Stopped Flow Fourier Transform Nuclear Magnetic Resonance Spectroscopy. An Application to the α-Chymotrypsin-Catalyzed Hydrolysis of *tert*-Butyl-L-phenylalanine

John J. Grimaldi and Brian D. Sykes*

Contribution from the Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138. *Received August 14, 1974*

Abstract: A stopped flow Fourier transform nuclear magnetic resonance spectrometer capable of monitoring reactions with half-times as short as 1 sec is described. By utilizing the Fourier difference spectroscopy technique described by Ernst, this spectrometer (1) can be used to detect the weak resonances of reacting species in the presence of a strong solvent resonance and (2) allows the signal averaging of transients obtained at the same time after mixing in successive transients. Various pulse sequences for obtaining stopped flow FT spectra are given and stopped flow FT nuclear magnetic resonance is used to measure the α-chymotrypsin hydrolysis of L-phenylalanine *tert*-butyl ester. The capabilities of stopped flow FT nuclear magnetic resonance are discussed in detail.

The development of Fourier transform (FT) techniques in nuclear magnetic resonance (nmr) spectroscopy has focused attention on the possibility of using nmr to follow time-dependent phenomena such as irreversible chemical reactions or chemically induced dynamic nuclear polarization.[1-5] Obviously, having the nmr spectrum of a reaction mixture including *reactants, intermediates, and products* at several times during a reaction would provide a very powerful tool for elucidating reaction mechanisms. We have previously described the construction of a stopped flow nmr spectrometer and its use to study the acid-catalyzed dissociation of $Ni(NH_3)(H_2O)_5^{2+}$ [1] and conformational changes produced in Concanavalin A by the binding of Mn^{2+}, Ca^{2+}, and α-methyl-D-mannoside.[2] Both of these reactions involved following a single resonance, thereby allowing the observation of reactions with very short half-times by sacrificing the requirement to observe the complete spectrum. We have also used FT nmr methods to follow the relatively slow cleavage of cell wall tetrasaccharide by lysozyme.[3]

In the present paper we describe the application of stopped flow Fourier transform nmr techniques to the α-chymotrypsin-catalyzed hydrolysis of the *tert*-butyl ester of L-phenylalanine (PABE). Through this application we are able to assess the capabilities and limitations of stopped flow FT nmr. The success of this technique depends on overcoming two practical problems of FT nmr. First, under most reaction conditions, a strong solvent resonance will create dynamic range problems and will interfere with the observation of the much weaker resonances of the reacting species. This is especially true for biochemical reactions which are normally studied in water. Second, a lock system which is not affected by pushing and mixing is needed to allow averaging of transients. These two problems were simultaneously solved in the present application by using the Fourier transform difference spectroscopy technique described by Ernst.[6] The basis of this technique is that in the presence of one very strong resonance such as water, the free induction decay (FID) can be approximated by a single carrier frequency which is modulated by the difference frequencies between the strong resonance and each weak resonance. The free induction decay of the difference frequencies can be extracted by envelope detection with a diode detector followed by removal of the water signal by a high pass filter. The great advantage of this technique is that it allows very weak signals to be observed in the presence of a strong solvent signal, and at the same time allows successive transients, which are now independent of magnetic field fluctuations, to be added together for signal averaging. The drawback is that FT difference spectroscopy does not differentiate between resonances upfield or downfield of the strong resonance.

The stopped flow Fourier transform nmr experiment can be described by the pulse sequence

$$[\text{PUSH–DP}[[\text{PULSE–AT–DT}]_{NT}\text{–DK}]_{NK}]_{NP} \qquad (1)$$

where AT is the spectral aquisition time; DT, DK, and DP are respectively the delay times between transients, between blocks of transients, and directly after the push; and NT is the number of spectra per block, NK the number of blocks per push, and NP the number of pushes. The maximum time resolution achievable in stopped flow FT nmr, *i.e.*, the number of spectra obtainable per reaction half-time, is governed primarily by the restriction that $AT \ll \tau_{1/2}$ which assures that the spectral line shapes remain undistorted by the reaction. However, for many reactions, the practical limitations imposed by relaxation times (T_1 and T_2), spectral resolution desired, and sensitivity will further restrict the time resolution.

Experimental Section

Stopped flow Fourier transform experiments require a rapid mixing system coupled with a computer controlled pulsed nmr spectrometer. The flow system used in these experiments has been described previously.[2] It consists of a rapid mixing cell located in a modified Varian V-4331 probe and standard driving and stopping syringe blocks.

