A versatile rapid-mixing and flow device for X-ray absorption spectroscopy

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A low-temperature rapid-mixing and flow system has been designed and implemented to monitor catalysis involving metal ions by X-ray absorption spectroscopy at the ID-18 beamline of the Advanced Photon Source, Argonne National Laboratory. The system will allow examination of biological metallo-intermediates at dilute metal ion concentrations by the detection of X-ray fluorescence. The instrument can be cooled to sub-zero temperatures, thus lengthening the life time of a reaction intermediate. A portable UV–visible spectrometer is integrated with the flow system to monitor the sample optically. The system can also be used as a continuous-flow device to minimize radiation-induced sample damage by reducing sample exposure to the X-ray beam. The integration of the stop-flow instrument with the synchrotron beamline and X-ray fluorescence detector systems makes it unique for time-resolved X-ray absorption studies of dilute biological reactions. The results of the initial testing of the system are presented.

Keywords: stop-flow/mixing device; time-resolved spectroscopy.

1. Introduction

Rapid mixing of two reactants is a widely used technique in spectroscopic investigations of the interaction between metalloenzymes and substrates in biology (Chance, 1974). The initiation of the reaction through rapid mixing in combination with techniques that can monitor the formation and breakdown of intermediates provides insight into the mechanism of catalysis. However, most fluorescent and visible absorption measurements of such reactions do not provide direct information on the structure of intermediates. In addition, many metalloenzymes of interest contain a catalytic zinc ion which is spectroscopically silent. Application of X-ray absorption fine structure (XAFS) to the rapid mixing of two reactants is a logical extension since the experiments can be performed in the solution state, and can provide both kinetic and structural information on the intermediates. It can also monitor zinc-catalyzed reactions directly since it is not dependent on a metal having an unfilled *d*-shell. Studies using a rapid-mixing device have been carried out previously at synchrotron sources on concentrated ($\sim$100 m*M*) samples using X-ray transmission detection (Inada *et al.*, 1997; Abdul Rahman *et al.*, 2002). However, the rapid-mixing technique has not been widely used at synchrotron beamlines for studying reaction intermediates of dilute ($\sim$1 m*M*) biological samples (Zhang *et al.*, 1995). The majority of biological XAFS measurements on such intermediates carried out to date have been restricted to those on freeze-quenched intermediates. The lack of data on intermediates in solution is partly due to the fact that XAFS measurements on dilute systems require a long time for data acquisition, with each data set (*i.e.* an energy scan) taking minutes to complete at most of the synchrotron beamlines. The long data-acquisition time coupled with the requirement for large sample volumes and high enzyme concentrations has largely precluded the use of rapid-mixing techniques to reactions of biochemical interest.

The advent of high-intensity and high-brightness third-generation synchrotron sources now permits the exploration of structure/function relationships at lower enzyme concentration and at faster data-acquisition times. Typically, the beam intensity at an insertion-device beamline, such as the ID-18 beamline at the APS, is about 10^{13} photons s^{-1} into a bandwidth of 1×10^{-4} (Fischetti *et al.*, 2003), which is more than a 100-fold increase over existing second-generation bend-magnet beamlines. In addition, the intense photon flux of an undulator beamline can be focused into a spot as small as 30 µm vertically and 150 µm horizontally, using advanced focusing optics. These advances allow for fast data acquisition on a very small sample volume, which are the prerequisites for time-resolved XAFS investigations. Herein we report the implementation of a rapid-mixing and flow device for examination of reaction intermediates by X-ray spectroscopy at beamline ID-18 of the Advanced Photon Source.

