(balance of cobalt material is **5**). However, when 1 equiv of PPh_3 (0.016 mmol) is added to a benzene solution of ethyl diazoacetate (0.027 M) and **5** (0.027 M), the diazoacetate is slowly consumed over the course of 4 days and an 84% yield of cobaltacyclobutene **9** is observed by ^1H NMR spectroscopy.[14] The isolated metallacyclobutene **9** is stable to ethyl diazoacetate in benzene at room temperature. However, when a benzene-d_6 solution of **9** and ethyl diazoacetate (1.5 equiv) is heated at 50 °C, diene complexes **6-8** are formed in the same ratio as that observed from reaction of **5** with ethyl diazoacetate.[10]

The dramatic difference in product distribution for **5** vs **1** is presumably related to the stability of the unsaturated cobaltacyclobutene intermediate. The bulky trimethylsilyl group inhibits the formation of bimetallic products, and the electron-withdrawing sulfone substituent stabilizes the intermediate toward subsequent reaction with additional carbene. When the reaction of **1** and ethyl diazoacetate is repeated in the presence of PPh_3, no metallacyclobutene product is observed by ^1H NMR spectroscopy. Detailed mechanistic studies are currently underway to determine the specifics of metallacyclobutene ring formation.

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Supplementary Material Available: Characterization data for **5-9** and **11**; listings of fractional coordinates, bond distances, bond angles, hydrogen atom coordinates, and thermal parameters (8 pages); table of observed and calculated structure factors (10 pages). Ordering information is given on any current masthead page.

(14) In a related NMR experiment, a 93% yield of **9** was achieved by further addition of ethyl diazoacetate after ~80% conversion.

Detection of an Enzyme-Intermediate Complex by Time-Resolved Solid-State NMR Spectroscopy

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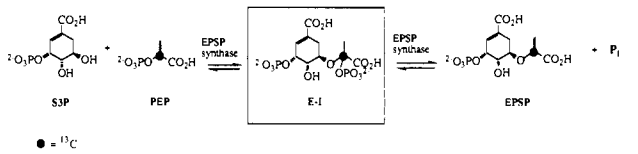
The elucidation of the structure of an enzyme-substrate complex at various points along the reaction coordinate is one of the most sought after goals of enzyme chemistry. The two techniques that generate the most structural information, X-ray crystallography and NMR spectroscopy, also are among the slowest and most insensitive methods for the data collection.[1] In this communication, we report the first application of a new NMR method, which we call time-resolved solid-state NMR spectroscopy, to the direct detection of the transient enzyme-intermediate complex of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, a key enzyme in the aromatic amino acid biosynthetic pathway. Unlike X-ray diffraction of enzyme crystals, which has difficulty defining

Scheme I



intermediate structures due their intrinsic motions, time-resolved solid-state NMR spectroscopy focuses primarily on the intermediate. This provides a generally applicable technique for "mapping out" intermediate structures as a function of time.

Time-resolved solid-state NMR spectroscopy is complementary to X-ray crystallography, which to date has achieved time resolution of the order of 0.5 h with cryoenzymological methods[2] and 1-3 s with Laue diffraction methods,[3,4] although in theory Laue methods can achieve time-resolutions in the millisecond regime.[5] However, the limitations of time-resolved Laue diffraction methods that arise from substrate diffusion and transient lattice disorder give rise to diffuse electron density maps, and this has been improved only to an extent by using caged substrate molecules.[6] Disordered substrates are not too surprising, since during catalysis the enzyme-bound substrate is dynamic and undergoing significant molecular motion, which is where NMR spectroscopy is useful. Although NMR spectroscopy has been used to study rapid reactions in solution, in particular by continuous-flow and stopped-flow methods,[7] the time-resolution to date is at very best ca. 20 ms and in practice of the order of 200 ms to >10 s. The method of time-resolved solid-state NMR spectroscopy which we introduce here has a time-resolution achievable on the order of ca. 2 ms.

We have examined a well-characterized enzyme, 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (EC 2.5.1.19), which catalyzes the penultimate step in the aromatic amino acid biosynthetic pathway in higher plants and bacteria. EPSP is formed from shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) (see Scheme I). The enzyme is a monomer with molecular weight M_r = 46 000, and the cloned *E. coli* gene has been used to generate a hyperexpressing strain,[8] so the bacterial enzyme is available in gram quantities. Furthermore, EPSP synthase is the primary site of action of the herbicide glyphosate[9] or *N*-phosphonomethylglycine.

This enzyme has been extensively studied by kinetic and biophysical methods in the last five years and is one of a limited number of enzymes for which the full kinetic and thermodynamic profile has been determined.[10] The direct observation of the enzyme-intermediate E·I complex was first reported by our laboratory[11] and later confirmed by another laboratory.[12] The E·I

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(1) The technique of electro-spray ionization mass spectrometry [e.g., Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C. M. *Science* **1989**, *246*, 64] is, in principle, amenable to use in time-resolved studies of enzymes, and experiments using this method are currently underway (Goodlett, D.; Shuttleworth, W. A.; Edmonds, C. G.; Smith, R. D.; Evans, J. N. S., unpublished results).

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complex is unusually stable, which makes it a particularly attractive system for developing time-resolved solid-state NMR of enzyme–intermediate complexes.

