NMR Spectroscopy

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Time-Resolved Dynamic Nuclear Polarization Enhanced NMR Spectroscopy**

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High-resolution NMR spectroscopy is routinely used for the characterization of small molecules, as well as for the elucidation of structure and functional mechanisms of biological macromolecules.[1,2] Nuclear spins are only weakly aligned even in a high magnetic field, which makes NMR spectroscopy a rather insensitive technique. Consequently, the most common applications of high-resolution NMR spectroscopy are on samples under equilibrium conditions, where extensive signal averaging can be applied to acquire one- or multidimensional spectra over the course of hours and days. A notable way of increasing the available signal in the acquisition of a single NMR scan is hyperpolarization,[3–7] where an independent physical means is used to bring the nuclear spin system into a non-equilibrium, polarized state before the measurement. Applied to biological molecules, for example, hyperpolarized xenon has been used for the identification of hydrophobic cavities in proteins,[8] while chemically induced dynamic nuclear polarization has been used for the characterization of protein-folding intermediates.[6,7] Recently, dynamic nuclear polarization (DNP),[9] a different hyperpolarization technique, has been demonstrated to provide a gain in the signal-to-noise ratio of a factor of approximately 10^4 for single-scan liquid-state NMR spectroscopy.[3] DNP has the advantage that it in principle allows nuclear spins in any small molecule to be polarized, while retaining the advantages of carrying out high-resolution NMR measurements in the liquid state. Once spins have been hyperpolarized, the acquisition of an NMR spectrum can be achieved rapidly, requiring only a time sufficient to distinguish the frequencies of different resonance lines in the spectrum, which can be as low as 10 ms. On the other hand, obtaining an equivalent signal-to-noise ratio by means of conventional signal averaging would typically take several months.[2,3] As hyperpolarization makes this same high signal-to-noise ratio available in one instant, NMR spectroscopy in this modality seems ideally suited for the study of a dynamic process in real time. Here, we demonstrate the potential of time-resolved NMR spectroscopy by using compounds polarized by DNP for the example of monitoring enzyme kinetics. As a model reaction, we have chosen the hydrolysis of N_α-benzoyl-L-arginine ethyl ester (BAEE) by the enzyme trypsin, a well-characterized serine protease.[10,11]

The ^{13}C nuclei, which were present at natural abundance, of a small aliquot of BAEE were polarized by solid-state DNP (see Experimental Section). After dissolution of this aliquot in buffer, whilst maintaining spin polarization, the sample was taken up in a specifically designed sample injector (Figure 1) and then rapidly injected into the adjacent NMR spectrometer. Admixing of a small volume of nonpolarized enzyme solution already present in the NMR spectrometer took place concomitantly with sample injection. The mixing triggered the start of a reaction in which BAEE was converted into N_α-benzoyl-L-arginine (BA; Scheme 1).

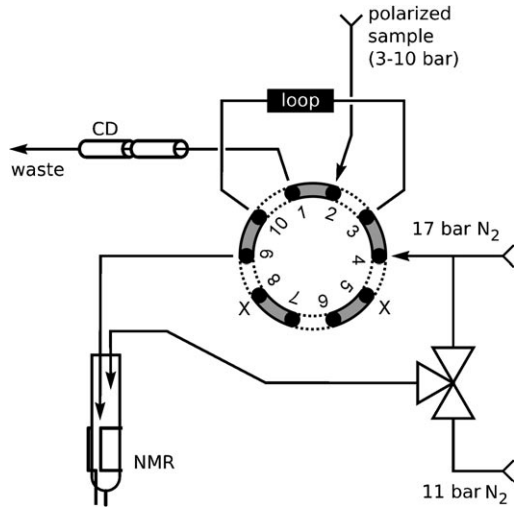
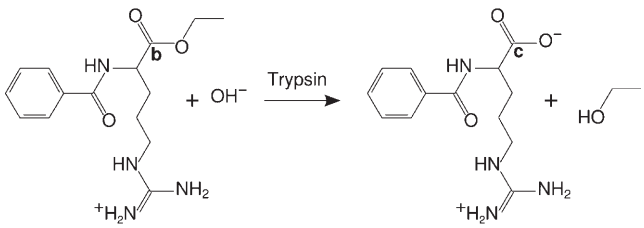


Figure 1. Sample injection system. The dashed lines designate the flow path during the loading phase, and the solid lines with gray shading designate connections during the injection phase.