2. Design rationale

The basic design of the stop-flow device is adapted from our earlier work at beamline X9 of the NSLS at Brookhaven National Laboratory (Zhang *et al.*, 1995) and previous experience with the design of a low-temperature stopped-flow instrument for visible absorption and fluorescence studies (Hanahan & Auld, 1980). The use of separate motor drives for the two drive syringes provides an accurate and flexible sample delivery system. Two important aspects of the design of the instrument for XAFS studies is the capability of monitoring rapid reactions and determination of the state of the integrity of the reactants during the reaction. The stop flow is designed to operate at ambient to sub-zero temperatures. At low temperatures, the lifetime of a reaction intermediate can be lengthened allowing monitoring of the reaction over a longer time frame. Salt and other antifreeze compounds may be added to protein solutions (Auld, 1993) to allow operation below 273 K as has been demonstrated for the proteolytic enzyme carboxypeptidase A (Auld *et al.*, 1984). A second emphasis is the ability to monitor visible optical properties associated with a biological reactant during the XAFS experiments. Optical spectroscopy has the potential to provide additional information about the reaction kinetics and the nature of intermediates. In addition, continuous monitoring of the optical spectrum can also provide an indication of sample integrity under the conditions of the intense X-ray beam radiation.

One of the challenges for synchrotron-based biological research, especially at the third-generation sources, is X-ray-induced damage of protein samples. It is believed that the damage is caused by the generation of free radicals. Measurement of samples in frozen solution state can reduce the damage. Often, antifreeze compounds, such as glycerol, are added to the protein solution during low-temperature spectroscopy measurements to reduce the diffraction artifacts caused by ice crystals. However, the addition of antifreeze itself can accelerate the degradation of the samples (Penner-Hahn, 2002) and in some cases can inhibit an enzyme-catalyzed reaction. One simple solution for maintaining the sample integrity while performing measurements in the solution state is to flow the sample continuously in the X-ray beam. In this regard we have adapted our stop-flow system for time-resolved XAFS to be usable as a flow device. The combination of the flow and optical monitoring technique will be essential for obtaining data of the highest quality.

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3. Implementation of the mixing and flow device

The flow and mixing device is shown in Fig. 1(*a*). Two driving systems, mounted on top of the device (a third drive can be easily added), are connected to the drive syringes. The drive syringes are embedded in an aluminium support frame to provide structural stability and good thermal contact. The system is enclosed in a Plexiglass environmental chamber with mylar windows. Two sets of motors are interchangeable to deliver the sample volume as fast as 2 ml s^{-1} or as slow as 0.1 µl s^{-1} for a single syringe. Solenoid valves can be opened or closed within 15 ms. The synchronized motion of the valves and the drive syringes provide rapid delivery of samples at the initial acceleration phase of the motor drives.

The enzyme and substrate flow through separate Teflon tubes to a mixer mounted on top of the observation chamber (Fig. 1*b*). The amount of each sample delivered is under the control of a computer. The mixing and observation system is designed to minimize the total sample volume needed for a measurement. A Burger ball mixer is directly embedded on top of the entrance to the observation chamber that is constructed with a Teflon frame and mylar windows. This sample chamber has a volume of the mixer plus the observation chamber equal to 50 µl. The minimum mixing time is 12 ms for the present configuration. For an even smaller mixing volume, we have obtained a commercial turbulence mixer that is used in high-pressure mixing devices. The total volume required for one observation can be as small as 25 µl by connecting this mixer to a 1 mm-inside-diameter polyamide tubing as the observation chamber. In this case the mixing time is close to 6 ms when the sample syringes are driven at highest speed. When the instrument is used for continuously flowing the sample through the X-ray beam the concern is not the maximum speed but the slowest speed at which a non-turbulent flow is sustainable. We have observed such a smooth flow of sample at a speed of 0.1 µl s^{-1} up to 100 µl s^{-1} when used with 1 mm-diameter tubing.

