A Stopped-Flow Apparatus with Light-Scattering Detection and Its Application to Biochemical Reactions

(laser excitation/filtration/biopolymers/amplitudes)

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ABSTRACT A stopped-flow apparatus utilizing light-scattering for following the progress of a reaction is described. The method is applicable to all reactions that result in a significant change of the average molecular weight. It was possible due to several modifications of a conventional stopped-flow system to obtain a sensitivity comparable to that of commercial instruments for static light-scattering measurements. Experiments on three reactions are reported: association and dissociation of mercury ligands with DNA, dissociation of the dimers of DNA-dependent RNA polymerase, and complex formation of tRNA^{Ser} (yeast) with the cognate aminoacyl-tRNA synthetase. The changes in the intensities of the scattered light are calculated and compared with the measured amplitudes.

In most fast kinetic experiments the changes in concentration of the reactants are monitored by optical absorption, fluorescence intensity, or, in some cases, conductivity (1). If the reaction of interest proceeds without affecting these physical properties then indirect methods using labeled reactants or indicators can be tried. However, this approach can complicate the experiments seriously, and it has to be demonstrated that the reaction is unaffected by such a procedure.

In biochemistry considerable interest is attached to studies of interacting macromolecules, such as interactions between proteins and nucleic acids, the association and dissociation of subunits of proteins, and the binding of small molecules to nucleic acids. These reactions, which are often not accompanied by changes in the spectroscopic properties mentioned above, are associated with considerable changes in the distribution of molecular weights. Therefore, the intensity of Rayleigh light-scattering, which depends upon molecular weight, can be used for following the progress of such reactions.

Until now the use of light-scattering for following the kinetics of reactions has been confined to micelles and membrane suspensions (2, 3). In these experiments the intensity of the scattered light and the relative changes during the reaction are very high and no serious experimental complications have been reported. However, in experiments in homogeneous solution, in which for example complexes of a molecular weight of about 10^5 are involved, the intensity of the scattered light is several orders of magnitude lower than in the experiments on micelles.

In this paper, we describe a stopped-flow apparatus utilizing detection by light scattering, which is sensitive enough to investigate interactions between macromolecules to a molecular weight of 25,000 and to a concentration of about 1 mg/ml.

Various examples of biochemical reactions are given, and the limitations of the method are discussed.

MATERIALS

The buffers were prepared from reagent-grade chemicals and triple-distilled water. Mercuric acetate was purchased from Merck; 3,6-bis-(acetatomercurimethyl)-dioxan (AMD) was prepared according to Edsall *et al.* (4). Calf-thymus DNA, type V from Sigma, was degraded by a Branson sonifier for at least 15 min and further purified by phenol extraction (5) before dialysis against 0.02 M sodium acetate buffer (pH 5.5). The molecular weight of DNA was calculated by the equation of Doty, McGill, and Rice (6) from the viscosity measured in a low-shear viscometer (7). Seryl-tRNA synthetase and tRNA^{Ser} from bakers yeast were kindly donated by A. Pingoud; the preparation is described elsewhere (8). DNA-dependent RNA polymerase from *Escherichia coli*, prepared essentially as described by Burgess (9) and by Nueslein and Heyden (10) was a gift from Drs. J. Hoggett and C. Stein.

METHODS

The basic equipment of a stopped-flow apparatus with light-scattering detection is very similar to that with fluorescence detection; however, the wavelength of the incident light and the scattered light are the same. A simplified scheme of the instrument is depicted in Fig. 1.

Flow System. The flow system was a modified version of the

FIG. 1. Schematic diagram of the stopped-flow apparatus.

Abbreviation: AMD, 3,6-bis-(acetatomercurimethyl)-dioxan.

FIG. 2. Diagram of a driving syringe.

conventional Durrum-Gibson system (11). The two reactants were injected from the driving syringes through a mixing chamber containing eight tangential jets into the observation cell; the flow was stopped when the piston of the stop syringe hit the trigger switch. The observation cell was a quartz tube of 2.8-mm inside diameter; it was mounted nearly perpendicular to (see below) the incident light beam. The syringes were driven either by hand or by use of a pneumatic drive (Durrum). The details of the syringes can be seen in Fig. 2. The diameter of the circular groove that holds the O-ring of the moving piston was fitted very precisely, to ensure that the system was leakproof, but also so that the pistons were easily movable and did not form too much dust by abrasion.

Purification System. Carefully purified solutions were needed to perform sensitive light-scattering experiments, since dust particles produce an extremely high noise signal. All parts of the flow system could easily be demounted for cleaning. During a set of experiments the driving syringes were not exchanged because the whole system could be flushed continuously with water or buffer, which was filtered between the reservoir bottle and the driving syringes (Fig. 1). This cleaning process and eventual freedom from dust particles was checked by recording the intensity of background scattering. After the Teflon stopcocks, which were on the unfiltered side, were opened, solutions for the experiment were drawn into the driving syringes through the same filters that were used for the purification. Microscopic air bubbles, a source of serious perturbations, were avoided by use of degassed solutions. Generally poly(vinylchloride) (PVC) membrane filters (Sartorius Membranfilter, Goettingen, Cat.-no. 00128, pore size $0.2\ \mu\text{m}$) were used for buffers and DNA solutions, and filters made from regenerated cellulose (Sartorius, Cat.-no. 00116, pore size $0.6\ \mu\text{m}$) were used for protein solutions. Best results were obtained when the system was flushed with buffer each time before the driving syringes were refilled with solution.

