A stopped-flow apparatus for infrared spectroscopy of aqueous solutions

Andrew J. WHITE,* Kevin DRABBLE and Christopher W. WHARTON†

School of Biochemistry, University of Birmingham Edgbaston, Birmingham B15 2TT, U.K.

IR spectroscopy has been widely applied in the study of photo-activated biological processes such as photosynthesis, but has not been applied to the study of reacting systems which require rapid mixing of aqueous solutions. This has been due in part to the high pressure needed to make an aqueous solution flow rapidly through the 50 μm optical pathlength between the plates in an IR cuvette suitable for use with $^2\text{H}_2\text{O}$ and the high viscosity of the concentrated protein solutions required to generate measurable IR signals. An apparatus, based largely on conventional stopped-flow technology, is described which achieves mixing well within the time-resolved performance (≈ 40 ms) of modern Fourier-transform IR (FTIR) spectrometers, since the dead time of the mixing device is ≈ 15 ms. It has proved possible to achieve efficient mixing by using a simple six-jet mixing device. This is probably at least in part because of the high back pressure which develops when aqueous fluid is passed rapidly through the

short pathlength of the cuvette and which promotes turbulent flow. Several examples of measurements of the deacylation of acylchymotrypsins are provided which demonstrate the operation of the apparatus in conjunction with a spectrometer capable of scanning at four scans/s. For cinnamoyl-chymotrypsin, isotope-edited spectra have been obtained which show somewhat lower resolution than is achieved by conventional scanning methods, since some smoothing has to be applied to the spectra. Difference spectra of the acylation of chymotrypsin by glycylglycine *p*-nitrophenyl ester have been obtained by averaging ten stopped-flow shots and show good signal-to-noise ratio without smoothing. It is predicted that this apparatus is likely to find a variety of applications in the study of enzyme-catalysed reactions, since the spectra are relatively rich in structural information, and isotope editing greatly enhances the interpretability of the spectra.

INTRODUCTION

An extensive protein-structural database has been provided by X-ray crystallography and, more recently, NMR has been widely applied to the determination of the structure of small proteins in solution. Information relevant to mechanism has been obtained by a study of bound inhibitors, products and putative transition-state analogues. All of this information refers to essentially static non-reacting systems, but group and segmental mobility can be estimated from temperature factors in crystallography and relaxation measurements in NMR spectroscopy. Neither of these central structural methods is easy to adapt to the study of reacting systems, although time-resolved crystallography using synchrotron radiation has had some success and promises much in the future [1].

UV-visible spectroscopic methods are well suited for time-resolved measurements, and these can be made in the subpicosecond range if lasers are used. They suffer from the disadvantage that little structural information is provided, although in favourable cases, such as in studies of bacteriorhodopsin, where the intermediates of the photocycle show distinct and accurately interpretable spectral characteristics, these methods have proved very valuable [2].

Vibrational spectroscopy provides much more structural information in terms of bond strengths and mobility than the UV-visible spectroscopic methods, but is more complex to implement in time-resolved mode. Raman spectroscopy has proved to be more versatile than IR spectroscopy in this respect, and nanosecond-time-resolved Raman spectrometers are well established, with picosecond instruments becoming available [3].

Resonance enhancement, which greatly amplifies Raman scattering, permits the method to be focussed on a part of the molecule of interest that possesses an accessible electronic transition. Photoexcited phenomena can be studied by pump-and-probe techniques. Where the molecule of interest, such as a protein-bound ligand, does not possess an accessible chromophore, resonance enhancement cannot be applied and Raman scattering is very weak.