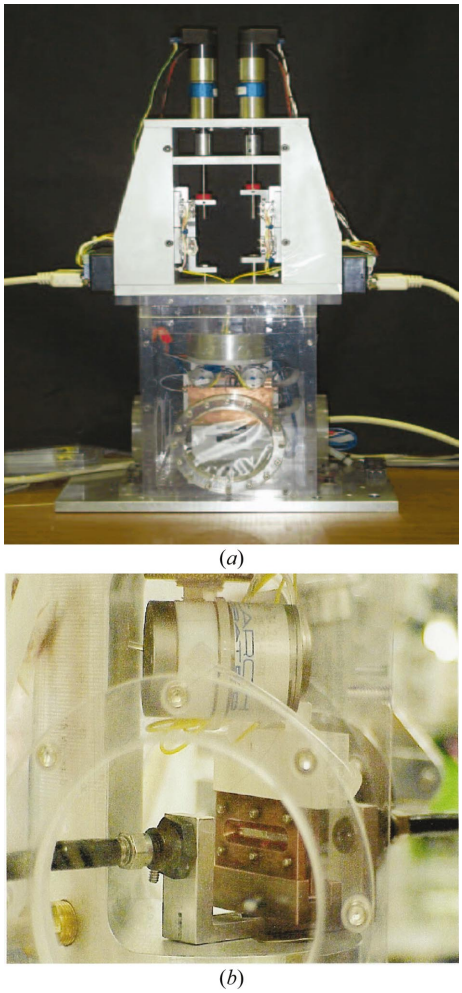

Figure 1
(*a*) Photograph of the stop-flow instrument in its environmental chamber. (*b*) Photograph of the stop-flow mixing jet and flow assembly mounted on the sample cell with the optical monitor in place.

Cold nitrogen gas can be supplied to the environmental chamber that hosts the stop-flow system (Fig. 1*a*). The temperature can be modulated by a Thermofoil heater mounted on the aluminium frame. In this manner the temperature on the massive aluminium support frame can be accurately controlled within about 0.5 K. Thus, the whole sample volume will be at the equilibrium temperature. This is desirable when either a high flow speed or frequent mixing is required. The temperature of fast reactions is controlled to within about 0.1 K. Alternatively, a thermoelectric cooler, which regulates the temperature of a small sample reservoir and the mixer, can be mounted on the aluminium frame and connected to the driving system. This arrangement is adopted when a small mixing volume is involved. The combined cooling system allows the sample temperature to be maintained at as low as 233 K during measurements.

The collection of optical spectra, so called 'on-line and at the spot', provides very valuable information about the nature of the sample in addition to X-ray spectroscopy information. An external light source is used which provides light in the 200 to 800 nm UV–vis range. The entering beam is transmitted through fiber optics to the sample. The transmitted light of the sample is collected by an optical lens and fed through another optical fiber to the spectrometer that disperses the light *via* a grating across a multi-element liner CCD array detector. A thin optical fiber (about 200 µm in diameter) and lenses are used to maintain a light spot on the sample similar to the dimension of the X-ray beam to ensure that both techniques examine the same sample volume of the reaction chamber. Mylar windows are used in the reaction cell when optical measurements are made.

The synchronization of stop-flow mixing and the data-acquisition system is a key for fast time-slice/kinetic experiments. Currently, the best time resolution achievable using the ID-18 Struck scaler (model SIS3801) is about 1 ms at 200 MHz count rate. We use a dedicated personal computer (PC) and a PC-based controller to control the flow system. The communication between the beamline control computer and the computer controlling the flow system is through I/O lines of the controller. The control program is written in the C++ programming language with a user-friendly graphical user interface. Three operation models are allowed: (i) stop-flow operation with multiple syringes; (ii) mixing/continuous flow with multiple syringes; and (iii) continuous flow with a single syringe. In each of the flow modes the sample volume, the flow rate and the delay time can be specified for each individual syringe. In the stop-flow mode the mixing of reactants can be repeated until the drive syringes are depleted of sample. When operating under a continuous-flow mode, the flow direction and the number of repeats can be specified. The program controls the opening and closing of the solenoid valves in synchrony with the driving system.

The program also controls the optical components for spectroscopic data collection. The optical data are fed into the computer through an A/D converter. Dark current and 100% transmission of the solution in the absence of sample are recorded and stored for each set of experimental conditions. Repeated measurements with ms resolution are carried out in synchrony with the driving system. X-ray

data collection at a single wavelength is performed with the same control software and a PC-based A/D board. In this case the only communication needed between the beamline computer and the stop-flow controller is the triggering of the beamline photon shutter in synchronization with the mixing operation. This is highly desirable when monitoring rapid reactions.