Optical System. The source of light was a He-Ne laser (Siemens LG 641, wavelength 632.8 nm) operated from a highly stabilized power supply. It was mounted on an optical bench in a position where the polarization was in the horizontal plane. The laser beam crossed the axis of the observation cell at a distance of about 8 mm from the outlet side of the mixing chamber (Fig. 1). The diameter of the beam was about 1.4 mm, which was about half the inside diameter of the cell. It was possible to avoid multiple reflections of the laser beam in that part of the solution where the observation is performed by inclining the beam at an angle of 15° (α in

FIG. 3. Optical alignment of the detection system.

Fig. 1) with respect to a plane perpendicular to the cell axis. Also the axis of the multiplier detection system was inclined by an angle β (Fig. 1) to this plane to minimize the influence of the light that was reflected or scattered on the cell wall. The image of the scattering volume was formed in a small aperture in front of the multiplier, as shown in Fig. 3. Thus, it was possible to eliminate most of the light from sources outside the scattering volume. Further precision was achieved by inserting the slit aperture on the top of the cell.

Determination of the Dead Time. The dead time depends upon the position where the laser beam passes through the observation cell; therefore, it was determined without any alteration of the optical adjustment. The reaction:

was used, which is sufficiently fast and can be followed by an absorbance change at the laser wavelength of 632.8 nm (12). The conditions were the following: 1 mM MnO_4^- , 5–10 mM $[\text{Fe}^{\text{II}}(\text{CN})_6]^{4-}$ in phosphate buffer of pH 6.3 and ionic strength of 0.1 M at 22° . Under these conditions the reaction was pseudo-first order. The dead time was found to be 3–4 msec.

RESULTS AND DISCUSSION

Linearity

Before the instrument was applied to kinetic measurements, the sensitivity of the detection system was tested with aqueous solutions of poly(vinylpyrrolidone) (molecular

FIG. 4. Intensity of the scattered light (in volts) versus concentration of poly(vinylpyrrolidone).

FIG. 5a. Oscillogram of the complex formation of DNA with AMD (for conditions see text). Oscilloscope settings: 20 msec/unit, 100 mV/unit, 1-msec rise time. The final intensity refers to 1.52 V.

FIG. 5b. Oscillogram of the dissociation of Hg-DNA complex. Oscilloscope settings: 5 sec/unit, 100 mV/unit, 1-msec rise time. The final intensity refers to 0.88 V.

weight 24,500). It is a water-soluble synthetic polymer and does not aggregate measurably up to a concentration of 50 mg/ml. As shown in Fig. 4 the measured intensity of scattered light was proportional to the concentration. The ordinate intercept (0.12 V) is due to background intensity. From corresponding measurements with solutions of this polymer in a static light-scattering apparatus, the contribution of the scattering from pure water was calculated to be about 40% of the background intensity.

Interactions of DNA with mercury ligands

As examples of the reaction of small molecules with macromolecules the complexes of HgAc_2 (13, 14) and AMD with calf-thymus DNA (molecular weight 250,000) in 0.02 M NaAc buffer (pH 5.5) were investigated. Both mercury ligands are bifunctional and the molecular weights of their DNA complexes for $r = 1.0$ (r = added mol of mercuric compounds per mol of base pairs) are 30% (for HgAc_2) and 75% (for AMD) higher than that of the uncomplexed DNA.

Reaction time

Fig. 5a shows an oscillogram of the association of DNA (concentration = 0.5 mg/ml) with AMD to a final r - value of 1.0. The curve could be fitted to the time course of a second-order reaction with an association rate constant k_r of $1.4 (\pm 0.3) \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$, when moles of base pairs and moles of AMD were taken as concentrations and the possible influence of cooperative binding was neglected. Within the limits of error the same value was found when the reaction under otherwise identical conditions was followed by optical absorption at 280 nm. The corresponding complex formation with HgAc_2 was markedly faster, and more than 80% of the

FIG. 6. Calculated (open symbols) and measured (filled symbols) amplitudes of the complexes of DNA with mercuric acetate (circles) and AMD (squares) versus r (see text). The scattering of the calculated points is due to experimental values of dn/dc .

FIG. 7. Dissociation of 0.35 mg/ml of polymerase in 0.55 M KCl. Oscilloscope setting of the two beams: lower beam: 50 msec/unit, 200 mV/unit; upper beam: 0.5 sec/unit, 200 mV/unit. The upper beam is triggered 50 msec later than the lower one. The difference in the ordinate of both beams is 1.8 units. The final intensity is shown by the straight lines and refers to 2.8 V.

total reaction was completed within the dead time of the instrument.

