

# Stopped-Flow Apparatus for X-ray Scattering and XAFS

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A stopped-flow apparatus is described for X-ray scattering and X-ray absorption studies. It can be successfully operated over a wide temperature range down to 253 K with highly viscous solutions (20 cP). The characteristics of the stopped-flow apparatus and its application to studies of biological reactions are reviewed.

**Keywords:** stopped flow; X-ray scattering; protein-folding studies; X-ray absorption.

## 1. Introduction

It is essential to be able to perform time-resolved studies on biomacromolecules. Kinetic studies with several probes such as optical absorption, fluorescence and circular dichroism have contributed to the elucidation of the structure and function of biomolecules (Chiba, Ikai, Kawamura-Konishi & Kihara, 1994; Kawamura-Konishi, Kihara & Suzuki, 1988), but lack of direct conformational information leads to ambiguities in the interpretation of the results. Instead, direct probes indicating structural information are necessary in the study of biomolecules, and X-ray scattering, X-ray diffraction and X-ray absorption studies are of particular interest. In this paper, we describe stopped-flow and rapid-quenching apparatus for X-ray scattering and X-ray absorption. These time-resolved methods reveal many dynamical features of protein function and structure.

The first stopped-flow apparatus for X-ray scattering was reported by Moody *et al.* (1980), who used a Gibson–Durrum mixer. Subsequently, we have reported two versions of a stopped-flow apparatus for X-ray scattering (Nagamura, Kurita, Tokikura & Kihara, 1985; Tsuruta *et al.*, 1989). In this article, we review the characteristics of the previous two versions of stopped-flow apparatus, as well as those of two new versions.

## 2. Stopped-flow apparatus

Stopped-flow X-ray scattering (SFXS) methods have been applied to the elucidation of protein folding (Eliezer *et al.*, 1993; Semisotnov, Kihara *et al.*, 1994), subunit reorientation such as allosteric transitions (Kihara *et al.*, 1987; Tsuruta *et al.*, 1990, 1994), conformational change (Arakawa *et al.*, 1994), and subunit association and dissociation (Moody *et al.*, 1980; Fowler *et al.*, 1983; Inoko, Kihara & Koch, 1983; Kihara *et al.*, 1987; Wang *et al.*, 1989; Sato *et al.*, 1994). In particular, it is useful to obtain X-ray scattering patterns of intermediate or transient states in the kinetic pathway. To undertake such analyses, it is necessary to obtain sufficiently stable scattering patterns in short time

periods. Thus, synchrotron radiation is essential to time-resolved X-ray scattering.

To use as many photons as possible, large windows are necessary for collecting all the photons at the sample position. The beam size is usually  $1.5 \times 3 \text{ mm}^2$  at the Photon Factory, and hence windows larger than this size are required. The requirements for a stopped-flow apparatus can be summarized as follows. (1) The window should be strong, transparent and large (*ca*  $3 \times 4 \text{ mm}^2$ ), as described above. We have used quartz windows of  $50 \mu\text{m}$  thickness. Recently, we have employed sapphire of the same thickness, because sapphire is more stable and transparent than quartz, and also adheres to ceramics (the support material of the window) faster. (2) The stopped-flow apparatus should be capable of operation at subzero as well as ambient temperatures. The solvent is so viscous at subzero temperatures that mixing efficiency should be high at high viscosity. (3) In protein-folding studies, the unfolded protein is usually dissolved in several moles of urea or guanidine hydrochloride. The urea or guanidine hydrochloride should be diluted to one sixth or so when the protein becomes refolded. This necessitates a special mixer with which 1:6 mixing (one volume of a solution is mixed with six volumes of a second solution) is available. (4) The proteins used in the experiments are concentrated, so the volume used for each experiment should be as small as possible. At present, the syringe is capable of delivering a  $100 \mu\text{l}$  'shot' of protein. With this type of stopped-flow apparatus, we are able to deliver 100 shots without needing to refill the reservoirs.

### 2.1. Version 1

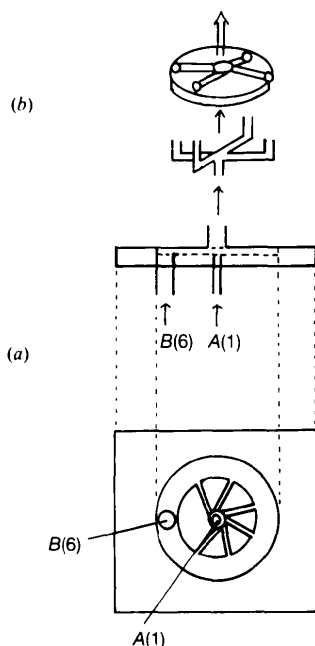
To make the window large, we developed a slit-type mixer, with which we were able to mix two solutions along a line at the same time (Nagamura *et al.*, 1985). This has been successfully applied to many biological reactions. However, the quantity of scattered photons was not sufficient to follow enzyme reactions in physiologically important time resolution, *i.e.*  $10^{-4}$ – $10^{-3}$  s. As the next step, we tried to slow down the reactions by lowering the temperature to subzero temperatures.