4. Results

The BioCAT beamline of ID-18 at the Advanced Photon Source, Argonne National Laboratory (Rosenbaum *et al.*, 2003; Fischetti *et al.*, 2003), was used for the initial tests of the stop flow. A double-crystal monochomater with Si(111) orientation was used, which focuses the beam horizontally down to 400–800 μm at the sample position using the sagitally focusing second crystal. The dimension change of the horizontal focus during a scan of a few hundred eV is negligible compared with the beam size due to the small convergence angle of the focused beam. A focus of 100 μm in the vertical direction is achieved through a cylindrically bent mirror, which is also used for harmonic rejection. The harmonic contents (third harmonic in particular) is largely eliminated by placing the angle between the mirror and the orientation of the beam just above the critical angle for the X-ray energy. The beamline was operated in a rapid-scan mode, *i.e.* data collection 'on-the-fly', which utilizes a Delta Tau PMAC controller (multi-axis motion) and Struck scaler (32 channel on-board memory arrays). The minimum collection time per data point is 1 ms, and a scan can be completed as fast as 1 s. The time for return of the monochromator to its initial energy is about 3–5 s for the experiments described herein. The beamline shutter can be closed during the return time period to reduce radiation damage.

The sample concentrations used for the tests are about 0.2 to 1 m*M*. The measurements are performed in the fluorescence mode. The detectors used for the data collection are a multilayer analyzer array detector (Zhang *et al.*, 1998; Zhang, 2003) or a fluorescence ionization chamber. The energy resolution of the multilayer detector at the zinc $K\alpha$ is typically 300–350 eV. When using a fluorescence ion chamber, an appropriate filter and solar slits are used to improve effective signal counts (Stern & Heald, 1983). The effective photon count rate is generally over 1×10^6 s^{-1}. When taking 5 s scans over a 100 eV region, the effective photon counts per data point is about 50 000.

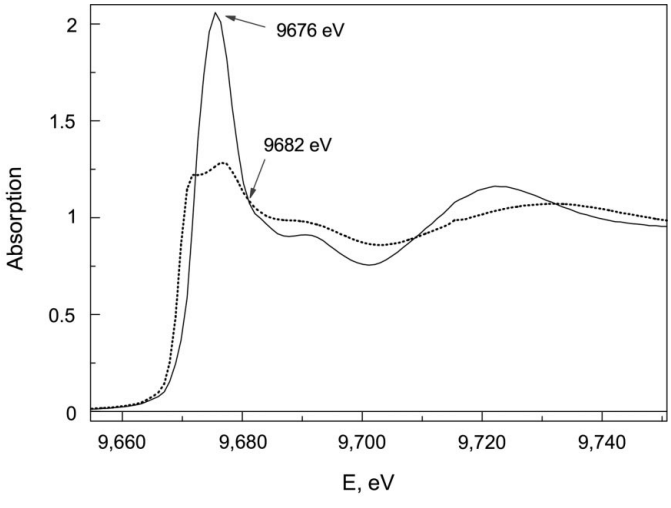

Figure 2
Near-edge spectra of $Zn(D\text{-}PEN)_2$ (dash) and ZnEDTA (solid) in 50 m*M* Hepes at pH 7.1 under a continuous-flow mode.

4.1. Continuous flow and mixing tests

XAFS spectra are obtained for the zinc complexes of D-pencillamine, D-PEN [$Zn(D\text{-}PEN)_2$], and ethylenediaminetetraacetic acid, EDTA (ZnEDTA). The data are collected in a continuous-flow mode of the stop-flow device in order to minimize possible radiation damage to the sample. The sample is continuously flowed in a 1 mm-diameter polyamide tubing in and out of the X-ray beam during the measurement. A flow rate of 10 μl s^{-1} reduced to 1 μl s^{-1} produced no time-dependent spectral changes. Fig. 2 shows the near-edge spectra of $Zn(D\text{-}PEN)_2$ compared with ZnEDTA at pH 7.1 with a flow rate of 1 μl s^{-1}. The spectra differ markedly, inverting in intensities at the photon energies 9676 and 9692 eV. These spectra provide information on the starting and endpoint for complete conversion of a $Zn(D\text{-}PEN)_2$ complex into a ZnEDTA complex.