Scheme 1. Trypsin-catalyzed conversion of BAEE into BA. "**b**" and "**c**" refer to Figure 2.

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The progress of the catalyzed reaction was monitored in real time by observing a sequence of ^{13}C NMR spectra over the course of three seconds. For each spectrum, a fraction of the polarization that had previously been generated by the DNP mechanism, and that had been retained by the substrate and product molecules, was converted into NMR-observable spin coherence by a variable flip angle pulse.[12] From the spectra shown in Figure 2, the reduction in the intensity of the substrate resonance (BAEE; Figure 2a,b), as well as the appearance of product (BA; Figure 2a,c), can be seen. For a quantitative analysis, the signal intensities were normalized with reference values obtained from a measurement in the absence of trypsin, which was scaled so that the resulting intensity ratio extrapolated to $t = 0$ is equal to 1 (intercept in Figure 2d). This procedure removes the effect of signal loss arising from spin-lattice relaxation during the reaction time.

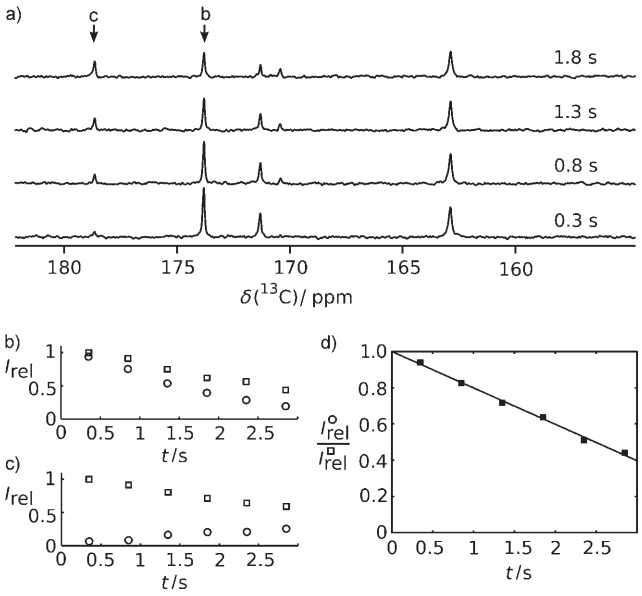

Figure 2. Kinetics of BAEE hydrolysis (3.3 mM) by trypsin (54 μM) in 50 mM potassium phosphate buffer, pH 7.6, at 27 °C. Time-resolved ^{13}C spectra (a) show loss of BAEE (resonance **b**), and growth of the BA resonance (**c**). In (b) and (c), intensities from the spectra of the reaction (○) are shown together with intensities from a reference spectrum (□; scaled as used for (d)). d) Linear regression of the ratio of intensities from (b), which yields $k_{cat} = 12.1\ s^{-1}$.

The normalized intensities are a linear function of time (Figure 2d), as is expected for a reaction where substrate concentration (3.3 mM) is much higher than the K_m value of the enzyme (35.5 μM[11]).[13] The rate of catalysis ($k_{cat} = 12.1 \pm 1\ s^{-1}$) can be obtained directly from the slope of this line by multiplication with the ratio of the substrate/enzyme concentration. The error was estimated from the spread of values obtained from different data sets, as well as from different methods for integration of the signals. To validate the DNP NMR measurements, we have compared the trypsin activity with a measurement by UV/Vis spectrophotometry, which yielded a rate constant of $12.5\ s^{-1}$ for the batch of trypsin used (25 °C, pH 7.6), and is also in good agreement with a published value under similar conditions.[11] While we chose a chromogenic substrate to enable this comparison, there are many naturally occurring substrates that are inaccessible to spectrophotometry, but that can still be directly observed by the DNP NMR measurements presented here.

The increase in the intensity of the resonance stemming from the reaction product was also linear (not shown). The apparent rate constant was $9.0\ s^{-1}$, which is lower than the rate of catalysis. The reason for this difference most likely is a shortening of the spin-lattice relaxation time when substrate is bound to the enzyme. This effect could potentially lead to additional information on the dynamics of the binding of different substrates.

