Fast mixing device for time-resolved synchrotron x-ray scattering studies of radiation sensitive proteins

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A fast mixing device apparatus has been constructed to study the kinetic structural reactions of radiation sensitive proteins by time-resolved small angle x-ray scattering. The sample cell has a volume of 0.75 ml and is translated through the synchrotron x-ray beam as the experiment progresses. The dead time of the device is 50 ms, and it is able to mix viscous liquids with ratios ranging from 1:1 to 1:250 with a measured precision of 0.1 μl. The device uses two motor driven syringes that are individually controlled. The whole instrument is thermostated and is remotely controlled. Cleaning of the cell is carried out remotely, removing the need to enter the x-ray hutch between each measurement. Commissioning of the apparatus was carried out by following the assembly of the radiation sensitive protein tubulin into microtubules induced by two different chemical jumps. © *1998 American Institute of Physics.* [S0034-6748(98)02201-1]

I. INTRODUCTION

Conformational changes and assembly play an essential role in the modulation of biological activity in proteins and nucleic acids. The high x-ray intensities generated by modern synchrotron radiation sources has enabled the study of a wide range of structural changes in systems as a function of time, in the fields of biology, chemistry, and material science. A variety of techniques available on such sources for these studies include small and wide angle scattering, dispersive EXAFS, QEXAFS, protein crystallography, and powder diffraction.[1–3] With the advent of third generation synchrotron sources and improvement in detection systems, time-resolved measurements on samples producing very weak signals can now be carried out. However, the techniques necessary to induce structural changes from an initial state to a final state are not widely available so that the full benefit of the time resolved x-ray scattering technology cannot be obtained.

Without a fast mixing device, the study at synchrotron radiation laboratories of the dynamics of protein interactions initiated by a chemical change of its environment is dependent on a number of parameters: the dead time associated with the filling of the cell, leaving the experimental x-ray hutch and the starting of the data acquisition system. The complete process can take about 3 min, during which time the sample is already undergoing its change and thus the initial phase of the reaction is missed, in addition this has the inherent risks of chemical manipulations inside a common working place. After every experiment the cell should be taken out of the holder and cleaned, with the result of a loss of expensive beam time.

Some stopped flow devices have already been designed for application in the synchrotron radiation environment.[4,5] Unfortunately, those devices cannot be used with radiation sensitive proteins. Tubulin is a typical radiation sensitive protein, tubulin contained in a cell like one of the devices built by Moody *et al.* and Tsuruta *et al.*[4,5] is permanently damaged after a few seconds of exposition to the x-ray beam at the Daresbury Laboratory.[6] Since the time needed for the assembly of tubulin to take place is of the order of tenths of seconds[1] a scanning sample cell needs to be used[6] for sensitive systems. Therefore, the previously constructed devices cannot be used in the kinetic studies of radiation sensitive systems like tubulin, since the large volumes needed to fill such a cell preclude the use of the conventional stopped flow devices (150–200 μl for Moody *et al.*[4] and 180 μl for Tsuruta *et al.*[5]).

II. NEW FAST MIXING APPARATUS FOR THE MEASURING OF KINETICS IN RADIATION SENSITIVE SAMPLES

To overcome the problem of following structural changes over many tens of seconds of radiation sensitive proteins, we have designed and constructed a chemical jump device (Fig. 1) capable of mixing two viscous solutions and to load them in a specially designed scanning x-ray cell (Fig. 2). The device is computer controlled and linked to the station computers. It is able to wash the measuring cell between two measurements making unnecessary to enter the measuring hutch as long as there is sufficient sample present in the syringes.

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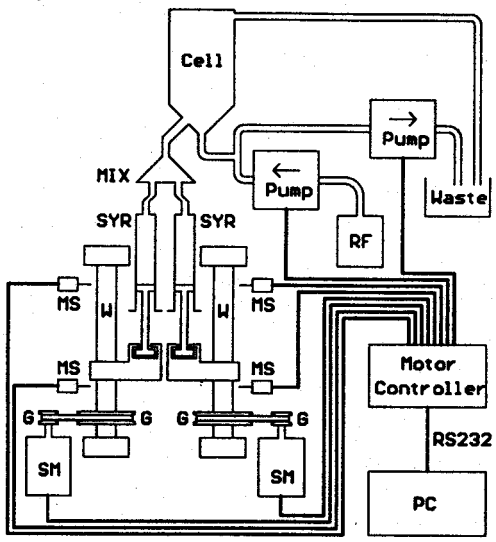