Rapid XAFS spectra are collected by stop-flow mixing of $Zn(D\text{-}PEN)_2$ with EDTA at a mixing ratio of 1:1 for the concentration of zinc and EDTA at 293 K (Fig. 3*a*). The scan range was about 150 eV in the near-edge region with a repeat scan time of 10 s. Spectral changes occur at the energy regions predicted by the measurements

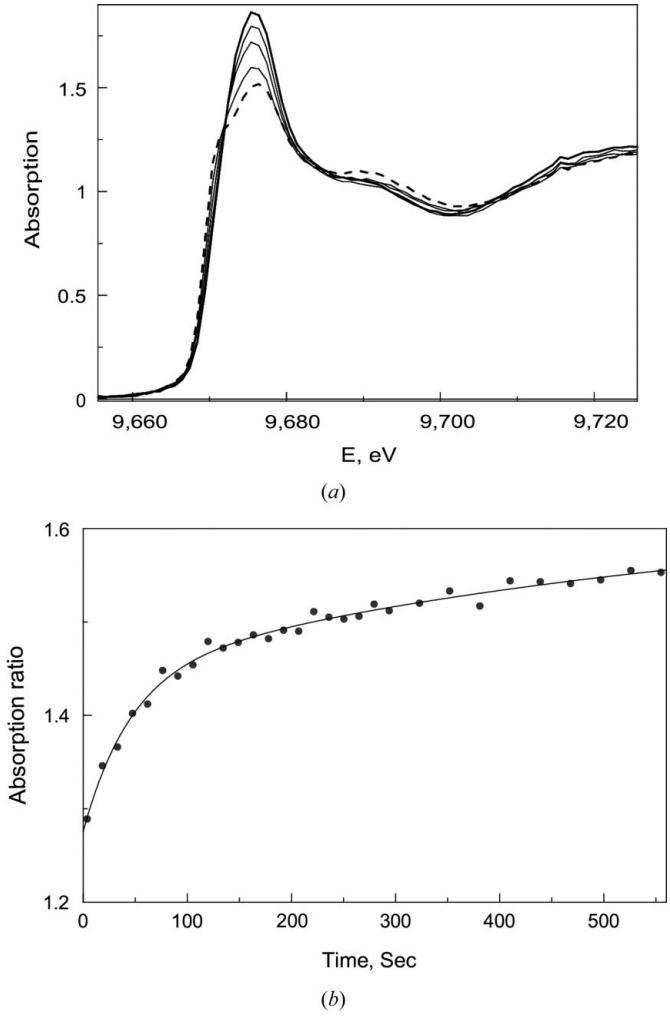

Figure 3
(*a*) A solution of 2.8 m*M* zinc in the presence of 7.1 m*M* D-PEN (ratio 1:2.5) in 50 m*M* Hepes buffer at pH 7.1 is mixed with 2.8 m*M* EDTA in the same buffer at 298 K. XAFS scans of the reaction are shown for 4 s (dashed line), 33, 106, 281 and 573 s after mixing. (*b*) The time course of the absorption ratio of the XAFS intensities at 9676 and 9682 eV (the latter is one of the isobestic points of Fig. 2), reflecting the replacement of D-PEN by EDTA in the D-PEN complex of zinc.

on the stable complexes (Fig. 2) confirming the conversion of Zn(D-PEN)$_2$ into ZnEDTA (Fig. 2). The rate of the reaction as measured by the absorption intensity ratio at 9676 and 9682 eV (one of the isobestic points of the spectra) is shown in Fig. 3(*b*). This time course may reflect the formation of an intermediate species that is converted into the final ZnEDTA complex. Further studies of the effect of the concentration of EDTA and D-PEN on the time course of the reaction should aid in dissecting the details of the reaction mechanism. Zinc ligand exchange processes are likely to occur in biological systems all the time but their mechanisms are not easily determined owing to the spectroscopically silent nature of zinc. XAFS is the only spectral technique that can directly detect the presence of such an intermediate in the conversion of one zinc complex into another.