## 2.2. Version 2

The second version was constructed to make operations possible at subzero temperatures and in highly viscous solutions (Tsuruta *et al.*, 1989). Reservoirs and mixing blocks are thermo-regulated by a Peltier device. The apparatus could be operated at 253 K. The mixing efficiency was improved by employing two-step mixing with two-jet and four-jet mixers; two solutions were mixed with a two-jet mixer, then the resultant mixed solution was divided into four, and the divided solutions were mixed again with a four-jet mixer. The volume ratio of the two mixed solutions could be changed from 1:1 to 1:3.5, depending on the experimental requirements.

## 2.3. Versions 3 and 4

We have constructed two new stopped-flow apparatus. The main improvements are: (1) For 1:6 mixing, we used a new mixing block which ensures thorough mixing (Fig. 1). (2) We employed a ruby-ball autoswitching system instead of the rotating valve system used in versions 1 and 2 (Figs. 2 and 3). When the two solutions are drawn from the reservoirs to the pistons, the channels between the pistons and the mixing block are closed by a ruby-titanium spring, and when the solutions are forced out of the pistons, the channels between the pistons and the reservoirs are closed (Fig. 3). With this system, we do not need rotating valve parts,



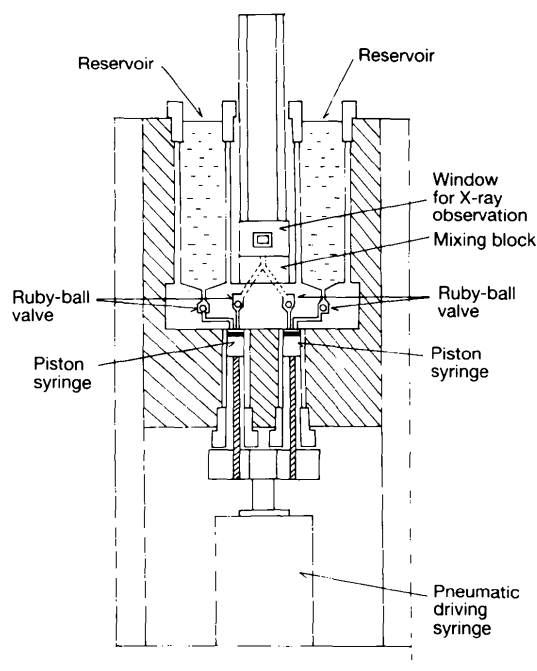
**Figure 1**

A schematic diagram of the mixing block. It consists of two parts, a 1:6 mixer and a four-jet mixer. (a) The 1:6 mixer. Solution A (one volume) flows through the central hole and solution B (six volumes) flows through the larger hole in the peripheral circular channel. Solution B then flows into the central mixing area through six narrow channels (0.5 mm diameter each) and is mixed with solution A. The mixed solution is forced upwards, divided again into four equal flows, and mixed with a four-jet mixer. (b) The four-jet mixer, the same type as used in previous studies (Tsuruta *et al.*, 1989).

which saves the time of rotation, and prevents mechanical problems accompanying the rotating mechanics. This is particularly useful in applications requiring multi-shots of about 100 shots. One problem with the ruby-ball system is that it may be affected by contaminated precipitates. We have had such a problem once. Care is therefore needed to keep the sample solutions dust-free, and to avoid the production of precipitates in the reaction. (3) Version 4 has large reservoirs (12 ml each). This ensures more than 100 shots without refilling the reservoirs. This is particularly useful for protein-folding studies.

Version 3 was constructed for general use, and version 4 was specially constructed for the study of protein folding. Their main parameters are summarized in Table 1.