FIG. 1. Fast mixing device for time-resolved synchrotron x-ray scattering studies of radiation sensitive proteins. PC, personal computer that controls the device; SM, stepper motors; G, gearwheel; MS, microswitch; W, worm wheel; SYR syringes; MIX, mixing chamber; RF, leaning solution reservoir. All parts are mounted on an aluminum block and the system is thermostated using a water bath.

Figure 1 shows the newly constructed apparatus. Two solutions A and B are stored in Hamilton glass syringes which can be of different volume. Syringe sizes can vary between 250 μl and 2.5 ml. These syringes are moved by stepper motors able to pump up to 1.5 ml/s of each of the syringes. The liquid moves towards the mixing chamber which lies immediately before the cell. The complete system is controlled via an RS232 interface by an IBM compatible computer, which is connected to the data acquisition system of the synchrotron x-ray station. The system includes a peristaltic pump capable of cleaning the cell if the solution conditions are going to be changed. This avoids the dismount of the cell between two measurements to clean it, increasing the amount of beam time available for effective measurements.

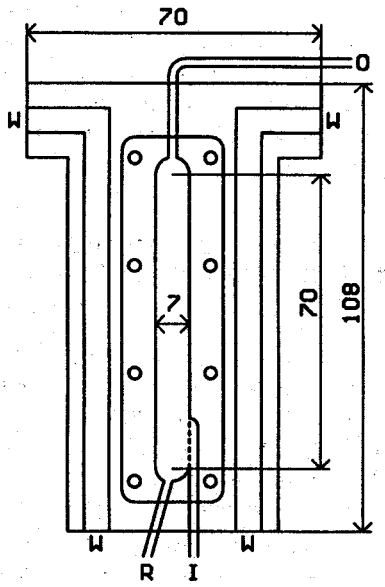

FIG. 2. Cell of the fast mixing device.

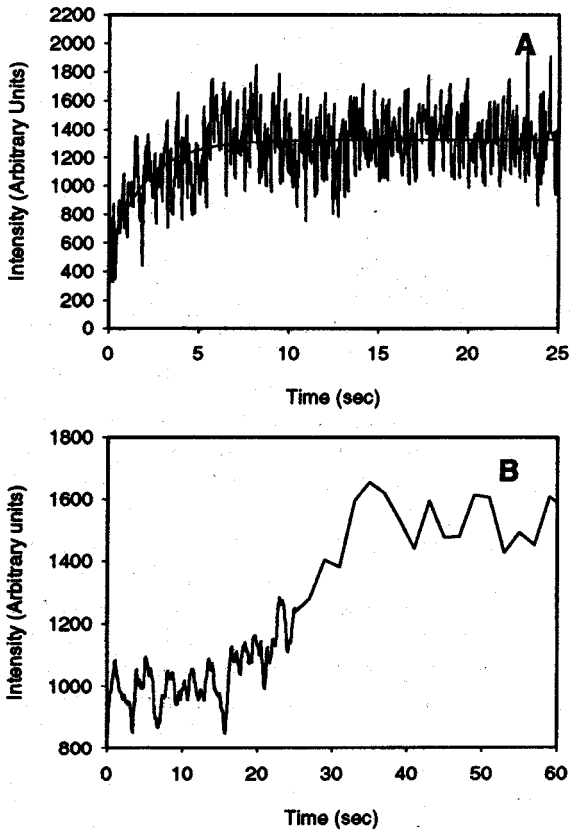

FIG. 3. (A) Time diagram of assembly of 50 μM tubulin in a 10 mM phosphate, 1 mM ethylenediaminetetraaceticacid, 1 mM 5′-guanosine triphosphate, 50 μM taxol buffer at pH 6.7 and 37 °C. The $MgCl_2$ concentration of the tubulin solution has been raised from 0 to 4 mM causing the assembly. The time resolution was 25 ms during the total run. The line is the fitting curve to a single exponential with an apparent rate constant of 0.5 s^{-1}. (B) Time diagram of assembly of 50 μM tubulin in a 10 mM phosphate, 1 mM ethylenediaminetetraaceticacid, 1 mM 5′-guanosine triphosphate, 4 mM $MgCl_2$ buffer at pH 6.7 and 37 °C. The taxol concentration of the tubulin solution has been raised from 0 to 50 μM causing the assembly. The time resolution was 0.1 s during the first 25 s of the run and 2 s from second 25 to second 60.

