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Stopped-flow x-ray scattering: The dissociation of aspartate transcarbamylase

(allosteric enzyme complex/kinetics/mercurial/synchrotron radiation)

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ABSTRACT **A combination of stopped-flow and x-ray scattering techniques was used to study the dissociation of aspartate transcarbamylase (carbamoylphosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2) with a 2:1 excess of *p*-chloromercuribenzenesulfonic acid (the ratio being calculated on a basis of reactive sites), in the presence and absence of the transition state analogue *N*-(phosphonacetyl)-L-aspartate. At 10 mg of protein per ml, the scattering curves allowed some details of the reaction to be followed with a time resolution down to 1 sec. The curves showed not only the dissociation of the enzyme complex but also the formation of the subunits. These results show that, with present facilities, x-ray scattering could be used to study dissociation or reassociation reactions with a time resolution of the order of 100 msec.**

Aspartate transcarbamylase (ATCase; carbamoylphosphate: L-aspartate carbamoyltransferase, EC 2.1.3.2) (reviewed in ref. 1), a 300,000-dalton complex consisting of six regulatory and six catalytic chains, is a widely studied allosteric enzyme. Its regulatory behavior is linked to a large conformational change (2–4), which we have shown to cause a substantial alteration in the x-ray solution scattering pattern (5). This provides a means of investigating the quaternary structure states of the enzyme in solution.

Despite the value of the x-ray solution scattering method in structural investigations (6, 7), its use in kinetic studies would have been inconceivable before exposure times were drastically shortened by two recent developments: position-sensitive detectors, and the availability of focusing cameras for the intense synchrotron radiation from storage rings. But many serious technical problems remain before its combination with the stopped-flow technique can be fully exploited: a well-collimated high-flux x-ray beam with low background must be available; the mixing device must be operated by remote control and be able to inject the solution into a cell that is transparent to the x-ray beam but resistant to pressure transients; the scattering patterns must be collected for many successive brief periods, stored rapidly, and evaluated for mixing artifacts before averaging; and new methods must be developed for obtaining the maximal structural and kinetic information from the data. We have reached a stage in this development at which we can study the dissociation of ATCase by mercurials. This reaction, which gives two active catalytic trimers and three regulatory dimers (8, 9), is of interest because of the theoretical connection between allosteric activation and dissociation (10) and the information about ATCase activation that has come from studies of its dissociation (11, 12).

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MATERIALS AND METHODS

Chemicals. ATCase (purified by a method used in this laboratory by which the heating step is avoided) was freed from dimer and from the species lacking one regulatory subunit as described (5, 13). It was dissolved (20 mg/ml) in 40 mM potassium phosphate, pH 7.0/1 mM sodium azide/0.1 mM phenylmethanesulfonyl fluoride and was dissociated by mixing with an equal volume of *p*-chloromercuribenzenesulfonic acid (PMBS) solution (1.6 mg/ml in 50 mM Tris·HCl buffer at pH 7.0). *N*-(Phosphonacetyl)-L-aspartate (PALA) was the generous gift of G. R. Stark. Its concentration was assayed by hydrolysis and aspartate analysis (13).

Reaction Conditions. In order to maximize the x-ray scattering, we needed a relatively high final ATCase concentration (10 mg/ml). To complete the dissociation, this would have required a concentration of *p*-chloromercuribenzoate (PMB), the mercurial used in previous studies (11, 12), exceeding its solubility, so we used PMBS instead. Furthermore, because of aggregation and eventual precipitation at high concentrations of mercurials, we were forced to use PMBS at the rather low final concentration of 0.8 mg/ml. Consequently, there were only two mercury atoms per reactive site, and the course of the reaction was no longer pseudo-first-order as in the earlier studies (11, 12), in which the ratio was 6:1.

