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An apparatus for stopped-flow X-ray scattering

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Résumé. — Cet article décrit un appareil de « stopped-flow » et son système de contrôle destiné à l'étude, par diffusion des rayons X, de cinétiques rapides de réactions en solution. Inspiré d'un appareil commercial utilisant les UV et la lumière visible, l'appareil à rayons X a un temps mort de 80 ms. Des résultats sont présentés pour la polymérisation de la protéine de conque du virus de la mosaïque du Brome par saut de pH, en utilisant l'installation de diffusion de rayons X à petits angles de Hasylab (Hambourg).

Abstract. — A stopped-flow apparatus and control system, designed for the study of rapid reaction kinetics in solution by X-ray scattering, is described. Inspired from a commercial stopped-flow unit used with UV and visible light, the X-ray device has a dead-time of 80 ms. Results are presented for the polymerization of the coat protein of Brome mosaic virus following a pH jump, using a small angle X-ray scattering instrument at Hasylab (Hamburg).

1. Introduction.

The intensity of synchrotron radiation X-ray sources is such that X-ray solution scattering can be used to monitor the kinetics of a reaction which substantially alters the scattering pattern. This is the case of dissociation or assembly processes, or to a lesser extent, of conformational changes of allosteric enzymes. The feasibility of such experiments has already been established by several studies [1-3]. When fast mixing is required to trigger the reaction — pH-jump, substrate addition, etc. — the stopped-flow method [4-5] is the most convenient way to achieve it in a time range from a millisecond to a second. A stopped-flow apparatus for X-ray scattering experiments has already been described [6], based on the design of Strittmatter [7] and Loverde and Strittmatter [12]. The apparatus we present here is of a different design, inspired, in its principle, from a stopped-flow system manufactured by Giken (Japan) to be used with visible or U.V. light. We give its main characteristics and show some results obtained on the small-angle set-up X33 of EMBL at Hasylab (Hamburg).

2. Description.

The kinetic study of a structural modification by X-ray scattering requires the creation of a homogeneous solution, obtained by mixture of several components, accessible at any instant during the structural change. This problem was solved in 1923 by Hartridge and Roughton using a technique based in the constant flow principle: a gas-pressure drives the reactants through the mixture chamber to the measuring cell at a constant rate.

The apparatus described here is based in the same principle. A schematic diagram of the apparatus is shown in figure 1 while the photograph in figure 2 shows a general view.

The two solutions to be mixed are placed in two separate 8 ml reservoirs V1, V2 under 1.5 bar of nitrogen pressure. A third, larger (40 ml) reservoir V3 contains adequate buffer for rising between shots. All three reservoirs are connected to the mixing chamber *via* electromagnetic valves EM1, EM2, EM3. The T-mixer T is followed by a mixer M (described in [9] and manufactured by Durrum). The measuring

Fig. 1. — Schematic diagram of the stopped-flow apparatus.

Fig. 2. — Photograph of the stopped-flow apparatus.

cell C is $8 \times 4 \text{ mm}^2$ in cross-section, 1 mm thick along the X-ray path, with mica windows about $20 \mu\text{m}$ thick. Viton gaskets pressed on the windows by two lateral pieces screwed on to the central piece ensure the watertightness of the cell.

With the exception of the measuring cell, made

out of Perspex, all the parts of the system in contact with the solutions are made out of chemically inert material: reservoirs and mixer parts are made out of Voltalef (trifluoro-chloro-ethylene, also known as Kel-F, manufactured by Viennot, Malakoff, France); a membrane of Viton prevents solutions from coming

into contact with the iron core of the valve (ref. 331 manufactured by T.H. France, St-Dié) ; the valve body is of PVFD, a compound with a chemical activity similar to PTFE ; connectors are standard 1/16" Chemitronix made out of polyethylene.

The three Volute reservoirs V1, V2, and V3 are put in tightly fitted copper sleeves which are screwed on to the vertical side of a large L-shaped copper plate, as well as all the other components up to the measuring cell. This plate is thermostated by means of Peltier elements. The whole apparatus is enclosed in a Perspex box with, on the inside, a 1 cm thick polystyrene coating. The temperature can be kept constant within 0.2 °C between 4 °C and 30 °C.

