Time-resolved solid-state REDOR-edited NMR detection of a transient enzyme–intermediate

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Time-resolved solid-state rotational echo double resonance-edited NMR has been used to detect transient enzyme–intermediates containing two dipolar coupled spins that are unique to the intermediate, so that even resonances buried in the natural abundance background can be detected.

The technique of time-resolved solid-state NMR spectroscopy was introduced by this laboratory for the direct detection of transient enzyme–intermediates, and first demonstrated for 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase,[1–4] and uridine diphosphate *N*-acetylglucosamine (UDP-NAG) enolpyruvyl transferase.[5] The time-resolved method[6] involves rapid freeze-quench of enzyme–substrate mixtures at discrete time intervals, enabling the pre-steady-state kinetic trapping of transient species as a function of time, followed by low-temperature solid-state NMR analysis of the samples at each time point. Enzymatic reactions which have either single or multiple intermediates can in theory be stopped along the reaction coordinate. When coupled with rotational echo double resonance (REDOR) solid-state NMR, enzyme active site distance information for discrete ground state species can be obtained as a function of time. This laboratory first demonstrated the direct detection of the intermolecular dipolar coupling in the transient enzyme–intermediate complexes of the enzymes EPSP synthase[7] and UDP-NAG enolpyruvyl transferase.[8]

The REDOR technique was introduced by Schaefer and coworkers,[9] and relies on the fact that the effect of the dipolar interaction between two spins on the rotational echo can be manipulated by π-pulses. The dephasing of magnetization of one spin involved in dipolar coupling to another heteronucleus in the presence and absence of these π-pulses, and subsequent refocusing as a function of the magic angle spinning frequency, leads to a variation of resonance intensities. This intensity variation is related to the dipolar coupling constant. The first spectrum is obtained using a standard cross polarization pulse sequence with π-pulses on the observed nucleus (*e.g.* ^{13}C) in the middle of the evolution period. During this period, the observable magnetization evolves under the influence of the chemical shifts and the heteronuclear dipolar interaction. The π-pulses refocus both interactions, leading to a signal S_0 during the acquisition period. The second spectrum is obtained with an additional train of π-pulses on the dipolar-coupled spin (*e.g.* ^{31}P). These pulses affect the observed signal by preventing rotational refocusing of the dipolar interaction. The magnetization is therefore not completely refocused, and the signal intensity drops by an amount ΔS. For weak dipolar coupling, the change in signal intensity is related to the distance between the coupled spins by[12] eqn. (1), where N_c is the number of

$$\Delta S/S_0 = KD^2N^2_c\nu_r^{-2} \quad (1)$$

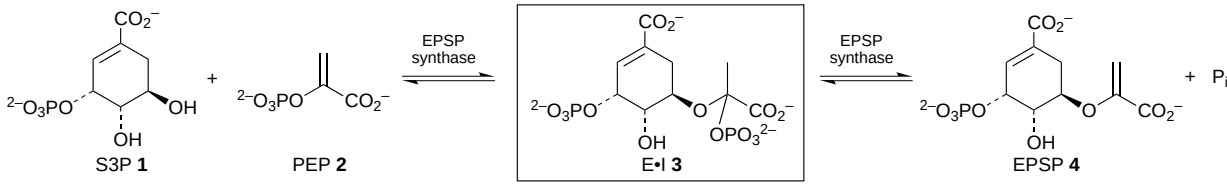
rotor cycles during the evolution period, ν_r is the spinning speed (in Hz), D is the dipolar coupling (in Hz), and K is a dimensionless constant (= 1.066). Measurement of D can be used to calculate internuclear distance (r) according to eqn. (2), where γ_I and γ_S are the gyromagnetic ratios for the

$$D = \left(\frac{\mu_0}{4\pi}\right)\frac{\gamma_I\gamma_S\hbar}{2\pi r^3} \quad (2)$$

two heteronuclear spins involved, and $\hbar$ is Planck's constant divided by 2π. This experiment therefore yields the internuclear distance, which for an isolated spin pair (*e.g.* ^{13}C–^{15}N or ^{31}P–^{13}C) can be determined to an accuracy of around ±0.1 Å. In addition to making quantitative distance measurements, the technique can also be used for qualitative measurements between spins within the distance dictated by the limits of the two nuclei involved in dipolar coupling.[7,8]

Here we report the first application of time-resolved REDOR-edited solid-state NMR spectroscopy to the detection of a reactive intermediate species trapped at the active site of an enzyme containing a strongly dipolar coupled spin pair unique to the intermediate, thereby editing an otherwise crowded solid-state NMR spectrum. To test this approach, we have used a well-characterized enzyme,[10–13] EPSP synthase (EC 2.5.1.19), which catalyses the penultimate step in the aromatic amino acid biosynthetic pathway in higher plants and bacteria. EPSP **4** is formed from shikimate-3-phosphate (S3P) **1** and phosphoenolpyruvate (PEP) **2** (Scheme 1). The enzyme is a monomer with molecular weight M_r = 46 kDa and the cloned *E.coli* gene has been used to generate a hyperexpressing strain,[14] so that the bacterial enzyme is available in gram quantities. Furthermore, EPSP synthase is the primary site of action of the herbicide glyphosate,[15] or *N*-phosphonomethylglycine. Schaefer and coworkers[16–19] have used solid-state REDOR NMR experiments to investigate the conformation of the bound inhibitor glyphosate in the ternary complex, and its relation to the enzyme active site. This is particularly significant, since all attempts to crystallize the ternary complex have failed thus far.