Stopped-Flow Equipment. The stopped-flow device was built at Heidelberg and gave no mixing artifacts when tested with thymol blue and acetic acid. It had a response time (between closing the drive switch and the appearance of the optical signal) of 90 msec. A pneumatic drive ejected 75- to 100-μl samples from each of two 5-ml syringes. After they were mixed in a Gibson–Durrum mixer (14), the solutions were passed into a 2-mm quartz x-ray capillary that was flattened to give parallel faces 1 mm apart. The area exposed to the x-ray beam was 5×2 mm^2 in Hamburg and 5×1 mm^2 in Orsay; and the capillary was oriented to match the beam's shape (i.e., it was vertical at Orsay and horizontal at Hamburg). In the direction perpendicular to the beam, the reaction was monitored with monochromatic light (252 nm), which was passed into and out of the cell holder by quartz light pipes; its intensity was measured with a photomultiplier and recorded with a storage oscilloscope. (The optical signal was satisfactory during the experiments at Orsay, and agreed well with the x-ray results.) Between shots the capillary was washed out with 0.5 ml of distilled water and cleaned by suction. Until exhaustion of the solutions in the sy-

Abbreviations: ATCase, aspartate transcarbamylase (EC 2.1.3.2); PALA, *N*-(phosphonacetyl)-L-aspartate; PMB, *p*-chloromercuribenzoate; PMBS, *p*-chloromercuribenzenesulfonic acid.

rings, all phases of mixing and washing were remotely controlled, and their proper execution could be followed from the oscilloscope trace.

X-Ray Scattering. Experiments were conducted at the two European synchrotron radiation laboratories equipped for small angle x-ray scattering: at Orsay, France, and the European Molecular Biology Laboratory outstation at Hamburg.

In the installation at Orsay (15–18), the singly focused monochromatic beam gave a spot about 1 mm wide at the sample, and about 0.8 mm wide and 5 mm high at the detector. This was a linear position-sensitive detector (19). The sample-to-detector distance was 859 mm, and the wavelength was 0.154 nm. With the storage ring run at 1.72 GeV and 200 mA, the flux of the focused beam was about 10^9 photons/sec. The counts were stored in the 4×10^3 memory of a Tracor Northern TN 1710 multichannel analyzer. For each experiment, 15 spectra of 256 points each were successively recorded in the following time pattern: 4 at 5 sec, 8 at 10 sec, 1 at 20 sec, and 2 at 40 sec. The recording was switched manually from one area of the memory to the next, and the data were stored on a disk after each shot.

The double-focusing optical system at Hamburg (20) gave a spot about 6 mm wide and 3 mm high at the sample and about 1 mm in diameter at the detector. This was of the same type as at Orsay but, because the sample-to-detector distance was 2300 mm, only one side of the diffraction pattern was recorded. The wavelength was 0.148 nm and, with the storage ring run at 4.7 GeV and 20 mA, the flux was about 10^{11} photons/sec in the direct beam and 3×10^{10} through the capillary. Sixty-four sequential spectra, each of 256 channels, were stored in a memory by using electronic time-slicing (21) with 1- or 5-sec slices. The data were stored on a disk after each shot.

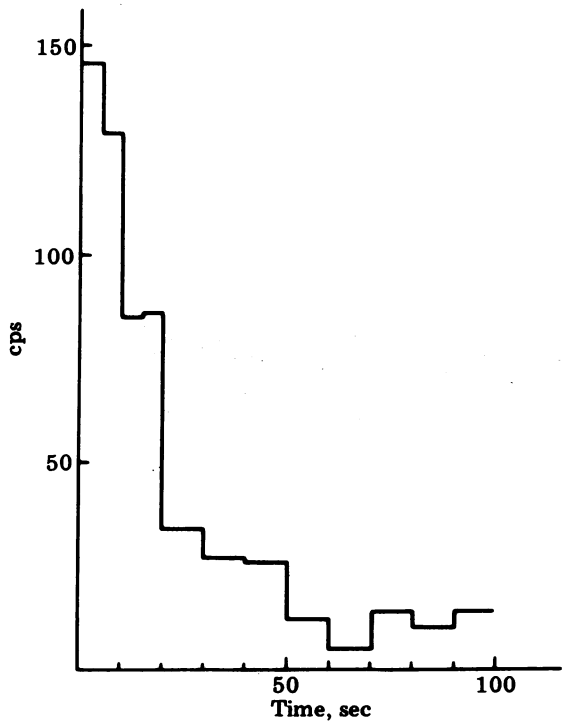

FIG. 1. Time course of the dissociation of ATCase 40% saturated with PALA (experiment shown in Fig. 2). The curve was calculated from the photons scattered into one side of the scattering pattern (channels 1–18) minus the curve for the last time-slice; this is equivalent to a 2.5-sec time-slice. cps, Counts per second.