The electronic command unit allows remote control of the apparatus, opening-time of the valves as well as temperature control. The valves have been calibrated at different N₂ pressures to determine the delivered volume *versus* opening-time. Typically, with a nitrogen pressure of 1.5 bar, 60 µl of solution are delivered in 50 ms. Valve operation is synchronized with data acquisition by TTL pulses as shown in figure 3.

3. Performance test.

The reproducibility of the volumes supplied by the electromagnetic valves for a fixed opening-time was better than 2 %. The volume supplied by a particular valve is proportional to the opening-time but different for each individual valve. Calibration of each valve is needed to know exactly the volume mixed.

In order to measure :

- the volume necessary for a maximal signal
- the filling time of the measuring cell
- the mixing time.

The dehydration of carbonic acid has been used as a testing reaction. Changes in absorbance after mixing equal volumes of 0.02 M NaHCO₃ and 0.01 M HCl

in the stopped-flow apparatus were monitored on a memory oscilloscope.

The volume necessary to obtain a maximal signal was 115 µl which corresponds to a volume 3 times greater than the dead volume plus the measuring cell volume (40 µl).

The filling time of the measuring cell was about 80 ms for a gas pressure of 1.5 bar.

Convolution of the mixing time in the chamber and filling time in the measuring cell gave a dead time of about 80 ms indicating that the mixing time is negligible compared to the filling time which is the limiting factor of the apparatus.

4. Experimental results.

The icosahedral capsid of Brome Mosaic Virus, a small plant virus, is made of 180 identical protein subunits of 20 278 molecular weight. The purified coat protein at neutral pH is solubilized as a dynamic equilibrium of monomers and dimers, with a small amount of higher oligomers ; at lower pH it is aggregated into capsids. Polymerization can be triggered by pH-jump, leading to the formation of capsids isomorphous to the virus [9].

The kinetics of the assembly process have been studied on the small-angle scattering set-up X33 of EMBL in Hasylab (Hamburg). The data acquisition system has already been described [10]. For each shot, 60 µl of a 8 mg/ml protein solution in neutral buffer (10 mM sodium cacodylate buffer pH 7.0, 500 mM KCl and 5 mM MgCl₂) were mixed with an equal volume of acetate buffer so as to bring the pH down to 5.0. Scattering spectra were recorded according to the time sequence shown in figure 3. 430 shots were recorded by groups of about 20. Each group, stored on a big disk unit of a PDP11/44 was checked for possible artefacts unnoticed during the experiment. All valid subsets (amounting to the quasi-totality of

Fig. 3. — Synchronization of valve operations with data acquisition. The time sequence for data acquisition shown here has been used for the experiments presented in the last section.

the data) were then added together to produce a final set of averaged data.

Figure 4 shows some spectra from the final set obtained at various times after mixing. The reaction can easily be followed by mere visual inspection: the first spectra (Fig. 4a), very flat and featureless,

Fig. 4. — X-ray scattering patterns of solutions of BMV coat protein taken at various times after pH-jump.

are characteristic of small globular particles such as monomers or dimers of the coat protein. Less than 1 s after mixing, a shoulder can be seen at about $0.04 \text{ \AA}^{-1}$ (Figs. 4b-4d), which turns into a distinct minimum and maximum in the next 2 s (Figs. 4e-4f). The minimum deepens further and the maximum increases during the following 40 s (Figs. 4g-4h). Minima and maxima are features observed with spherical particles. The positions of the minimum and maximum on our curves are very close to the ones observed with the intact virus.

Analysis of the evolution of the radius of gyration and of the intensity at the origin with time gives us information about the overall polymerisation process. In order to do any further, one needs a model to test our data. As yet, no theoretical model is available to account for the polymerisation. Extra information can only be provided by other experimental approaches.

We thus performed parallel experiments using light scattering and electron microscopy. Light-scattering monitoring of the reaction yields limited (one parameter) but high quality data on the kinetics of the reaction. Electron microscopy on frozen-hydrated samples [11] provides structural information concerning possible intermediary states. From these results we derive a model, compute theoretical curves and use least-square procedures to fit the model parameters to our experimental data. The interpretation is currently under way.

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