By using the present injection system, the first data point could be acquired with an initial delay of 300 ms. Further improvements of the apparatus will include the design of an NMR flow cell, to increase the time resolution close to the theoretical minimum of about 10 ms.[14]

The gain in signal provided by DNP enabled the measurement in a single scan of ^{13}C spectra without the need for isotopic enrichment (^{13}C concentration of 36 μM at 1.1% natural abundance). A conventionally acquired NMR spectrum of a standard solution of 25 mM BAEE under Ernst-angle conditions[2] yielded a signal/noise (S/N) ratio of 31:1 in 10.7 h. It is extrapolated that a spectrum equivalent to the first spectrum in the hyperpolarized data set (S/N of 62:1 in first scan), but using conventional signal averaging would require over 100 days. The observation of reaction kinetics would thus be impossible. This comparison illustrates the value of using DNP-enhanced NMR spectroscopy for enabling the measurement of rapid processes that would otherwise be inaccessible to NMR spectroscopy.

With the present sensitivity, a reaction can be detected at enzyme concentrations on the order of 10 μM, which are typical for biological tissue.[15] Hyperpolarization selectively enhances the signal of the polarized substrate by several orders of magnitude over any background from nonpolarized molecules. This property may prove particularly useful for measurements in cells or in crude cell extracts, which contain a large number of substances that would otherwise mask the substrate to be observed, but where reaction kinetics may be dramatically different from those under purified in vitro conditions.[16] Additionally, the ability to observe individual molecular sites by NMR spectroscopy, further enhanced by the large chemical shift range of ^{13}C nuclei, can give simultaneous kinetic information on reactions that occur in parallel. In more complicated situations, direct observation of saturation transfer[17] from an individually addressed nuclear spin in the substrate to the product may also allow the determination of reaction mechanisms by this method.

In conclusion, we have demonstrated that the increased sensitivity provided by hyperpolarized NMR spectroscopy enables time-resolved observation of enzymatic reactions under near-physiological conditions. This approach is, however, not limited to enzyme kinetics; other applications include the study of unidirectional chemical and biochemical processes as diverse as polymerization reactions or protein folding. DNP-enhanced time-resolved NMR spectroscopy is valuable where conventional NMR spectroscopic observation would require signal averaging, a situation that in practice is

often encountered because of the low intrinsic sensitivity of NMR spectroscopy. Under these circumstances, the gain in signal obtained from hyperpolarization directly translates into a reduction of the lower limit of the observable timescale.

Experimental Section

For DNP polarization, the sample of BAEE (EMD, Gibbstown, NJ) was mixed with the radical tris{8-carboxyl-2,2,6,6-tetra[2-(1-hydroxymethyl)]-benzo(1,2-d:4,5-d′)bis(1,3)dithiole-4-yl}methyl sodium salt ("OX63"; Oxford Instruments, UK) and a glassing mixture of 60% ethylene glycol (Mallinckrodt Baker, Phillipsburg, NJ) and 40% water to yield a 20 μL aliquot of 500 mM BAEE and 15 mM OX63. 5 mM [^{13}C]Urea (Cambridge Isotope Laboratories, Andover, MA) was added as an internal standard. This aliquot was polarized using a DNP polarizer (Oxford Instruments, UK), at 1.4 K and 60 mW of 93.976 GHz mm waves for 120 min. The polarized sample was dissolved in hot buffer solution (3 mL; 90% H_2O/10% D_2O, 50 mM potassium phosphate, pH 7.6) delivered at 10 bar peak pressure. Sample injection into the 400 MHz NMR spectrometer (Bruker, Switzerland) took place in two steps (Figure 1). During a loading phase, sample was pushed from the DNP polarizer into a 1 mL injection loop connected to a two-position 10-port valve (C22-6180, VICI Valco, Houston, TX). As sample fluid exited the loop (port 1 in Figure 1), a contactless conductivity detector (CD) triggered switching of the valve to start the injection phase (modified from Ref. [18], see Figure S1 in the Supporting Information). The contents of the loop were pushed into a 5 mm NMR tube using N_2 (17 bar, against a back pressure of 11 bar) to yield 450 μL injected volume. Subsequently, the valve was switched back to the loading position, and an overpressure of 17 bar was applied. A trypsin solution (1 mM, 25 μL, pH 3) was present in the NMR tube prior to sample injection. The two solutions mixed as the polarized sample was injected (see Figure S2 in the Supporting Information). NMR data was acquired as a sequence of six spectra (see Figure S3 in the Supporting Information). The sample temperature was determined by measurement of a representative sample with a small thermocouple. Final substrate concentrations were verified by reverse-phase high-performance liquid chromatography. NMR data were processed and integrated using MatNMR.[19]

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