A block diagram of the pulsed nmr spectrometer is shown in Figure 1. The output of the transmitter section of the 40.5 MHz V-4311 radiofrequency unit is gated by a V-4357 radiofrequency gate, amplified by a V-4357 radiofrequency amplifier, and then applied to the transmitter coils of the V-4331 probe. The radiofrequency gate is turned on by a 74121 monostable multivibrator which is triggered by the NOVA 1220 computer. The receiver section of the V-4311 is operated in the diode detector mode. Its output is bandpass filtered by an Ithaco 4215 active filter, digitized by a Biomation 610B, and then transferred to the computer. Data handling is accomplished with a NOVA 1220 12K computer equipped with a Computer Operations LINC Tape drive for data storage and a Megatek BP 721 DA converter for scope display and hardcopy output. Fourier transformation is accomplished with a FFT program provided by Data General.

The spectral widths obtainable with this spectrometer are limited in two ways. First, as the sweep width is increased much beyond 250 Hz, the definition and appearance of the spectrum becomes

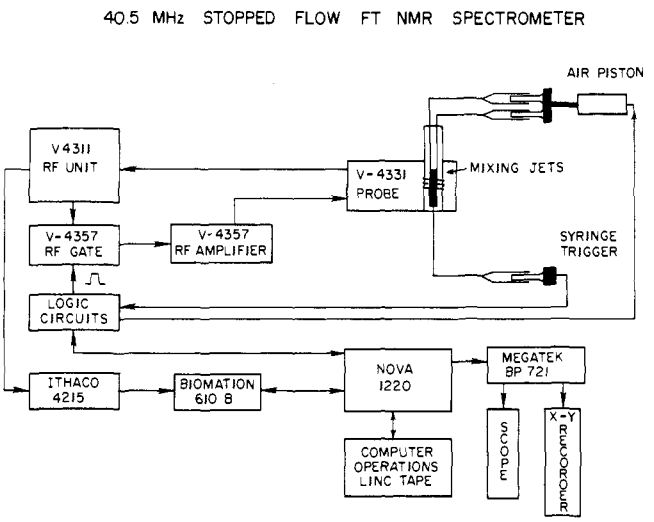

Figure 1. Block diagram of the 40.5 MHz stopped flow Fourier transform nmr spectrometer.

poor since the Biomation can store only 256 points. Second, the 6 bit dynamic range of the Biomation requires that the water response be removed by filtering prior to digitization. This prevents observation of frequencies below the cut-off frequency of the high pass section of the filter, which is typically of the order of 40–100 Hz. In addition, the filter introduces frequency-dependent phase shifts and amplitude attenuation in the spectrum. In the present case, the center frequency of the filter was chosen so that the phase shift was linear and the attenuation flat over the spectral range of interest.

The radiofrequency pulse length was adjusted to optimize the signal-to-noise ratio for the acquisition times and pulse delays used, and the T_1's of the particular sample. Typically the pulse length was near 38 μsec, corresponding to flip angles of approximately 50°.

The pulse sequence described by eq 1 is initiated by activating the hydraulic piston which pushes the driving syringes. When the stopping syringe strikes the stopping block, the computer is triggered. After a time DP, the computer initiates a radiofrequency pulse, delays, and then triggers the Biomation 610B to record the FID. During delay time DT, the new FID is transferred to the Nova 1220 and added to previously recorded FID's within the NT block. After completing each block, the computer delays time DK before acquiring the next block. After NK blocks are acquired, the computer waits for another trigger from the stopping syringe or returns to its idling mode if NP pushes have already been completed.

α-Chymotrypsin was purchased from Worthington Biochemicals (Type CDI, Lot No. 8LK). L-Phenylalanine *tert*-butyl ester was purchased from Cyclo Chemicals (Lot No. H3311) and was used without further purification. All buffers were 0.4 *M* K_2HPO_4 adjusted to the appropriate pH with concentrated HCl. The sample temperature was 23 ± 1°. Both of the reactants and the sample cell were allowed to equilibrate at this temperature before mixing.

Results

The stopped flow Fourier transformed spectra of the hydrolysis of L-phenylalanine *tert*-butyl ester by α-chymotrypsin are shown in Figures 2 and 3. Both sets of spectra were obtained by mixing 78 m*M* PABE pH 6.35 with 20 mg/ml α-chymotrypsin pH 8.5. The pH after mixing was approximately 6.9.[7] The spectra shown in Figure 2 were obtained from a single push (NP = 1) while those in Figure 3 are the average of eight pushes (NP = 8). Each spectrum in Figure 2 represents the average of four FID's (NT = 4) with the acquisition time AT = 0.512 sec (256 points), delay time DT = 0.703 sec, and DK = 0.001 sec. Therefore, the total time between each spectrum is 4.86 sec. The corresponding spectra in Figure 3 represent the average of 32 FID's ((NT = 4) and (NP = 8)). All spectra were taken in H_2O. The drift in the base line is caused by the initial distortion that occurs at the beginning of each FID due to filtering the water response.