4.2. Optical monitoring tests

A different zinc chelator exchange reaction is used to test the optical monitoring system of the stop flow. The visible absorption change in the chelator, 4-(2-pyridylazo)resorcinol (PAR), when it binds a metal is used to test the optical system (Chong & Auld, 2000). Rapid XAFS spectra are collected by mixing of 2 m*M* Zn(PAR)$_2$ with 100 m*M* D-PEN at a volume ratio of 1:3, resulting in a final zinc concentration of 0.5 m*M* and a D-PEN concentration of 75 m*M*. In this case, zinc transfer from the complex Zn(PAR)$_2$ to D-PEN can be monitored by the decrease in absorbance at 508 nm (Chong & Auld, 2000). Using a 200 µm optical fiber, light was delivered to the sample holder which is made with mylar windows. Once mixing is initiated, the stop-flow controller triggers both the XAFS and the optical measurements. Fig. 4 shows rapid-XAFS near-edge spectra with a scan time of 5 s (the return time of the monochromator to the starting energy is about 4.5 s) collected at 2, 12, 22, 32, 52, 72 and 112 s after the initiation of the reaction. Optical spectra collected in the same time frame are shown in Fig. 5. The time-dependent spectral changes can be fitted with a single exponential decay by following the change in signal at the energy 9668 eV for the XAFS spectra and the change in absorbance at 508 nm for the optical spectra, which yield first-order rate constants of 1.9 min^{-1} and 1.6 min^{-1}, respectively.

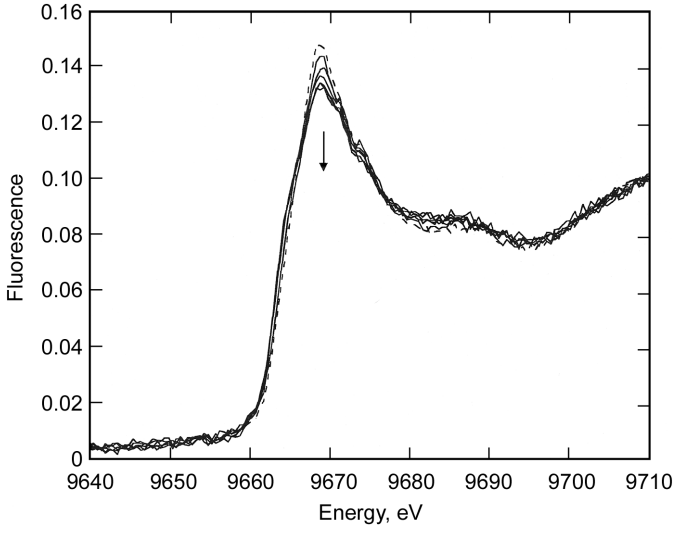

Figure 4
A solution of 2 m*M* zinc and 4 m*M* PAR in 50 m*M* Hepes, pH 7.4, is mixed with 100 m*M* D-PEN in the same buffer at a mixing ratio of 1:3. XAFS scans shown for the reaction at 2 s (dashed line), 12, 22, 32, 52, 72 and 112 s.