Stopped-flow apparatus is installed at beamline BL-15A1 of the Photon Factory of National Institute for High Energy Physics (Tsukuba, Japan). Vertical focusing mirrors and a single bent-crystal monochromator (Amemiya *et al.*, 1983) give a monochromatized beam of wavelength 1.50 Å, with approximately  $10^{11}$  photons  $s^{-1}$  when the positron storage ring is operated at 2.5 GeV and 160–320 mA. With these experimental conditions, if good statistics are required for intermediate scattering patterns, 100 ms is the upper limit of the time resolution, although this depends on many factors such as protein concentration,



**Figure 2**

Stopped-flow apparatus for X-ray scattering. Solutions A and B are first put into their respective reservoirs. Then the solutions are drawn from the reservoirs by a downwards movement of the piston syringes. During this stage, the ruby-ball valves between the reservoirs and the pistons are open, and those between the pistons and the mixing block are closed. The pistons are then raised, causing the open ruby-ball valves to close and *vice versa*. The solutions are thoroughly mixed in the mixing block, and then forced upwards, passing through the window area. All parts are thermo-regulated.

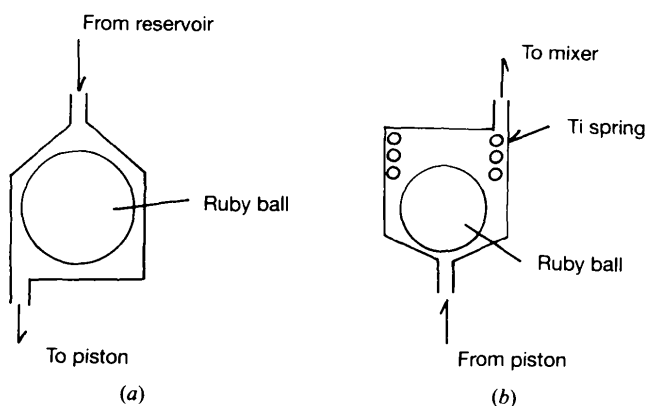
signal-to-noise ratio *etc.* At the sample position, the beam was typically 1.2 mm high and 2.6 mm wide. The scattering pattern was recorded by a one-dimensional position-sensitive proportional counter. In some cases, an imaging plate was also used as a detector (Tsuruta *et al.*, 1989).

The scattering patterns were recorded over increasing time intervals: *e.g.* 25 patterns of 0.1 s each, followed by 25 patterns of 0.2 s each, followed by four patterns of 5.0 s each. The same experiments were repeated and photons in each frame were accumulated to improve counting statistics.

Below we describe two examples of the applications of stopped-flow X-ray scattering.

### 3. Allosteric transition of aspartate transcarbamylase

Aspartate transcarbamylase (ATCase) undergoes T to R transition upon substrate binding. The transition is also affected by the binding of allosteric effectors such as CTP, ATP and CTP + UTP. We have performed extensive kinetic studies of the allosteric transition (Tsuruta *et al.*, 1994). This transition has a time constant of about 4 ms at 277 K, so the X-ray scattering pattern cannot be followed unless the reaction is slowed down. The reaction was followed by cooling the enzymes below 273 K in the presence of ethylene glycol. In such systems, we could monitor time-resolved scattering patterns with a time resolution of 100 ms. An example of the reaction is illustrated in Fig. 4. The SFXS study reveals that this transition is explained by a two-state model. No intermediate species are required for the interpretation of the time-resolved scattering pattern. The allosteric transition of ATCase was also monitored in



**Figure 3**

Ruby-ball valves. (a) Ruby-ball valve A between the reservoirs and the pistons. (b) Ruby-ball valve B between the pistons and the mixing block. When the pistons are moved downwards, ruby ball A is pressed onto the chamber floor allowing the flow of solutions from the reservoirs, whereas ruby ball B is pressed onto the bottom hole preventing the flow-out of solution from the bottom channel. In contrast, when the pistons are raised, ruby-ball valve A closes the connection between the pistons and the reservoirs, whereas ruby-ball valve B stays open. In order to make the bottom hole of ruby-ball valve B stay open, a titanium spring is attached to the upper part of the ruby ball.

**Table 1**

Characteristics of the stopped-flow apparatus.

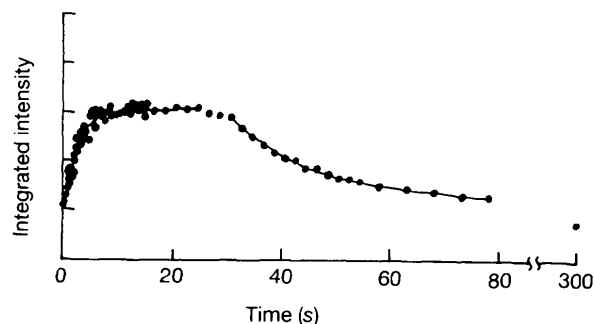
	Version 1	Version 2	Version 3	Version 4
Minimal sample volume ( $\mu$ l) for one shot (1:1)		180	100	100
Mixer type	Slit type	4 jet+2 jet	4 jet+2 jet	4 jet+2 jet (1:1) and 1:6 mixer (1:6)
Mixing ratio	1:1	1:1	1:1	1:1
Dead-time (ms)	13	10	5.5	6.2
Temperature control	Circulating water	Peltier	Peltier	Circulating water
Maximum viscosity (cP)		20		
Reservoir volume (ml)		3	3	12
Flow exchange	Rotating	Rotating	Ruby ball	Ruby ball

the presence of the substrate analogue, acetyl phosphate and carbamyl phosphate (Kihara *et al.*, 1987).

