storage ring. For instance, in each of the first ten time bins data from 12 synchrotron bursts were stored (see bottom two lines of Fig. 1). Hence, each of these first ten time bins represents time increments of 30.7 μsec after photolysis by the laser beam.

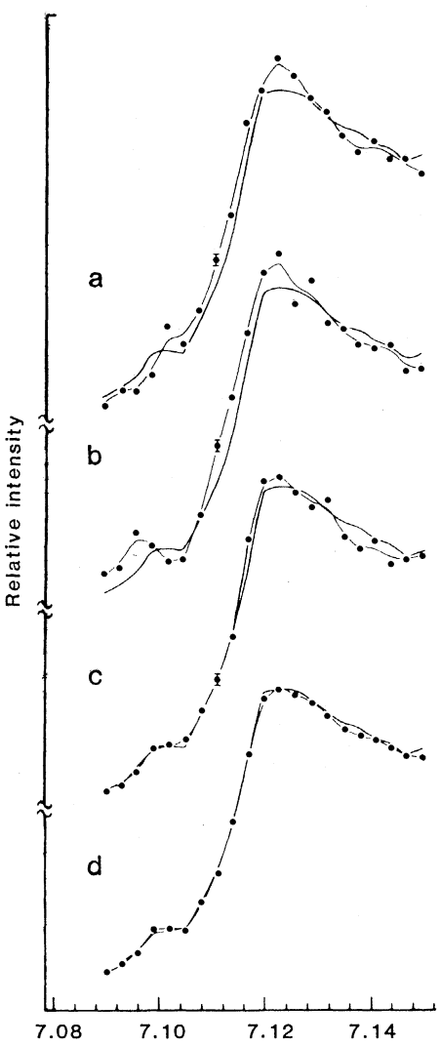

Fig. 2. Time-resolved x-ray absorption spectra of iron in myoglobin at four different time intervals after laser photolysis of 1 m*M* MbCO solution with other sample conditions the same as in Fig. 3: (a) 0 to 307 μsec; (b) 307 to 563 μsec; (c) 563 to 1,203 μsec; and (d) 1,203 to 21,683 μsec. In each case the time-resolved spectra (thin lines) are shown in comparison with the spectrum of the final time interval (dark line), which represents the spectrum of fully recombined MbCO. The dots are the actual data points of the time-resolved spectra; the thin line represents the results of smoothing the data and should be considered only as a guide for the eye. Error bars have been included on one data point each in (a), (b), and (c); in (d), because of the longer length of time interval 4, the error is actually less than the size of the data point. Throughout all the spectra the major source of noise was from Compton scatterred background.

In order to improve statistics, the data shown in Fig. 2a represent a sum of the first ten time bins since no statistically significant changes were observed during the first 300 μsec of data accumulation. Based on recombination times determined from earlier MbCO photolysis experiments with visible absorption changes (*2*), we constructed four additional time intervals to span the completion of the recombination process (see Fig. 1). Our five time intervals (Fig. 2) were 0 to 307, 307 to 563, 563 to 1,203, 1,203 to 20,480, and 20,480 to 42,163 μsec. The last time interval was chosen to cover a time regime when all the photolyzed Mb had recombined with CO. Consequently, the data in the last interval could be used for the reference (recombined MbCO) spectrum. Hence, at a given x-ray energy, the spectra of both the photolyzed and the recombined states were collected. This technique ensured that any variations observed between the data from the five different time intervals could be attributed solely to differences in the state of photolysis of the samples as a function of time and not to experimental artifacts. With this method, we were able to construct an x-ray absorption spectrum for each time interval and the spectra thus obtained could be compared with confidence.