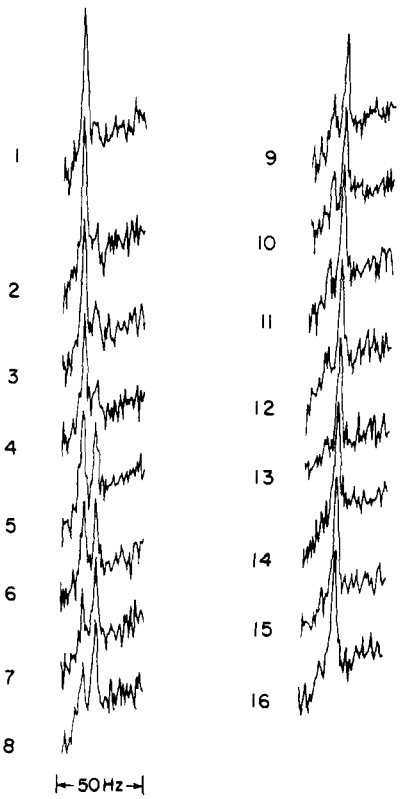

Figure 2. Stopped flow Fourier transform nmr spectra obtained by mixing 78 m*M* PABE with 20 mg/ml α-chymotrypsin (final pH 6.9[7]). Spectral width shown in 50 Hz. The parameters of eq 1 were NP = 1, NT = 4, NK = 16, DP = 1 msec, AT = 0.512 sec, DT = 0.703 sec, DK = 1 msec. The filter bandpass was 100 to 200 Hz. The delay between the radiofrequency pulse and beginning of acquisition was 11 msec. The total time between each spectrum is 4.86 sec.

The low- and high-field peaks in each spectrum are the *tert*-butyl protons of the ester and alcohol, respectively. The ester peak occurs at 137.4 Hz from water, and the chemical shift difference between the two peaks is 7.6 Hz at 40.5 MHz. No chemical shift change of either resonance was observed during the course of the reaction.

The spectra in Figures 2 and 3 show the decrease in intensity of the ester peak with time and the concomitant increase of the alcohol peak. A plot of the intensity of each peak as a function of time is shown in Figure 4. In order to obtain the initial velocity for the ester hydrolysis, the initial slope of each curve in Figure 4 was calculated. The first point was not included since the magnetizations had not reached their "equilibrium" values (see Discussion) and thus were not on scale with the remaining points. Since each slope is measured in intensity units per second, the intensity at some point during the reaction has to be related to concentration. For the ester peak the intensity is extrapolated back to time zero and set equal to 39 mm. The intercept is 98.5 mm. This gives a slope of 2.47 mm/sec which is equivalent to 0.99 m*M* ester hydrolyzed per second. For the alcohol, the intensity levels off at 70 mm. This gives a slope of 1.65 mm/sec which is equivalent to 0.92 m*M* alcohol formed per second.

Discussion

The maximum time resolution in stopped flow FT nmr is obtained with AT as short as possible, NT equal to one, and DP, DT, and DK as close to zero as possible, subject to the interrelated limitations imposed by relaxation times (T_1