IR spectroscopy is generally difficult to implement in fast-time-resolved mode, but picosecond time resolution has recently been achieved in the study of photoexcited biological samples where laser mixing techniques have been used [4]. Spectra of transient species can be constructed rather laboriously by tuning, for example, a CO laser across a range of rotational lines that cover the mid-IR frequency range [5]. Dispersive (grating) IR spectrometers scan rather slowly, but can be used to measure time-resolved events at a single frequency (at a time), although the throughput is poor and the sensitivity accordingly indifferent. FTIR spectrometers have high sensitivity, but the time-resolved performance is limited by the scan rate, unless step-scan or stroboscopic methods are used for the study of events that can be repetitively photoactivated [6]. Scanning is achieved by modulating the light which passes through the spectrometer with a Michelson interferometer, and this requires mechanical motion of a moving mirror, which has to follow a very accurate path. This mechanical requirement (and probably also the high precision analogue-to-digital conversion of the interferometric data required) limits the scanning speed of FTIR spectrometers to about 100 s^{-1} at 8 cm^{-1} resolution and defines the maximum time-resolved performance that can be achieved. At 2 cm^{-1}

Abbreviations used: pH* refers to a pH-meter reading in $^2\text{H}_2\text{O}$ solution; FT, Fourier-transform.

* Present address: Biology Division, School of Sciences, Staffordshire University, College Road, Stoke on Trent ST4 2DE, U.K.

† To whom correspondence should be addressed.

resolution, which is required to resolve overlapping bands characteristic of conformers of enzyme-bound ligands, the scan rate is reduced to about 25 s^{-1} . The transient light-induced behaviour of rhodopsin has been studied at nanosecond time resolution by exploiting a repetitive step-scan methodology in which an acceptable signal-to-noise ratio is achieved by accumulation of flash-step-scan cycles [6,7].

Most enzyme-catalysed reactions are not amenable to photolytic initiation, although photolytic decaging of substrates to give the active form has been used to good effect in studies of muscle contraction and DNA repair [8,9]. The other methods which can be exploited with FTIR spectroscopy to gain time-resolved spectra are continuous and stopped-flow mixing [10,11]. The latter has been widely exploited in the study of enzyme-catalysed reactions in conjunction with UV-visible, fluorescence and, more recently, CD spectrometers.

We have expended considerable efforts to establish continuous mixing, since it has the desirable property of permitting continuous scanning at a fixed distance from the mixing point (and hence time delay after mixing) so that low-noise spectra can be accumulated. In experiments where it is desired to obtain difference spectra of bound ligands in the form of enzyme reaction intermediates against the strong background absorbance of the enzyme it is necessary to use a high ($\approx 2\text{ mM}$) enzyme concentration. Despite evaluating many designs, it proved impossible to avoid enzyme denaturation in the mixing process. This was largely due to the difficulty of mixing the viscous enzyme solution with the substrate solution at the relatively low velocities available in a continuous mixing system when the reactant volumes available are limited. Fortunately stopped-flow mixing proved to be much more amenable and an effective apparatus has been constructed which has a dead time for mixing of $\approx 15\text{ ms}$. Several of the design features of the apparatus are unusual in view of the short pathlength of the IR cell ($50\text{ }\mu\text{m}$), which necessarily means that high pressures are generated during the mixing process. In the present paper we describe the design, construction and operation of an apparatus for use with aqueous solutions as well as some applications to the measurement of time-resolved spectra of acylchymotrypsin deacylation.

DESIGN, CONSTRUCTION AND OPERATION OF THE APPARATUS

The stopped-flow drive and valve systems

A primary consideration in the design of the apparatus is the high pressure that develops during the flow phase through the IR cuvette, since the pathlength is only $50\text{ }\mu\text{m}$, and concentrated protein solutions are viscous. This consideration, and the desire to minimize the dead volumes involved, led us to adopt HPLC technology for the interconnections, since we could be sure that any pressure likely to be generated in the drive system would be tolerated. An overall view of the apparatus is shown in Figure 1. The pneumatic flow-inducing system is largely conventional for stopped-flow applications. The length of the flow pulses is defined by a barrel ratchet mechanism that allows the syringe contents to be discharged as a fixed number of shots depending on the pitch of the latching stops on the barrel. We use a barrel with ten latches together with 0.5 ml Hamilton gas-tight syringes that have Teflon plungers, which means that $50\text{ }\mu\text{l}$ of each reagent is used per shot. We have successfully used the apparatus with one syringe replaced with a $100\text{ }\mu\text{l}$ syringe, since the dead volume is about $25\text{ }\mu\text{l}$ (see below). As the pneumatic driving ram operates, a caliper pushes the drive barrel forward. On the return (reset) stroke the spring-loaded caliper opens and retracts over the latch on the barrel ready for the next drive stroke. An adjustable optical-position-sensitive detector cuts off the air