Because of a variety of interrelated experimental constraints such as the need for near-complete laser photolysis and prolonged x-ray exposures without sample deterioration and the necessity that the sample have a concentration compatible with both visible and x-ray absorption cross sections, a flow sample cell was constructed (*3*). The cell had a 1-mm path length through which the 1 m*M* Mb solution flowed. A flow speed was chosen so that the entire sample volume in the x-ray beam (1 mm by 12 mm) was renewed after several laser pulses. At the low sample concentrations that had to be used, a much better signal-to-noise ratio could be realized by collecting the fluorescent x-rays emitted during de-excitation of the iron atom rather than by measuring the absorption coefficient in a transmission geometry. Even when utilizing this fluoresence method of data collection and using appropriate manganese filters to minimize the background signal in our NaI detectors, we found that the largest source of noise throughout the entire spectrum was due to Compton scattered radiation from the solvent containing the Mb.

The deoxy Mb and MbCO solutions were prepared by standard techniques (*2*) from purified sperm whale skeletal muscle Mb (Sigma Corporation). Ultraviolet-visible absorption spectra were taken of all solutions to demonstrate the deoxy and CO character of the samples. In addition, ultraviolet-visible absorption measurements were also made before and after the x-ray experiments in order to ensure that no sample degradation had taken place.

Two static near-edge spectra recorded at CHESS of 10 m*M* deoxy Mb and MbCO are shown in Fig. 3. The prominent differences between the spectra are the more pronounced pre-edge feature ($E \approx 7.104$ keV) and a shift to higher energy of the iron edge in the Mb upon CO ligation. The pre-edge feature in MbCO is generally assigned to a 1*s*-3*d* bound transition, whereas the edge shift to higher energy is attributed to an alteration in the electronic charge distribution around the iron due to an iron-ligand interaction (*4*). Differences in both the pre-edge features and the iron edge position were observed as a function of time after photolysis in our time-resolved measurements. However, in this report we concentrate on the alterations in the edge position because of the greater signal-to-noise ratio in this region due to the greater production of fluorescent x-rays at and above the absorption edge.

The time-resolved spectra will be presented in terms of the five time intervals described above. Data collected during each of the first four intervals will be compared to data collected during the fifth and final reference interval. As in the static measurements of the deoxy and MbCO samples (see Fig. 3), the position of the x-ray absorption edge

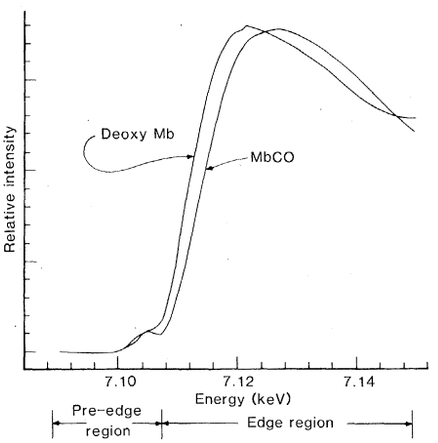

Fig. 3. Static spectra of 10 m*M* solutions of deoxy Mb and MbCO at *p*H 7.6 in 0.1*M* phosphate buffer and an excess of sodium dithionite. The major differences in the spectra are the pre-edge feature at ≈ 7.104 keV and the ≈ 3-eV shift to higher energy of the liganded MbCO as compared to the unliganded deoxy Mb.

in the 0- to 307-μsec time regime after photolysis (Fig. 2a) has a ≈ 3-eV shift to lower energies relative to that for MbCO; this result indicates that at this time a significant amount of the photolyzed CO has not yet recombined with the Mb. As can be seen from the magnitude of the edge shift in Fig. 2b, a sizable fraction of the CO has still not recombined with the Mb. The first indication of near complete recombination is seen in Fig. 2c (the 563- to 1203-μsec time interval). The data collected during the fourth and fifth time intervals are identical (Fig. 2d), demonstrating complete recombination.

Attempts have been made to obtain time-resolved x-ray absorption spectra in the temporal regime that we have considered. These experiments (*5*) were not successful, and it was suggested that there may be accelerator instabilities that precluded such time-resolved experiments with storage rings. Our data on the flash photolysis and recombination of MbCO demonstrate the feasibility of such time-resolved techniques. We believe that these data represent the first x-ray absorption spectroscopic observation made with the use of synchrotron radiation of dynamic charge and structural redistribution on a time scale of much less than a millisecond. The ability to perform such experiments on this time scale, which is consistent with many biological and chemical processes, bodes well for the general application of such atom-selective time-resolved techniques for the determination of oxidation state and structural alterations in the active sites of proteins and other chemical systems. We see no inherent reason why this scale cannot be decreased by several orders of magnitude.