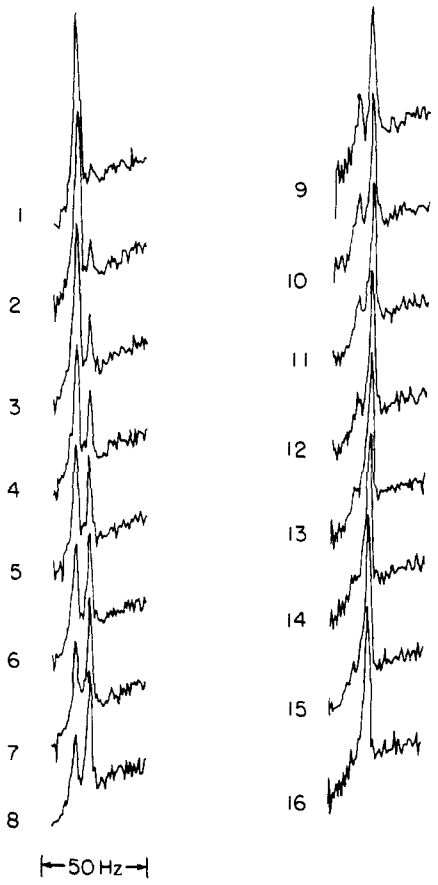

Figure 3. Stopped flow Fourier transform nmr spectra obtained by mixing 78 mM PABE with 20 mg/ml α-chymotrypsin (final pH 6.9[7]). Spectral width shown in 50 Hz. The parameters of eq 2 were: NP = 8, NT = 4, NK = 16, DP = 1 msec, AT = 0.512 sec, DT = 0.703 sec, DK = 1 msec. The filter bandpass was 100 to 200 Hz. The delay between the radiofrequency pulse and the beginning of acquisition was 11 msec. The total time between each spectrum is 4.86 sec.

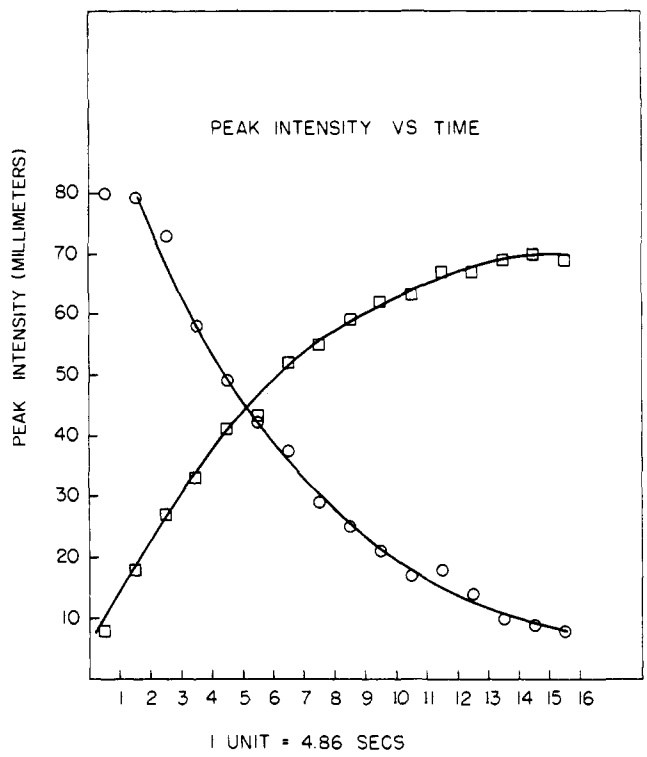

Figure 4. Plot of peak height as a function of time for the *tert*-butyl protons of PABE (○) and alcohol (□). Data from Figure 3. The alcohol curve does not go through the origin since a small amount of alcohol exists in the ester solution prior to mixing.

and T_2), the resolution desired, and the sensitivity which are discussed below. The sensitivity determines whether or not one can operate with NT = 1. If the single transient sensitivity is not sufficient to observe the species of interest, three alternatives are possible: (1) increasing the number of spectra per block while decreasing the number of blocks per $\tau_{1/2}$, (2) increasing the concentrations of reactants, or (3) increasing the number of pushes. Since the first alternative decreases time resolution and the second can decrease $\tau_{1/2}$, the obvious solution is to increase the number of pushes if sufficient volumes of reactant are available. The stopped flow FT nmr spectrometer described above is sufficiently reproducible to permit the averaging of free induction decays obtained at the same time after mixing in successive stopped flow pushes. If sufficient volumes are available, acceptable signal-to-noise ratios can be achieved without sacrifice in time resolution. In the absence of sensitivity restrictions, the maximum possible time resolution depends in detail on which spectral feature (*e.g.*, peak intensity, line width, chemical shift) is being followed with time.

If the intensities of well-separated peaks are being monitored, the maximum possible time resolution is determined by the spin-lattice relaxation times of the peaks. At first sight this would seem to require that the time between successive free induction decays (DT + DK + AT for NT = 1) be several times longer than the T_1's of all of the species involved so that the intensity of each peak is determined solely by the concentration. There are three possible approaches to this potentially rather restrictive limitation. The first is the use of special sequences such as DEFT[8] to return the magnetization toward equilibrium between free induction decays. Second, if relative concentrations of the species involved are not required but only the concentrations of each species as a function of time, and if the T_1's of each species remain constant during the reaction, then the delay time can be much shorter. Under conditions of constant T_1, the intensity of each peak will reflect the time-dependent change in concentration of the reaction species; however, comparison of the intensities of different peaks is no longer straightforward. The analysis is identical with that for the FT progressive saturation T_1 experiment[9] and requires (1) that $T_2^* \ll$ DT + DK + AT (for NT = 1), and (2) that the observation cannot begin until the quasi-equilibrium magnetization is established (~3 (DT + DK + AT)). Here T_2^* is the time constant characterizing the decay of the FID. Obviously, for pulse delays less than T_1, sensitivity is sacrificed since the observed magnetization will be decreased. Third, the alternate pulse sequence

$$(\text{PUSH–DP}_1\text{–PULSE–AT–DK})\text{–}$$
$$(\text{PUSH–DP}_2\text{–PULSE–AT–DK})\text{–} \quad (2)$$

can be used where DP is varied to obtain spectra at successive times after mixing and DK $\gg T_1$.