RESULTS

Although the scattering curves given by individual stopped-flow shots were very noisy, the decrease in central scattering was always clearly visible (Fig. 1). To see better the changes occuring during the reaction, the curves from all satisfactory shots (comprising >90% of the total) were summed, and the scattering curve averaged over the last 40 sec (Fig. 2) or last 10 sec (Figs. 3 and 4) was subtracted from all the others. The resulting curves are displayed, without smoothing, as three-dimensional plots. These are shown for three experiments: at 40% PALA saturation (Orsay) in Fig. 2, at 0% PALA (Hamburg) in Fig. 3, and at 100% PALA saturation (Hamburg) in Fig. 4. The first two experiments used time-frame intervals of 5 sec (or more, in Fig. 2) and therefore were not adapted to detect reactions as fast as shown in Fig. 4, in which 1-sec intervals were used. In all figures, the signal/noise ratio was large enough to allow investigation of the reaction's time course and to give some information about the composition of the reaction mixture at different times.

The first difference scattering curves become negative around their middle channels (seen most clearly in channels 30–35 in Fig. 2 but also around channel 100 in Figs. 3 and 4). This dip arises from the fact that an average of the last few scattering curves was subtracted from all the others and indicates that the later curves must show detectable scattering over the same angular range as the dip in the first curves. Thus, x-ray scattering can follow not only the disappearance of ATCase but also the appearance of its subunits. Information on this last point would be unavailable in a light-scattering stopped-flow study.

The slowly falling later phase of the reactions seen in Figs. 2–4 is presumably due to the relatively small excess of PMBS. Furthermore, the initial time slices used in the experiments of Figs. 2 and 3 were too long to give an accurate value of the

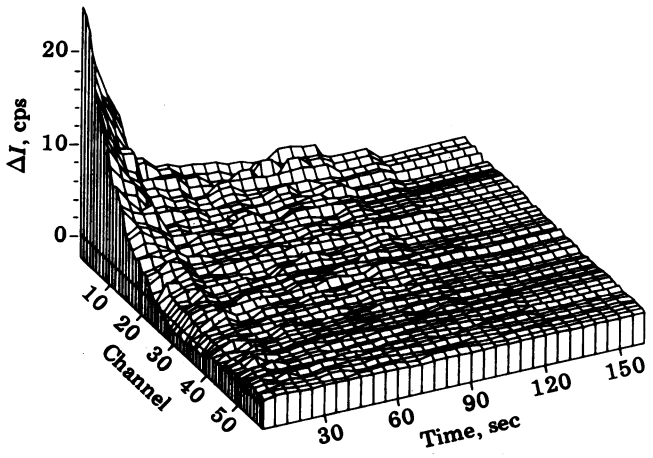

FIG. 2. X-ray scattering curves obtained at Orsay for ATCase dissociation. ATCase at 20 mg/ml in one syringe was mixed with PMBS at 1.6 mg/ml in the other syringe; 40% of active sites were saturated with PALA (0.16 mM). Because of the different lengths of the original time frames, the data have been represented at 5-sec intervals by putting together two identical (scaled) copies of a 10-sec frame, four copies of a 20-sec frame, etc. The curves shown are the sums of scattering curves from 29 shots, and the scattering curve for the last 40 sec has been subtracted from all the others (see *Appendix*). To facilitate comparison, the difference intensities have been plotted in counts per second (cps). To obtain the counts recorded in each channel of a frame in the summed data shown here, multiply both by the appropriate time interval and by twice the number of shots. (The extra factor of 2 arises from the averaging of the left and right sides of the pattern.) Channel number 1 corresponds to a spacing of 2 sin $\theta/\lambda = 0.0036$ Å^{-1}, and a difference of one channel corresponds to a separation of 2.27×10^{-4} Å^{-1}.