4.3. Rapid mixing and kinetic measurements

The hydrolysis of Co(III)ethylenediaminedichloride complexes are readily monitored by visible absorption spectroscopy (Pearson *et al.*, 1955). X-ray spectroscopy changes are also expected since water displaces a chloride ion in the reaction. We have synthesized the *cis* and *trans* complexes. The rate of the reaction can be controlled by the pH of the buffer chosen as well as by the temperature. Rapid XAFS spectra are measured immediately after mixing of *cis*-[Co(III)en$_2$]Cl$_2$ in 2 m*M* HNO_3 with 100 m*M* TAPS buffer at pH 8.6 and 295 K. A series of near-edge spectra are collected with a scan time of 2 s and an additional 4 s for the monochromator to return to the initial conditions. Fig. 6 shows the 1st (dashed line), 3rd, 5th, 10th and 20th scans. As can be seen, changes occur on the white line of the spectra (7728 eV), with an isobestic at 7734 eV. Changes also occur beyond 7734 eV but these are smaller than those at the edge. Time-slice

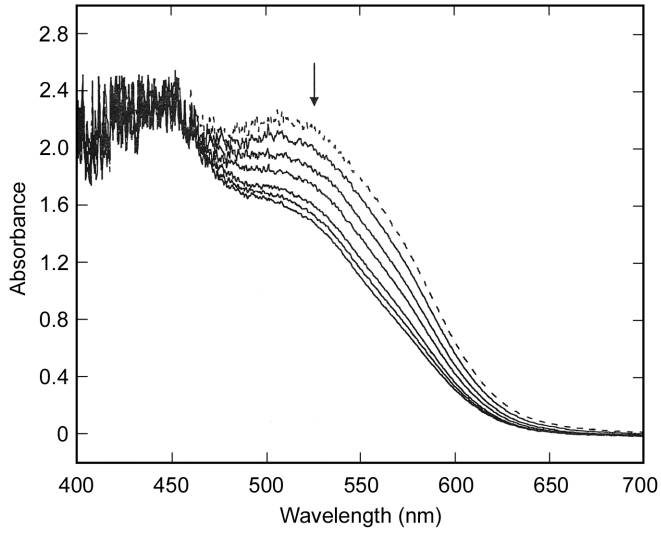

Figure 5
The reaction of the Zn(PAR)$_2$ complex with D-PEN is followed through the change in the visible absorption of the Zn(PAR)$_2$ complex by an on-line optical monitoring system. Visible absorption spectra are shown for the reaction at 2 s (dashed line), 12, 32, 52, 72 and 112 s.

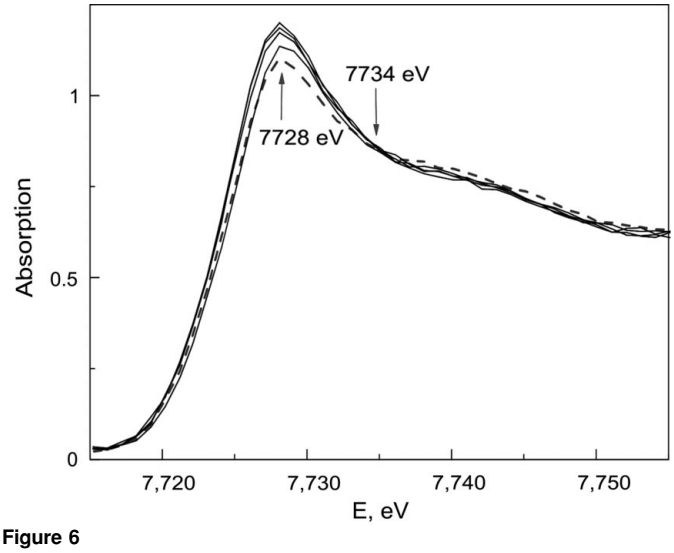

Figure 6
Quick-XAFS spectra for the reaction of 10 m*M* *cis*-[Co(III)en$_2$]Cl$_2$ in 2 m*M* HNO_3 mixed with a solution of 0.2 *M* TAPS, pH 8.6, at 295 K. The spectra are recorded at 1, 13, 25, 55 and 115 s after mixing.