High power stepper motors (power 100 W, max. torque 110 N/cm) are employed to drive the syringes rather than a pneumatic system so that there is sufficient power to have a large range of mixing conditions for highly concentrated protein samples (up to 100 mg/ml) in viscous solvents. This system has been satisfactorily tested with bovine serum albumine samples contained in 60% glycerol. This system has the additional advantage of allowing mixing ranges rating from 1:1 to 1:250.

The use of motor driven syringes instead of a pneumatic system has the disadvantage of having a lower flow rate and thus a longer dead time. However, the high pressures produced by pneumatic devices (around 7 bar) would preclude the use of thin mica sheets (20–40 μm) as windows of the cell, and force the use of quartz windows like the ones used

TABLE I. Characteristics of the fast mixing device.

Sample volume	from 0 to 6000 μl regulated in 1 μl steps
Mixing ratio	from 1.0:1.0 to 1.0:250.0
Precision of the ratio	0.1 μl using 250 μl syringes
Dead time	50 ms with a 750 μl sample volume using 2.5 ml syringes
Temperature range	from -20 to 100 °C

in Refs. 4 and 5. The quartz windows have a much larger x-ray absorption than the thin sheets of mica we have used therefore, the resulting scattering signal would be much weaker. This fact increases the time needed to acquire a decent signal by at least a factor of ten, which results in a poor time resolution [the 25 ms of Fig. 3(a) can be compared with the 1 s of Ref. 4], that ruins all benefits obtained from the smaller dead time.

The scanning cell usually employed for radiation sensitive samples[6] has been modified to allow the use of the fast mixing device. The cell is made out of brass and is gold coated, the windows of the cell are of thin mica sheets (20–40 μm) to reduce x-ray absorption. In order to optimize the absorption to exposed volume ratio the path length of the cell was set to 1 mm. The total volume of the cell is approximately 0.8 ml, although the volume irradiated by the x-ray beam at any given moment is only ~ 5 μl. The cell is fitted to a stepper motor, and the elongated window allows the cell to be translated vertically with respect to the beam during the course of an experiment. Hollow contours are on the side of the cell so that its temperature is thermostated.

The schematics of the cell is shown in Fig. 2. The modifications were done as follows, first the inlet of the measuring solution (A) has been divided into two parallel inlets at both sides of the scanning cell. This has been done to avoid the formation of air bubbles in the filling process, since the original inlets used to trap air bubbles due to a fountain effect, i.e., the solution filled only the central part of the cell. This problem precluded the reducing of the dead time in the first prototypes of the cell. Second an additional bottom inlet (B) has been introduced to allow a cleaning solution to be introduced into the cell allowing a peristaltic pump to clean the cell between two experimental conditions. Third, an outlet (C) has been constructed to allow the used solutions to be discarded.

The precision of the system and the efficiency of the mixing has been checked spectroscopically using a stock solution bovine serum albumine (Sigma). The precision was found to be better than 0.1 μl when 250 μl syringes were used. The mixing was found to be good even at the 1:250 mixing ratio. The dead time was estimated from the dead volumes of the system and found to be around 25 ms at the maximum speed of the larger syringes (2.5 ml). The test experiments showed that the value should be between 25 and 50 ms, so the last value was taken as practical dead time.

The complete system is thermostated with a water circulating bath that allows the use of temperatures ranging from -20 to 100 °C depending on the fluid employed. If the sample is temperature sensitive, the syringes and the cell can be thermostated at different temperatures in order to cooldown the syringes during the measurement of a sample at a higher one.

The total dimensions of the apparatus (excluding the control devices and the cell) are $40\times30\times15$ cm. The total weight is 10 kg.