The dissociation of both DNA complexes was performed by complexing the mercury ligand with 0.05 M EDTA in 0.02 M NaAc buffer (pH 5.5). Whereas the dissociation of the DNA-AMD complex proceeded in several steps, that of DNA-mercury followed a single exponential (Fig. 5b). The half-life of the latter reaction was $5.4 (\pm 0.3)$ sec and was independent of the r -value between 0.2 and 1.0.

Amplitudes

In order to evaluate the kinetic experiments it was necessary to demonstrate that the measured amplitude A truly corresponded to the difference in the weight-average molecular weights at the beginning and at the end of the reaction. The amplitudes can be calculated from Eq. [1], which is derived from the basic expressions for light-scattering (15):

$$A = K' \times \left[\sum_i M_i c_i \left(\frac{dn}{dc} \right)_i^2 - \sum_j M_j c_j \left(\frac{dn}{dc} \right)_j^2 \right] \quad [1]$$

Subscripts i and j represent reactants and products, respectively, and M , c , and dn/dc are the molecular weight, concentration, and refractive index increment. The constant K' was taken from calibration experiments with poly(vinylpyrrolidone). Eq. [1] is valid as long as the diameter of the molecules is smaller than a tenth of the wavelength of the incident light, the change in the second virial coefficient can be neglected, and no significant depolarization occurs. In Fig. 6 the amplitudes for both types of DNA complexes are calculated according to Eq. [1] and compared with the values obtained from the corresponding final values of association and dissociation experiments. From Fig. 5a and b, it can be seen that for these reactions the total processes were observed in the kinetic experiments.

Dissociation of dimers of DNA-dependent RNA polymerase

As an example of the application of this method to protein-protein interactions, we report preliminary experiments on the dissociation of dimers of RNA polymerase. The dimers of DNA-dependent RNA polymerase of *E. coli* (molecular weight 9.8×10^5) are stable in low-salt concentrations (<0.1 M KCl), whereas only monomers (molecular weight 4.9×10^5) are present in salt concentrations above 0.5 M KCl (16). Fig. 7 depicts the process of dissociation of the polymerase after a salt jump from 0.05 to 0.55 M KCl. This experiment, together with further measurements (J. Hoggett,

unpublished results), showed that the dissociation is not a one-step reaction. Two steps, one in the 50-msec and the other in the second range, were observed (Fig. 7). A faster process was only measurable in lower salt concentrations (J. Hoggett, unpublished results).

Complex formation of seryl-tRNA synthetase with tRNA^{Ser}

As the third example, the complex formation of tRNA^{Ser} (molecular weight 25,000) with its aminoacyl-tRNA synthetase (molecular weight 100,000) was studied. The structure of tRNA^{Ser} can be changed in a reversible process from its denatured to its native conformation, when the concentration of free Mg⁺⁺ is increased from zero to about 5 mM (17). According to measurements of Pingoud (8), the fluorescence of the synthetase is only quenched when the native tRNA^{Ser} is used. Therefore, either the denatured form does not bind at all to the enzyme or the binding does not affect its fluorescence. In our light-scattering experiments a solution of 5 μ M denatured tRNA in 0.03 M phosphate buffer [0.5 mM EDTA (pH 7.0)] was mixed with a solution of 5 μ M synthetase in the same buffer but containing 10 mM MgCl₂. A slow increase in the intensity of the scattered light was observed, with a time constant of 40 sec at 35°C; the same kinetics were found in corresponding fluorescence experiments. These results are interpreted as follows: the initial intensity of the scattered light after rapid mixing shows that the denatured tRNA^{Ser} does not bind to the synthetase; under the influence of Mg⁺⁺, the tRNA renatures slowly and binds to the synthetase. Therefore the increase in intensity of the scattered light and the decrease in the fluorescence intensity occur simultaneously. The rate-limiting process in this experiment is the renaturation of the tRNA, since the binding of the native tRNA to the synthetase is much faster (8).

CONCLUSIONS

The experiments reported in this paper have not been undertaken to clarify the reaction mechanisms in any detail, but to demonstrate that light-scattering detection can be used for stopped-flow investigations on biochemical reactions. It is applicable in general to all reactions of macromolecules that result in a change of molecular weight of at least 10% and that can be followed in sufficiently high concentrations. For a molecular weight of about 25,000, the lowest acceptable

concentration was about 1.0 mg/ml; in this case the measured amplitude of the scattered light arising from the macromolecules was twice the value of the background intensity. After optimal adjustment, about 60% of the background intensity, when measured with filtered degassed water, was due to the light from reflections in the observation cell. In this respect the sensitivity of this instrument is in the same range as that of commercial instruments for static light-scattering measurements.

An obvious advantage of this method is that evidence from the amplitudes about the change in molecular weight during a reaction can be used to help formulate reaction mechanisms.

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