supply at the end of the drive stroke. The refilling operation involves simply the separation of the spring-tensioned caliper and drawing back the barrel and drive plate to the first shot position. The syringes are refilled by drawing back manually. The valve system is electrically interlocked to prevent attempts to refill when the flow system is directed through the reaction cell. The 2.54 cm drive ram is operated at 552 kPa (80 lbf/in^2), which potentially produces an hydraulic pressure in the drive system of 19.32 MPa (2800 lbf/in^2). The actual pressure during steady-state drive is likely to be somewhat less than this, owing to the motion in the system.

The components of the system that caused most problems during development were the valves that permit refilling of the drive syringes with reagents. Several types of rotary microvolume valves, including Hamilton HPLC-type valves were tried, but all proved to leak during the pressure pulse. A slider valve operated by a pneumatic ram and constructed from blocks of Teflon proved successful in that the sliding element of the valve could be clamped very tightly between the outer segments while sliding movement could still be attained by operating the pneumatic ram at 552 kPa (80 lbf/in^2). The low frictional properties of Teflon sliding over O-rings which provide the seals in the valve have meant that long-term operation has been possible without damage.

Since it is necessary to maintain dry-air-purge conditions inside the spectrometer sample chamber, a good seal is required at the point where the reagent tubes enter the spectrometer. This has been achieved by arranging that the Tefzel HPLC tubes (Anachem, Luton, Beds., U.K.; 0.8 mm internal diameter), which carry the reagents in and products out, are interfaced to the chamber by a slotted plate which slides into the side of the sample chamber. Zero-dead-volume plastic HPLC connectors are screwed into each side of this interfacing plate to carry the tubes through the plate.

The mixing system and IR cuvette

Figure 2(a) shows the mixing and cuvette assembly, while Figure 2(b) shows a close-up of the mixing-jet system. A prime concern in the design was the desire to keep the dead volume as low as possible and, accordingly, the mixing-jet assembly is mounted into the front plate of the cuvette in contact with a Teflon washer which abuts directly on to the drilled (1 mm diameter) CaF_2 inlet plate of the cuvette. The dead volume from the drive syringes to the mixer is about 0.25 ml for each line, the dead volume between the mixer and the sample space is $\approx 25\text{ }\mu\text{l}$, and the sample observation space ($1\text{ cm} \times 2\text{ cm} \times 50\text{ mm}$) requires a volume of $10\text{ }\mu\text{l}$. In FTIR spectrometers the beam is circular, softly focused to a diameter of about 0.8 cm , so only 25% of the sample space is interrogated by the IR beam. It is possible that the dead space prior to mixing might be reduced by the use of smaller-internal-diameter HPLC tubing, but the pressure in the drive system will increase and may cause problems with valve leakage or the flow rate. It is important that the drive system is vibrationally isolated from the spectrometer, the only connection being the flexible Tefzel tubing, otherwise the spectrometer is seriously affected by the shock of the drive pulse. The cuvette is much more robust than standard liquid-sampling cuvettes in that a carefully (flat) surfaced 9 mm front plate is used together with six bolts to clamp the assembly together. Teflon gaskets ($100\text{ }\mu\text{m}$) are used to seal the CaF_2 windows to the front and back plates and a $50\text{ }\mu\text{m}$ Teflon spacer separates the windows. The clamping bolts have to be very tight to prevent leakage and have to be tightened very carefully and systematically as with a motor-car cylinder head. It is unfortunately rather easy to break the expensive CaF_2 plates

Figure 1 General schematic diagram of the stopped-flow and IR cuvette system

The stopped-flow drive system is mounted on a table or trolley that is vibrationally isolated from the spectrometer, the only connection being the HPLC tubes that conduct the reactants to the mixing system. The IR cuvette is viewed from above. The interface plate that carries the reactant tubes through the wall of the sample chamber is not shown in this diagram, but is described in the text. The various components that comprise the system are described in detail in the text.

during tightening, so the cell should be dismantled as infrequently as possible.