The characteristics of the apparatus allow its use in any other technique (without any need to modify the device for its use with stopped flow techniques) in which a relatively large (~ 1 ml) volume is needed to fill a measuring cell therefore, making kinetic studies accessible to conventional apparatus. The device is especially useful in the cells that are not easily accessible in the measuring position since the tubes that connect the syringes with the mixing chamber can be very easily extended and thermostated. The device has been successfully tested in filling a measuring cell placed into a superconductor magnet where it is almost unaccessible.

III. X-RAY SCATTERING STUDY IN THE ASSEMBLY OF TUBULIN INDUCED BY CHEMICAL JUMP

The self-association reaction of purified tubulin to form microtubules can be induced by a temperature jump from 4 to 37 °C. This assembly process has been studied previously,[1] and has provided a large insight in the way the microtubules are formed. Nevertheless, the function of microtubules in the cell is not regulated by changes in temperature. Their function is mainly modulated by the binding of nucleotide (5′ guanosine triphosphate),[7] as well as by the concentrations of the Mg^{+2} and other ions,[8] the binding of those ligands should imply conformational changes that regulate the function of the protein. Its function can also be altered by the use of antimitotic drugs that bind specifically to tubulin, like colchicine[9] or taxol.[10]

The purified tubulin solutions were prepared as described in Ref. 1. The time resolved x-ray scattering measurements to 3 nm resolution were made at station 2.1 of the Daresbury Laboratory Synchrotron Radiation Source, and data were processed as previously described.[11–13]

We have successfully followed the kinetics of assembly induced by a chemical jump in concentration of Mg^{+2} and taxol using this apparatus. The results are displayed in Figs. 3(a) and 3(b). These graphs show the change in the intensity of the central scattering of the tubulin solution (integrated from 0.011 to 0.031 nm^{-1}) versus time. The increase in central scatter reflects the assembly of tubulin into microtubules. It is clearly seen that the rate of assembly to microtubules is different, indicating that the method of assembly is not the same.

The device is now operating at the Daresbury Laboratory, with the help of this device the study of structural

changes at radiation sensitive samples will become possible at conditions much more realistic than before.

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[1] J. F. Díaz, J. M. Andreu, G. Diakun, E. Towns-Andrews, and J. Bordas, Biophys. J. **70**, 2408 (1996).
[2] B. R. Dobson, S. S. Hasnain, M. Neu, C. A. Ramsdale, and L. M. Murphy, J. Appl. Phys. **32**, 192 (1993).
[3] J. Wong, M. Fröba, and R. Frahm, Physica B **208**, 249 (1995).
[4] M. F. Moody, P. Vachette, A. M. Foote, A. Tardieu, M. H. J. Koch, and J. Bordas, Proc. Natl. Acad. Sci. USA **77**, 4040 (1980).
[5] H. Tsuruta, T. Nagamura, K. Kimura, Y. Igarashi, A. Kajita, Z. X. Wang, K. Wakabayashi, Y. Amemiya, and H. Kihara, Rev. Sci. Instrum. **60**, 2356 (1989).
[6] J. M. Andreu, J. García del Ancos, D. Starling, J. L. Hodgkinson, and J. Bordas, Biochemistry **28**, 4038 (1989).
[7] J. J. Correia, L. T. Baty, and R. C. Williams, J. Biol. Chem. **262**, 17278 (1987).
[8] J. C. Lee and S. N. Timasheff, Biochemistry **16**, 1754 (1977).
[9] R. C. Weisenberg, G. G. Borisy, and E. Taylor, Biochemistry **7**, 4466 (1968).
[10] J. F. Díaz and J. M. Andreu, Biochemistry **32**, 2747 (1993).
[11] J. M. Andreu, J. Bordas, J. F. Díaz, J. Garcia de Ancos, R. Gil, F. J. Medrano, E. Nogales, E. Pantos, and E. Towns-Andrews, J. Mol. Biol. **226**, 169 (1992).
[12] J. M. Andreu, J. F. Díaz, R. Gil, J. M. de Pereda, M. García, V. Peyrot, C. Briand, E. Towns-Andrews, and J. Bordas, J. Biol. Chem. **269**, 31785 (1994).
[13] J. F. Díaz, E. Pantos, J. Bordas, and J. M. Andreu, J. Mol. Biol. **238**, 214 (1994).