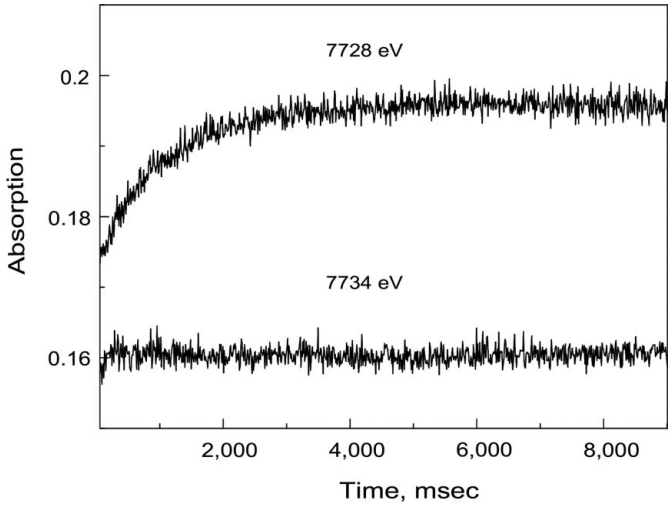

Figure 7
Time-slice XAFS for the reaction of *cis*-$[Co(III)en_2]Cl_2$ and base at the maximum intensity change, 7728 eV, and at the isobestic energy, 7734 eV. The time resolution is 10 ms. Other conditions are given in the caption to Fig. 6.

measurements at a single energy are performed on *cis*-$[Co(III)en_2]Cl_2$ in 2 m*M* HNO_3 mixed with 100 m*M* TAPS buffer at pH 8.6 and 295 K (Fig. 7). In this case the stop-flow control computer triggers the beamline control computer while initiating the mixing after a slight time delay. Data collection is through the Struck scalar, which has 1024 buffers to store data with a collection time of 10 ms for each sliced time period. Energies at 7728 and 7734 eV are selected for measurement based on the rapid-scan XAFS observations (Fig. 6). The signal change at the energy 7728 eV increases in an exponential manner dependent on a rate constant of 0.943 s^{-1} ($t_{1/2}$ = 0.74 s). As expected, no change in intensity occurs at the isobestic energy of 7734 eV. The combination of lower temperatures and monitoring at a single energy should allow the interconversion of ES (enzyme/substrate) complexes, that occur on the millisecond to second time scale, to be followed.

5. Discussion

Stop-flow devices have become more available from commercial venders. These devices are largely oriented towards optical spectroscopy techniques, which may not be suitable for XAFS applications. In order to accommodate a large number of experimental protocols (*e.g.* X-ray fluorescence detection, low temperature, optical monitoring *etc.*), commercial devices will need to be redesigned and integrated with beamline hardware and software. The design of the stop flow and the control software described herein produces a very flexible and user-friendly device that can benefit many users for experiments of enzyme structure/function studies. It is also useful in reducing radiation damage in routine XAS measurements, thus circumventing the problem of obtaining reliable data in the solution state at high-intensity beamlines for some sample systems. The apparatus has been used for a time-slicing experiment for the detection of a coordination change in the catalytic zinc ion in a bacterial alcohol dehydrogenase (Kleifeld *et al.*, 2003). The present configuration of the instrumental system is flexible and is able to accommodate a wide range of experimental protocols owing to its sample delivery and other options. Thus with conventional pressured piston drives it is not possible to flow the solution smoothly at low speeds and for extended time periods, which is essential for reducing sample damage during the measurement of relatively slow reactions and static systems in solution. The individual motor drives allow for mixing reactants with multiple volume ratios providing a wide range of sample concentrations and ratios of reactant concentrations.

The capability of operating at sub-zero temperatures will lengthen the lifetime of reaction intermediates. The on-line optical monitoring can be used to provide additional information for the reaction when chromophoric probes are part of the chemical reaction, as well as monitoring sample damage. Thus it can provide both XAFS and optical data in the same data set for metals that have unfilled *d*-shells. Time-resolved spectra obtained during a reaction can be examined when the instrument is operated in the stop-flow mode. Very rapid mixing can be accessed using the device with an optimized mixer and sample cell design. With an integrated data-collection system, the device can examine reaction intermediates in the order of milliseconds. Importantly, the design of the device can take full advantage of the small and intense beam provided by the third-generation sources for time-resolved as well as routine spectroscopic experiments.

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