The output side is uncritical, since the short pathlength of the cuvette means that the flow resistance is sufficient to arrest the flow rapidly after the drive pulse ceases. We have tested the system with inclusion of a stop syringe, but have found that this is an unnecessary refinement in the reaction systems we have studied.

A linear potentiometer connected as a potential divider which abuts the drive ram has been used to measure the dynamics of flow in the drive side of the system. Differentiation of the movement profile gives the velocity profile which shows that the maximum fluid flow velocity is reached in < 5 ms, while deceleration also takes < 5 ms. The stop is sufficiently fast that no material mixed at less than the maximum velocity reaches the observation chamber. The stop can, of course, be made more abrupt by use of a stopping syringe, which may be required for the study of reactions which are faster than those reported below.

The design of the mixer (see Figure 2b) is a simple six-jet device. Several mixing devices were assessed prior to adoption of this design, including a ball mixer as well as two- and four-jet tangential configurations. The Gibson eight-jet tangential mixer [10] worked well, but was difficult to construct. It is perhaps surprising that such a simple mixing device works satisfactorily, since tangential injection into the mixing barrel is usually deemed necessary for effective mixing. The mean mixing jet velocity is ≈ 2 ms $^{-1}$, the mean velocity in the barrel of the mixer is ≈ 1 ms $^{-1}$ and the velocity in the observation chamber is ≈ 17 ms $^{-1}$. We assume that the high back pressure exerted on the mixer by the IR cell aids turbulent flow and efficient mixing.

Testing of the stopped-flow cuvette

The IR stopped-flow cuvette was mounted in the optical beam line of a Durrum UV-visible stopped-flow spectrophotometer. Mixing was evaluated by using pH indicators (e.g. Phenol Red) and appropriate buffer solutions, which were used to provide a

jump in pH and hence a colour change. Despite the short pathlength, changes in absorbance were easily monitored and showed that, with non-viscous solutions, the dead time was ≈ 15 ms. To evaluate the effect of increased viscosity, a buffered solution of 100 mg/ml chymotrypsin was used to jump the pH. The dead time increased to ≈ 20 ms under these conditions. Similar results were obtained in measurements of the chymotrypsin-catalysed hydrolysis of *p*-nitrophenyl acetate. The measured dead times are not critical in the applications reported here, since the minimum IR scan time (250 ms) that we have been able to use is slow by comparison with the measured dead times.

The IR spectrometer and the time resolution that can be achieved

The stopped-flow system is interfaced with a Nicolet 60SX high-performance spectrometer. This instrument is capable of scanning spectra at a rate of 4 s $^{-1}$ at a resolution of 2 cm $^{-1}$. This is considerably slower than the latest instruments (up to 25 s $^{-1}$), but still allows the rapid-mixing facility to be usefully exploited. The maximum rate of reaction depends upon the experimental protocol. The spectral quality and the time resolution can be enhanced if scans can be accumulated from sequential shots of the stopped-flow system and cross-added at each scan interval after the trigger for data collection is applied. In this way the noise can be reduced by co-addition of spectra at a specific time after mixing; the spectral quality is dependent upon the number of shots used, and hence on the amount of material available. For example, if ten scans are required for an acceptable signal-to-noise ratio, then ten stopped-flow shots are used. This procedure will, for example, allow the measurement of the difference spectra of reaction mixtures (where the end point is subtracted from previous spectra) with a half-time of ≈ 1.5 s and an absorbance change in the region of 0.01. For a smaller absorbance change, more scans will be required. As an approximate guide, scans can be accumulated during the period in which a 10% change in concentration occurs; outside this range, significant distortion, caused by product features, will occur. It is