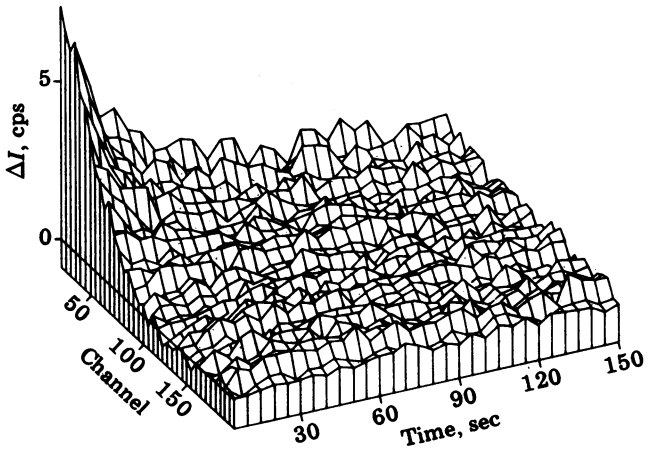

FIG. 3. X-ray scattering curves obtained at Hamburg for the dissociation of unligated ATCase, using 5-sec frame intervals. ATCase at 20 mg/ml in one syringe was mixed with PMBS at 1.6 mg/ml in the other syringe. The curves shown are the sums of scattering curves from 30 shots, and the scattering curve averaged over the last 10 sec has been subtracted from all the others. To facilitate comparison, the difference intensities have been plotted in counts per second (cps). To obtain the counts recorded in each channel of a frame in the summed data shown here, multiply both by the appropriate time interval and by the number of shots. Channel number 1 corresponds to a spacing of $2 \sin \theta/\lambda = 0.0029$ Å^{-1}, and a difference of one channel corresponds to a separation of 3.5×10^{-5} Å^{-1}.

initial rate. For both these reasons, the x-ray time courses do not show the striking effect of PALA on the reaction course (12). However, in optical kinetic studies of dilute solutions, we have found that PALA increases the initial rate of the reaction with PMBS to about the same extent as with PMB. These studies have also shown that the reaction with PMB is about 50% slower than that with PMBS, so that our results are difficult to compare with those published earlier (11, 12).

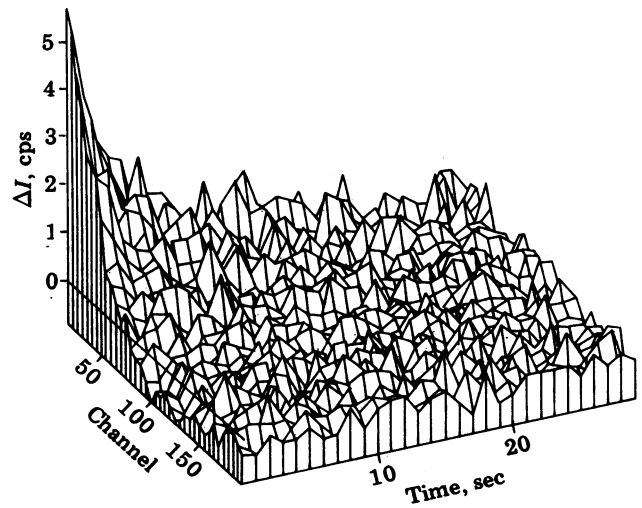

FIG. 4. Scattering curves obtained at Hamburg with 1-sec frame intervals for the dissociation of ATCase with its active sites completely saturated with PALA (concentration, 0.41 mM). ATCase at 20 mg/ml in one syringe was mixed with PMBS at 1.6 mg/ml in the other syringe. The curves shown are the sums of scattering curves from 46 shots, and the scattering curve averaged over the last 10 sec has been subtracted from all the others. To facilitate comparison, the difference intensities have been plotted in counts per second (cps). To obtain the counts recorded in each channel of a frame in the summed data shown here, multiply both by the appropriate time interval and by the number of shots. Channel number 1 corresponds to a spacing of $2 \sin \theta/\lambda = 0.0029$ Å^{-1}, and a difference of one channel corresponds to a separation of 3.5×10^{-5} Å^{-1}.