The Mb absorption edge results we have obtained so far are consistent with data obtained by other techniques (*2*) at room temperature. Thus, in terms of the edge shift, we observed a deoxy conformation immediately (0 to 307 μsec) after the laser flash. Our initial data also suggest that the pre-edge bound state transition (1*s*-3*d*) may be changing its position by ≈ 5 eV on a time scale shorter than the MbCO recombination time inferred from the shift in the edge position. Future experiments should be aimed at accurately determining the energy position of the near-edge peak in order to allow detailed comparisons with calculations. We also plan to extend these investigations to lower temperatures and higher solvent viscosities. This work not only will be considerably less demanding experimentally because of the slower time scales involved but also should provide further information on the intermediate states in MbCO recombination. Additional extensions of the technique to the region beyond the edge, in which fine structure is observed on the x-ray absorption spectrum, will allow transient structural studies of the active site to be made by extended x-ray absorption fine-structure analysis. These experiments should eventually yield important time-resolved information on the chemistry and interactions of the active site atoms and also dynamic structural information with a resolution of better than 0.05 Å.

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6. We thank Vic Pollack for the design and construction of the computer interface. We thank B. Batterman for his encouragement during the course of this investigation. We would also like to thank Don Middendorf for help in data collection and the entire CHESS staff for their cooperation.

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A Common *onc* Gene Sequence Transduced by Avian Carcinoma Virus MH2 and by Murine Sarcoma Virus 3611

Abstract. *A common cellular sequence was independently transduced by avian carcinoma virus MH2 (v-*mht*) and murine sarcoma virus (MSV) 3611 (v-*raf*). Comparison of the nucleotide sequences of v-*mht *and v-*raf *revealed a region of homology that extends over 969 nucleotides. The homology between the corresponding amino acids was about 95 percent with only 19 of 323 amino acids being different. With this example, 5 of the 19 known different viral* onc *genes have been observed in viruses of different taxonomic groups. These data indicate that (i) the number of cellular proto-*onc *genes is limited because, like other viruses of different taxonomic groups, MH2 and MSV 3611 have transduced the same* onc *gene-specific sequences from different cell species and (ii) that specific deletion and linkage of the same proto-*onc *sequences to different viral vector elements affect the oncogenic potential of the resulting viruses. The difference in transformation capabilities of MH2 and MSV 3611 serves as an example.*

The transforming *onc* genes of retroviruses consist either of sequences derived entirely from cellular genes, termed proto-*onc* genes, or more often from sequences derived from cellular and essential retroviral genes (*1*). Thus, proto-*onc* genes have obvious oncogenic potential as progenitors of viral *onc* genes. However, despite the presence of proto-*onc* genes in all vertebrate cells and despite the presence of retroviruses in many animal species, transduction of proto-*onc* sequences by retroviruses is a rare event. The probable reason is that, since sequence relationships are often lacking between essential retroviral and proto-*onc* sequences, nonhomologous recombination is necessary to transduce proto-*onc* sequences. It is now believed that proto-*onc* genes also have oncogenic potential on their own as a result of mutations, rearrangements, or enhanced expression (*1–4*). The risk of transformation for a given cell by a proto-*onc* gene would, in each of these cases, depend on the number of its proto-*onc* genes.

It is difficult to estimate the number of proto-*onc* genes, because retroviruses with *onc* genes are rarely isolated from tumors. Nevertheless, the following arguments suggest that the number of proto-*onc* genes is limited. (i) Transduction of proto-*onc* sequences by retroviruses appears to depend on nonhomologous recombination, and therefore any cellular sequence can be a candidate for transduction. Since only about 19 different *onc* genes have been found in retroviruses to date, the number of proto-*onc* genes appears to be small. (ii) Sequences of the same or very closely related proto-*onc* genes have been found in indepen-