Figure 2 Cuvette and mixing jets of the stopped-flow system

(a) Shows a close-up side view of the IR cuvette. (b) Shows a drawing of the mixing-jet assembly, which was machined from a single piece of Teflon. The marked dimensions are correct, but the diagram has been partly rescaled in order to assist visualization.

sometimes the case that product features fall outside the spectral range of interest; in such cases they do not affect the spectral profile, only the intensity is decreased. The time-resolution and noise performance are much reduced if only a single stopped-flow shot is used and early scans are averaged prior to subtraction of the end-point spectrum. It is often possible to manipulate the conditions of an experiment in such a way as to allow useful information to be obtained despite the limited time resolution currently available. The parameters that can most easily be manipulated are pH and temperature. We have used pH variation

Figure 3 Timing arrangements for the synchronization of the stopped-flow mixing process and the forward-data collection-mirror sweep of the Michelson interferometer of the FTIR spectrometer

Synchronization and triggering of the drive for the flow system based on the 'take data' ('TKDA') signal emitted by the spectrometer to indicate its scanning status is achieved by using a counter-timer as described in the text.

extensively in our studies of acylchymotrypsins and this has allowed us to examine acyl groups of varying specificity [12–14]. It remains the case that highly specific chymotrypsin substrates can only be studied at low pH using these methods, well below the pH optimum of the enzyme-catalysed reaction. This will be true even if the fastest-scanning instrument was available.

Triggering and data acquisition

An important consideration in the attainment of optimal performance concerns the triggering of data acquisition following the stopped-flow pulse. In order to avoid mirror wobble during acceleration of the moving mirror from a stationary state, the spectrometer is designed for continuous scanning. This means that the mirror is constantly in motion, and this motion must be synchronized with the stopped-flow shot such that data acquisition commences after the mixing dead time and that this coincides with the start of a mirror stroke. If this is not done, the information from the first scan is lost. A counter-timer is triggered by the start of the reverse stroke of the moving mirror (i.e. the reset stroke that returns the mirror to the start position for a forward scan in which data are taken) when the 'take data' ('TKDA') output signal changes level signifying the end of data acquisition for that scan (see Figure 3). The stopped-flow drive is triggered after a set delay that will allow the flow-stop drive cycle to be complete by the time the moving mirror has turned round and is ready to commence the forward stroke. This time is measured by the counter-timer and the dead time is added on. It is necessary to allow ≈ 5 ms latitude in these values, as the mechanical drive system is subject to a little jitter in the time it takes to complete the drive cycle. The drive time is dependent upon the air pressure used to drive the rams and is 30 ± 5 ms in our system when driven at 552 kPa (80 lbf/in²). The mirror reverse stroke takes 100 ms, so there is a delay of ≈ 60 ms between the mirror turnaround and the triggering of the drive system, since we allow at least 10 ms dead time to allow for jitter.

Figure 4 Time courses for the deacylation of cinnamoyl-chymotrypsin at pH* 8.45

(a) Shows the spectra for the deacylation of the $^{12}\text{C}=\text{O}$ compound, whereas (b) shows the spectra for the $^{13}\text{C}=\text{O}$ variant. (c) Shows the difference between (a) and (b), i.e. $^{12}\text{C}=\text{O}$ minus $^{13}\text{C}=\text{O}$. All features that arise from perturbation of the enzyme spectrum by the presence of the ligand are eliminated by this subtraction. The experiments were conducted by mixing cinnamoyl-chymotrypsin (100 mg/ml in unbuffered $^2\text{H}_2\text{O}$ at pH* 4.0), prepared as described by White et al. [13] with 100 mM pyrophosphate buffer, pH* 8.5. The mixed solutions (50 μl of each reactant per shot) had a final pH* of 8.45. Spectra were scanned at 4 s^{-1} in blocks of 50 at 2 cm^{-1} resolution and were processed as described in the text. The difference spectra were created by subtracting the spectrum of the products at the reaction end point from each of the single-beam acyl-enzyme spectra taken at the given times after initiation of the reaction. Baseline correction was applied such that a flat zeroed baseline was achieved between 1900 and 1760 cm^{-1} . In order to achieve an acceptable signal-to-noise ratio, all spectra were subjected to a 25-point dynamic smoothing process.