DISCUSSION

More detailed experiments, with a range of reactant concentrations and combined with good optical records, are clearly needed before the reaction can be investigated satisfactorily by this new technique. But, besides demonstrating the feasibility of such a study, our results suggest what additional information we might expect from better data.

The main reason for developing the x-ray kinetic method lies in the hope of studying reaction intermediates. The presence of an intermediate can be tested for by determining whether all the scattering curves can be represented as linear combinations of the initial and final curves (see *Appendix*). Although the noise level in our data is not so high as to prevent this sort of analysis, the possibility of artifacts in a new method complicates the interpretation of the results. With sufficiently good data, however, we might be able to approach the problem of obtaining the intermediate's scattering pattern. From x-ray scattering data alone, we could obtain only that component which is orthogonal to the patterns of the initial and final states; because the concentrations of these states are unknown, we cannot know the contributions of their patterns to the total scattering. But a knowledge of the concentrations of all the different components (derived from optical or quenching experiments) would allow the scattering pattern of an intermediate to be calculated, and hence its quaternary structure to be investigated. There is biochemical evidence for an intermediate in the reaction with PMB (12, 22, 23), but structural information about intermediates would be extremely difficult to obtain by optical stopped-flow methods.

The quality of the data in Figs. 2–4 is much better than that needed merely for estimating the rate of a reaction. Fig. 1 shows half of a scattering curve from one of the experiments of Fig. 2, corresponding to 2.5 sec of counting time (i.e., $1/58$th of the total data in this experiment). Clearly, useful data could be obtained from a similar reaction that was 60 times faster (i.e., with 100-msec time-slices), even with the current apparatus. Yet this is by no means the limit of what is possible. The total flux received by the detector was only about 3000 photons/sec at Orsay and about 2000 photons/sec at Hamburg. Because such detectors, combined with a time-to-digital converter, can record over 100 times as many photons/sec, we are very far from saturating the detector; on the contrary, we need to increase the number of photons absorbed by it. These photons are available at Hamburg, where the direct beam flux was about 100 times greater than at Orsay. When we have improved the geometry of the experiment so as to make use of this additional flux (e.g., by using an annular detector), we should substantially improve the quality of the data and make the x-ray technique applicable to a wide range of fast reactions.

APPENDIX

Suppose that no intermediate molecular species is present in a detectable amount. Then there are only two basic scattering curves (those for the initial and final mixtures), and every scattering curve must be a linear combination of these two. In general, they can be considered to be the scattering curves at times $t = 0$ and at time $t = T$, without requiring that these times necessarily represent the start and finish points. The scattered intensity at time t and at an angle corresponding to s Å^{-1} ($s = 2 \sin \theta/\lambda$) is then given by

$$I(s,t) = x(t)I(s,0) + [1 - x(t)]I(s,T),$$

in which $x(t)$ is the extent of reaction. To find this function from

any angular range (s_1,s_2), we calculate

$$\int_{s_1}^{s_2}[I(s,t) - I(s,T)]ds = x(t)\left\{\int_{s_1}^{s_2}[I(s,0) - I(s,T)]ds\right\}.$$

Whatever angular range is chosen, the time course $x(t)$ will be identical, except for multiplication by the quantity in braces; and this is independent of time, although it depends on the chosen range (s_1,s_2). However, if the time course curves obtained from different angular ranges are substantially different, we can conclude that two scattering species are insufficient to explain the data, and the existence of an intermediate must be postulated. This test has not given an unambiguous result with the data reported here; additional testing is needed with an apparatus that yields a much higher recorded flux.

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