### 4. Protein folding

The SFXS method was also applied to the study of protein folding. One of the main objectives in protein-folding studies is to determine the protein-folding sequence. Three hypotheses concerning the initial events in this sequence have been reported so far. The first and the second hypotheses propose initial secondary structure formation. The first hypothesis additionally proposes that overall globulization takes place as a distinct second step, whereas the second hypothesis insists that globulization must occur simultaneously with secondary structure formation. The third hypothesis proposes the reverse, *i.e.* globulization occurs first, followed by secondary structure formation.

We have performed a series of folding experiments using the SFXS method. The proteins tested were bovine carbonic anhydrase (BCA), phosphoglycerate kinase (PGK) and apomyoglobin (Eliezer *et al.*, 1993). With BCA, we obtained a fast observable increase of X-ray scattering intensity (Semisotnov, Kotova *et al.*, 1994; Semisotnov, Kihara *et al.*, 1994). As the secondary structure was formed within the dead-time of the mixing apparatus, this would suggest that globulization takes place separately after secondary structure formation.



**Figure 4**

Kinetics of structural change of ATCase shown as the integrated intensity over the angular range  $s = 0.0141\text{--}0.0226 \text{ \AA}^{-1}$  for enzyme reaction of  $59.5 \text{ mg ml}^{-1}$  ATCase at 268 K with 44 mM carbamyl phosphate and 44 mM L-aspartate. Solid curves show single exponential fitting of the data (Tsuruta *et al.*, 1994).

## 5. Other topics

Our previous paper (Tsuruta *et al.*, 1989) summarizes literature reports of stopped-flow studies. Since then, protease trapping of macroglobulin (Arakawa *et al.*, 1994) and coupling of ATP hydrolysis of proton ATPase with its dissociation into monomers (Sato *et al.*, 1994) have been investigated.

## 6. Kinetic studies using X-ray absorption spectroscopy

Kinetic studies of X-ray absorption have been performed not only by stopped-flow methods but also by rapid freezing, continuous flow and other techniques. Combined use of the stopped-flow method with dispersive EXAFS (extended X-ray absorption fine structure) has been successfully applied to studies of the oxidation–reduction kinetics of iron hexacyanide (Matsushita, Oyanagi, Saigo, Kaminaga & Kihara, 1984). The stopped-flow technique combined with dispersive EXAFS has also been applied to laboratory-source EXAFS (Inada, Funahashi & Ohtaki, 1994). However, in most cases with proteins, X-ray photons are not sufficient for the analysis of time-resolved EXAFS, or, in a more general sense, XAFS (X-ray absorption fine structure) studies. X-ray fluorescence is more sensitive than X-ray absorption; however, dispersive XAFS techniques cannot be applied to X-ray fluorescence. Thus, rapid-freezing and continuous-flow techniques are more applicable to intermediate structure analysis studies.

Saigo has been continuously developing a rapid-freezing apparatus in cooperation with Nagamura of Unisoku Inc. (Saigo *et al.*, 1987; Saigo, Hashimoto, Shibayama, Nomura & Nagamura, 1993). They employed Bray's method, *i.e.* squirting the reacting solution into cold isopentane at 133 K. The ageing time, the period between mixing and quenching, can be varied by changing the length of the reaction tube. The reacting solution, after passing through the reaction tube, is squirted from a nozzle into cold isopentane. The minimum time between mixing and quenching was 5 ms.

This method has been applied to the reduction of cyanomet myoglobin with dithionite (Saigo *et al.*, 1993). The intermediate detected has a local structure similar to that of Mb<sup>+</sup>.CN<sup>-</sup> until the cyanide ligand is released.

Fluorescent XAFS can also be combined with a continuous-flow technique (Thiel, Livins, Stern & Lewis, 1993). Thiel *et al.* combined XAFS with rapid-flow laser spectroscopy. They excited a platinum compound by laser illumination, and measured the fluorescent XAFS of the intermediate species. They obtained microsecond time resolution with this technique.

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Amemiya (Photon Factory) and other collaborators. Dr Saigo is thanked for information on recent studies concerning rapid freezing.

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