Figure 5 Time course for the deacylation of dihydrocinnamoyl-chymotrypsin at pH* 8.45

The acyl-enzyme was prepared at pH* 4.0 as described by Johal et al. [14] and mixed with pyrophosphate buffer as described in the legend to Figure 3. In this case 25 scans were averaged to obtain each difference spectrum. The reaction-end-point spectrum was subtracted from the single-beam acyl-enzyme spectra taken at the given times to generate the difference spectra.

Some examples of time-resolved measurements made with the system

The general experimental methods are described in [12–14]. Figures 4(a), 4(b) and 4(c) show the results of some pH-jump isotope-edited difference spectroscopic experiments on cinnamoyl ($\phi\text{-CH}=\text{CH}\cdot\text{CO}-$)-chymotrypsin, measured at pH* 8.5. The cinnamoyl-chymotrypsin was prepared at pH* 4.0 and jumped to pH* 8.5 by the mixing process. The progress of deacylation is reflected in all the bands in Figures 4(a) and 4(b), where the features represent a combination of the acylcarbonyl-group absorbance and features which result from protein perturbation, the latter being dominant. The decrease in absorbance at 1695 cm^{-1} is well-fitted by a first-order rate process (results not shown). In Figure 4(c), where the spectra of $^{13}\text{C}=\text{O}$ -cinnamoyl-chymotrypsin have been subtracted from those of the $^{12}\text{C}=\text{O}$ variant, the absorbance centred on 1700 cm^{-1} arises solely from the acyl carbonyl group, since protein perturbation has been removed by the isotope-editing process; this also declines according to a first-order rate process [13]. The data were accumulated in blocks of 50 scans, and the means of some of these blocks were used to form difference spectra when the end point of the reaction was subtracted. Each block was acquired in 12.5 s at four scans/s, and therefore the maximum time resolution was 6.25 s with this protocol. The smoothing that was applied in order to achieve an acceptable signal-to-noise ratio somewhat degraded the resolution from 2 cm^{-1} such that the two bands characteristic of the productive and non-productive conformers of the bound acyl group and seen in conventionally collected spectra at lower pH* are not resolved in this experiment [12,13].

Figure 5 shows a series of difference spectra for the deacylation of dihydrocinnamoyl ($\phi\text{-CH}_2\text{-CH}_2\cdot\text{CO}-$)-chymotrypsin at pH* 8.5. This acyl group is an order of magnitude more specific than the cinnamoyl group, and it can be seen that the apparatus is capable of describing the deacylation process in reasonable detail. In this case 25 scans have been averaged for each difference spectrum shown. The $^{13}\text{C}=\text{O}$ spectra, equivalent to those shown in Figure 5, proved to be essentially identical, and when the $^{12}\text{C}=\text{O}$ spectra were ratioed against the $^{13}\text{C}=\text{O}$ versions, no

Figure 6 Difference spectrum of $^{12}\text{C}=\text{O}$ dehydrocinnamoyl-chymotrypsin minus the reaction end point at $\text{pH}^* 11.0$

Acyl-enzyme was prepared at $\text{pH}^* 3.0$ as for the other acyl-enzymes and mixed with 200 mM Caps buffer, $\text{pH}^* 11.5$, the resulting pH^* being 11.0. The first four scans (4 s^{-1}) from ten stopped-flow shots were averaged to give a mean acquisition time of 0.5 s and a total of 40 scans.

significant features remained in the difference spectra. This experiment has been independently repeated and gave the same result. At lower pH^* the $^{12}\text{C}=\text{O}$ -minus-enzyme spectra show features in the $1720\text{--}1680 \text{ cm}^{-1}$ region that are comprised both of protein perturbation caused by the presence of the ligand and of the acyl carbonyl group [13]. The isotope editing in the $^{12}\text{C}=\text{O}$ minus $^{13}\text{C}=\text{O}$ spectra removes the perturbation features. There is a clear, if rather broad, band centred at 1731 cm^{-1} , but this feature is apparently absent at high pH^* , as described above. There appear to be two possible causes of this unexpected behaviour. First, the band may have broadened to an extent that renders it invisible. Secondly, the failure to observe the band may be the result of the lower intensity of this carbonyl band as compared with that of the cinnamoyl group as deduced from studies of model esters. Neither of these explanations is wholly convincing, and further repetition of the experiment is required.