When line width or chemical shift changes are monitored, the restrictions derived for intensity measurements do not apply except for the above-mentioned sensitivity considerations. Here the important consideration is that the spectral acquisition time must be long enough so that either (1) the observed line widths are not broadened (AT $> T_2^*$) or (2) the resolution is sufficient to follow the chemical shift changes δ involved ($1/\text{AT} \ll \delta$), for the two types of measurements, respectively. Of course the condition $1/T_2^* \ll \delta$ must also hold if chemical shift changes are being followed.

In the particular example that we have examined, the intensities of the reactant and product peaks were monitored. Since the delay times used were shorter than the T_1's of the

species involved, the velocities calculated from the peak intensities as a function of time are valid only if the T_1's for the reactant and product peaks were constant, and the T_2^* was less than DT + AT. Given that the T_1 of the product *tert*-butyl alcohol can be assumed to have been constant during the reaction, and the resolution observed ($\Delta\nu^* \simeq 2$ Hz) gives $T_2^* = 0.15 \ll \mathrm{DT} + \mathrm{AT} = 1.215$ sec, the major concern is the T_1 of the reactant PABE. For this substrate the observed T_1 will be the fast exchange averaged T_1 for the free substrate (T_{1F}) and the substrate bound to α-chymotrypsin (T_{1B})

$$1/T_1 = (P_F/T_{1F}) + (P_B/T_{1B})$$

where P_F and P_B are the fraction of substrate free and bound, respectively.[10] Given that P_F and P_B will change with time during the reaction, the observed T_1 is expected to be time dependent. However, for the initial portion of the reaction where velocities are calculated, P_F is very close to one and $P_B \rightarrow 0$. Also T_{1F} and T_{1B} are not observed to be very different for similar α-chymotrypsin substrates.[10] Therefore the observed T_1 of PABE can be assumed to be constant for the initial velocity calculations. The deviation of the intensities in the first spectrum in Figure 4 reflects the time required for the establishment of the quasi-equilibrium magnetization.[9] In addition, the difference in intensity between the ester peak at time zero and the alcohol at time infinity reflects the difference in T_1's between the two species.

A final point which must be considered is the validity of grouping several FID's together in one time point. It has been shown that this is valid for first-order kinetics, if the acquisition time is kept constant,[11] and it is equally valid for the analyzed zeroth order steady state phase of the Michaelis–Menten enzyme kinetics observed in this manuscript. For higher-order kinetics acquisition of the data in this manner may not be generally valid and one will have to operate with NT = 1.

The initial velocity for the α-chymotrypsin-catalyzed hydrolysis of PABE obtained is in reasonable agreement with a value estimated from the work of Purdie and Benoiton.[12] These workers measured the rates of the α-chymotrypsin-catalyzed hydrolysis of the methyl, ethyl, and isopropyl esters of L-phenylalanine. From their data we estimate by extrapolation that k_{cat} and K_M for the *tert*-butyl ester at pH 6.9 would be 900 min^{-1} and 10 mM, respectively. This corresponds to an initial velocity of 4 mM/sec which is close to the stopped flow value of 1 mM/sec.

Conclusion

The stopped flow apparatus described above is sufficiently reproducible to permit averaging of transients obtained in successive pushes. This has allowed us to achieve a time resolution which must be close to the maximum possible for typical high-resolution nmr spectra. Reaction half-times as short as 1 sec can be measured if meaningful spectra can be obtained with acquisition times of the order of 0.1–0.2 sec and delay times nearly zero, *i.e.*, if a resolution of 5–10 Hz is acceptable. The extension of nmr methods down to $\tau_{1/2} \simeq 1$–10 sec greatly extends the number of reactions which can be studied. In addition, even if practical considerations limit accurate kinetic measurements, it is important to remember that the restrictions on *observing* the spectra of reaction intermediates are much less severe. These spectra will clearly be important in analyzing reaction mechanisms.

References and Notes

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