The rationale for selecting EPSP synthase for testing the time-resolved REDOR-edited NMR approach was as follows. Since the enzyme–intermediate has already been thoroughly characterized by our laboratory in solution[11,12] and in the solid-state,[1,2,4] and this enzyme–intermediate contains a unique dipolar coupled spin pair (*e.g* ^{31}P–^{13}C), this then provides an



Scheme 1

unusual opportunity to test whether this approach is amenable for application to other enzymes. Fig. 1 shows {^{31}P}^{13}C CP-MAS REDOR difference (ΔS) and full echo (S_0) time-resolved solid-state NMR spectra of EPSP synthase·S3P mixed with [2-^{13}C]PEP under pre-steady-state conditions, followed by lyophilisation at low temperature.† The substrates in these experiments were present in an 10-fold excess over the enzyme. At this stage lyophilised solids have been employed because of technical difficulties associated with performing REDOR measurements on frozen solutions. The enzyme–intermediate complex was trapped under time-resolved conditions (the time-resolution being 50 ms in this case), and the sample kept at low temperature (< 263 K) throughout the lyophilisation. The spectra were obtained using the REDOR pulse sequence with dephasing π-pulses applied to the ^{31}P nuclei and detection on ^{13}C. The rationale for this choice of spin pairs was guided by the fact that apart from enzyme-free or bound [2-^{13}C]PEP, none of the species present *except* the tetrahedral intermediate (**3**) contains strongly dipolar coupled ^{13}C and ^{31}P. Thus the spectrum is edited with respect to those ^{13}C atoms which are dipolar coupled to ^{31}P, and the conditions can be adjusted (determined by the number of dephasing π-pulses) so that *predominantly* C–O–P linkages are detected. In the P–C REDOR difference spectrum (ΔS in Fig. 1), in addition to the substrate [2-^{13}C]PEP and possibly product EPSP at δ 152.5 (both species have closely similar chemical shifts[4]), and their associated rotational sidebands (RSB), which of course also contains a C–O–P linkage, the enzyme–intermediate (E·I) complex is evident at δ 104. This is consistent with our previously published NMR data on the enzyme–intermediate of EPSP synthase both in solution[16,17] and in the solid-state.[1,2,4] With 32 ^{31}P dephasing π-pulses, the predominant interaction arises from C–O–P linkages and other species experiencing dipolar interactions within a radius of 5 Å. Examples of the latter case are evident in Fig. 1, where resonances due to dipolar coupling between the phosphate of PEP/S3P or EPSP and the ^{13}C natural abundance of tris buffer can be seen at δ 62.1, both of which were present in excess over the enzyme. Also evident are residual contacts to the enzyme backbone carbonyl resonances at δ 174.5.

These data establish the viability of this approach for picking out resonances due to enzyme–intermediates bearing C–O–P linkages, and indeed any linkage containing a pair of unique isotopic labels. It also provides further evidence that even the unstable transient intermediate of EPSP synthase, formed from the true substrate under pre-steady-state conditions, can survive in the lyophilised solid. Schaefer and co-workers[20,21] have recently reported using P–C REDOR to investigate species that form in very slow reactions catalysed by mutant forms of EPSP synthase in the solid-state, although it is unclear at this stage whether the species they are detecting have any relevance to the mechanistic course of the enzyme. These results therefore represent the first example of the application of time-resolved solid-state REDOR-edited NMR spectroscopy for the detection of transient enzyme–intermediate complexes. The stage is now set for the application of this approach to other enzymes in which a unique dipolar coupled spin pair occurs only in the putative intermediate, so that its mechanistic details may be delineated.

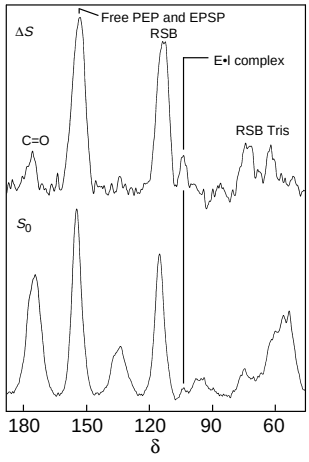


Fig. 1 9.4-T {^{31}P}^{13}C (P–C) CP-MAS REDOR difference NMR spectra of EPSP synthase plus shikimate-3-phosphate reacted with [2-^{13}C]PEP for 50 ms followed by lyophilisation at low temperature and obtained with and without ^{31}P dephasing. The full echo S_0 spectrum is without ^{31}P dephasing, and the ΔS spectrum is the difference spectrum of that obtained with and without ^{31}P dephasing.

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Footnotes

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† The time-resolved REDOR difference NMR spectra were obtained by rapid mix and freeze-quench (in 50 ms) of EPSP synthase (12.2 Units mg^{-1}) S3P with a solution of [2-^{13}C]PEP in 50 mM tris·HCl (pH 7.8) buffer to final concentrations of 5.8, 58 and 58 mM, respectively. The rapidly frozen sample was then lyophilised at < 263 K for 72 h. Approximately 70 mg of protein was used and the spectra were acquired at 233 K. The freeze-quench apparatus used is described in detail elsewhere (refs. 3 and 4). The EPSP synthase was isolated by literature methods (ref. 14). NMR spectroscopy was carried out as previously described (ref. 8).

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