Figure 6 shows a difference spectrum of dehydrocinnamoyl ($\phi\text{-C}\equiv\text{C}\cdot\text{CO}-$)-chymotrypsin minus enzyme at $\text{pH}^* 11.0$. In this case only the first four scans were used for averaging, and ten stopped-flow shots (i.e. 40 scans) were used to construct the difference spectrum. The half-life of the acyl-enzyme is 7.5 s, while the midpoint of the spectral acquisition occurred at 0.5 s. It is thus apparent that averaging of shots can give difference spectra with an excellent signal-to-noise ratio. Recently we have measured spectra of $\phi\text{-CH}_2\text{-CH}_2\cdot\text{CO}$ -chymotrypsin at high pH^* , where only the first scan (mean acquisition time 0.125 s) was used and 50 of these have been averaged. The resulting spectrum was very similar to that shown in Figure 5.

Figure 7 shows the result of a different type of experiment in which chymotrypsin was acylated with glycylglycine *p*-nitrophenyl ester at $\text{pH}^* 5.5$. The reaction was monitored for 25 s, at which time acylation will be complete, but deacylation insignificant, at this pH^* (A. White and C. W. Wharton, unpublished work). The noise level was achieved without smoothing by co-addition of 10 scans (2–11, 0.2–2.75 s) from each of 10 stopped-flow shots and subtraction of the endpoint spectrum [40 scans, $(15\text{--}25 \text{ s}) \times 10 \text{ shots} = 400 \text{ scans}$]. The band at $\approx 1770 \text{ cm}^{-1}$ which decreases represents the nitrophenyl ester carbonyl group. The carbonyl band(s) for the acyl-enzyme ester group is expected to occur at $< 1700 \text{ cm}^{-1}$, and either or both of the small new bands which appear at ≈ 1690 and $\approx 1674 \text{ cm}^{-1}$ may represent

Figure 7 Acylation of chymotrypsin by glycylglycine *p*-nitrophenyl ester at $\text{pH}^* 5.5$

Chymotrypsin (100 mg/ml, unbuffered at $\text{pH}^* 8.0$) was mixed with 4 mM glycylglycine *p*-nitrophenyl ester in 50 mM formate buffer, $\text{pH}^* 4.0$, containing 2.5% dimethyl sulphoxide, the resulting mixed solution having a pH^* of 5.5. Scans at 2 cm^{-1} resolution were accumulated at 4 s^{-1} for 25 s in each of ten shots. Each of the difference spectra shown were formed by adding ten scans (2–11, 12–21 etc.) from each of the ten stopped-flow shots and subtracting 40×10 scans taken for the end point of the reaction between 15 and 25 s. Glycylglycine *p*-nitrophenyl ester was prepared by treating benzyloxycarbonylglycylglycine *p*-nitrophenyl ester (1 g) (Bachem, Bubendorf, Switzerland) with 10 ml of HBr in acetic acid for 1 h, followed by washing with dry ether, filtering and drying in a vacuum desiccator. The stock solution (60 mM) was prepared in dry dimethyl sulphoxide.

this carbonyl group. There is a strong protein perturbation band in dihydrocinnamoyl-chymotrypsin at 1692 cm^{-1} [12,13], so it would be unwise to make assignments of these bands until such time as the isotope-edited experiments have been done. It is noteworthy that the noise level is highest in the region around $1630\text{--}1660 \text{ cm}^{-1}$, where the protein absorbance is strongest and that significant changes occur in the protein perturbation region ($1500\text{--}1700 \text{ cm}^{-1}$) as the acylation proceeds. Again isotope-editing is needed to positively identify perturbation features from potential changes in the substrate spectrum which may occur on acylation.

CONCLUSIONS

It is hopefully apparent from the description of the stopped-flow system that it is relatively unsophisticated and should be straightforward to construct. The drive part of the system could probably be replaced with any of the commercial versions available provided that they are tested to ensure that they can generate and withstand the pressure involved. The triggering arrangements can be implemented by a commercial counter-timer, although a custom-made unit can be assembled much more cheaply. It is probably wise to check that adequate mixing is provided in the particular system being studied, particularly if the mixed volumes are reduced. The eight-jet tangential mixer described by Gibson [11] is recommended if a more elaborate mixer is deemed necessary.

The examples presented here show that the system functions effectively on the time scales we have used. We will shortly interface the stopped-flow system with a spectrometer that will scan at a rate of 25 s^{-1} at 2 cm^{-1} resolution and confidently expect to be able to exploit the system more effectively with this spectrometer which is also an order of magnitude more sensitive. The very short pathlength (5 mm) required for the use of water solutions as a result of the strong absorbance of water at 1640 cm^{-1} means that an apparatus for use with water cannot be

designed to use a transmission cuvette. A design that employs attenuated total reflectance could probably be made to work, since the effective pathlength of the evanescent wave is very short. The transmission type of system described here should have wide applicability, since working with $^2\text{H}_2\text{O}$ is not normally a problem, and many enzyme reactions, such as those catalysed by dehydrogenases, esterases and proteinases can readily be followed by IR spectroscopy (C. W. Wharton, unpublished work). We would also foresee applications to problems of protein folding, since IR spectroscopy yields information that relates to protein secondary structure [15].

This work was funded by the Molecular Recognition Initiative of the Science and Engineering Research Council. We thank J. Harcourt and C. Hardman for their engineering design and construction skills.

REFERENCES

- 1 Singer, P. T., Smalas, A., Carty, R. P., Mangel, W. F. and Sweet, R. M. (1993) *Science* **259**, 669–673
- 2 Doig, S. J., Reid, P. J. and Mathies, R. A. (1991) *J. Phys. Chem.* **95**, 6372–6379
- 3 Ames, J. B., Ros, M., Raap, J., Lugtenburg, J. and Mathies, R. A. (1992) *Biochemistry* **31**, 5328–5334
- 4 Diller, R., Iannone, M., Cowen, B. R., Bogomolni, R. A. and Hochstrasser, R. M. (1992) *Biochemistry* **31**, 5567–5572
- 5 Dobson, G. R., Hodges, P. M., Healy, M. A., Poliakoff, M. and Turner, J. J. (1987) *J. Am. Chem. Soc.* **109**, 4218–4224
- 6 Gerwert, K. (1993) *Curr. Opin. Struct. Biol.* **3**, 769–773
- 7 Siebert, F. (1993) in *Biomolecular Spectroscopy Part A* (Clark, R. J. H. and Hester, R. E., eds.), pp. 1–54, John Wiley and Sons, Chichester
- 8 McCray, J. A. and Trentham, D. R. (1989) *Annu. Rev. Biophys. Biophys. Chem.* **18**, 239–270
- 9 Meldrum, R. A., Meaking, W. and Wharton, C. W. (1994) *Nucleic Acids Res.* **22**, 1234–1241
- 10 Chance, B. (1951) *Rev. Sci. Instrum.* **22**, 619–627
- 11 Gibson, Q. H. and Milnes, L. (1964) *Biochem. J.* **91**, 161–171
- 12 White, A. J. and Wharton, C. W. (1990) *Biochem. J.* **270**, 627–637
- 13 White, A. J., Drabble, K., Ward, S. and Wharton, C. W. (1992) *Biochem. J.* **287**, 317–323
- 14 Johal, S. S., White, A. J. and Wharton, C. W. (1994) *Biochem. J.* **297**, 281–287
- 15 Surewicz, W. K., Mantsch, H. H. and Chapman, D. (1993) *Biochemistry* **32**, 389–394