Solution-state NMR spectroscopy is well-established for studying enzymatic reaction mechanisms[11,13] and has been applied to E·I complexes at subzero temperatures.[14] However, the disadvantage of solution-state NMR at low temperature is that the cryosolvent required becomes viscous, resulting in increased line widths for the resonances from the enzyme-bound substrate. Also, the current molecular weight limit is around 40 kDa.[11] In contrast, with solid-state NMR spectroscopy,[15] anisotropic and dipolar broadening can be reduced by CP-MASS, and there is no known molecular weight limit. When coupled with a rapid freeze–quench method as has been employed in ESR studies,[16] short-lived enzyme intermediates can be trapped. Only the pre-steady-state kinetics for the enzyme need to be determined, without recourse to lengthy kinetic studies in cryosolvents.

The rapid freeze–quench method involves rapidly mixing enzyme and substrate together and freezing by spraying the mixture directly into a secondary cryogen such as liquid propane cooled to ~85 K. Concerns about the fate of the protein under these conditions of freezing have been addressed,[17] and at the protein concentrations and freezing rates (10^5 K s^{-1}) employed here, there is probably significant dispersal of the solute in the frozen water.[18] The frozen water is probably largely amorphous,[19] with the protein itself acting as a cryoprotectant,[19] thereby reducing the formation of hexagonal ice that is detrimental to the protein. We have found that the specific activity of EPSP synthase employed in the experiments reported here when assayed before and after rapid freezing was unchanged at these high protein concentrations.

Figure 1 shows ^{13}C CP-MASS solid-state NMR spectra of EPSP synthase·S3P mixed with [2-^{13}C]PEP under steady-state conditions (in the presence of the product, inorganic phosphate) and under pre-steady-state conditions where t indicates the time elapsed from the start of the reaction. The intermediate E·I is clearly visible at 104 ppm[11] under steady-state conditions, and its buildup is demonstrated as the reaction proceeds under pre-steady-state conditions. It is worth noting that the intensities of the E·I resonance correlate well with the concentrations observed by chemical quench methods.[10] On allowing the pre-steady-state reaction to proceed for a few minutes, the turnover of intermediate E·I to product E·EPSP is evident. In addition to the resonance due to the E·I complex, the resonance due to the E·EPSP product complex builds up at 155 ppm, and one tentatively assigned to the E·PEP substrate complex appears transiently at 151 ppm. Under the conditions these spectra were obtained, the free small molecules (substrate and product) are *not* detected due to their relative isotropic motion in frozen solution. We have found that spin locking fields that provide excellent cross-polarization for rapidly-frozen solutions of EPSP synthase and enzyme-bound species also provide very poor cross-polarization for PEP and EPSP. The control experiment in which EPSP synthase·S3P was rapidly mixed with unlabeled PEP showed no resonances at 104, 151, or 155 ppm. The other resonances in Figure 1 arise from natural abundance EPSP synthase and its rotational sidebands.

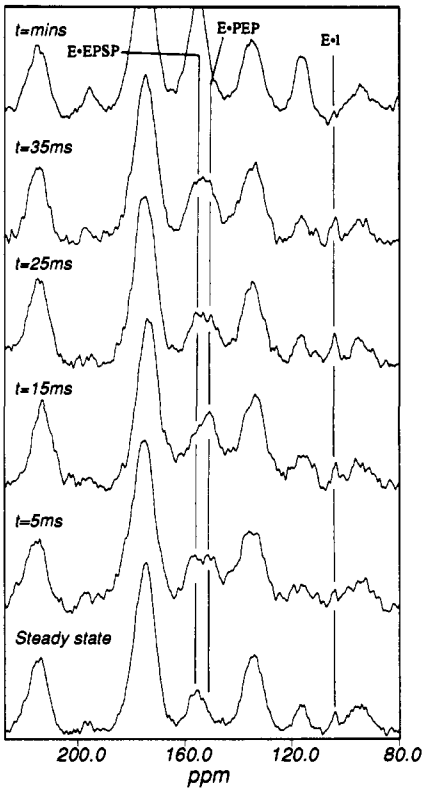

Figure 1. ^{13}C CP-MASS solid-state NMR spectra (9.4 T) of EPSP synthase at 233 K under conditions indicated steady-state (EPSP synthase (4 mM) in 20 mM phosphate buffer, pH 7.8, 15% D_2O in the presence of S3P (9.1 mM) and [2-^{13}C]PEP (7.6 mM), slow frozen in the NMR rotor over 90 s); rapidly mixed and freeze–quenched after time t (EPSP synthase (4 mM) in 50 mM tris buffer containing 5 mM β-mercaptoethanol, pH 7.8, in the presence of S3P (40 mM), and rapidly mixed with [2-^{13}C]PEP (40 mM) and sprayed into liquid propane at ~85 K using a freeze–quench apparatus with an instrument deadtime of 2.5 ms as determined by well-established methods[20] described in detail elsewhere[18]). EPSP synthase was isolated from *E. coli* BL21(λDE3)(pLysS)(pWS230) and purified by literature methods.[8] NMR spectroscopy was carried out on a Chemagnetics CMX-400 solid-state NMR spectrometer with a Doty DSI-368 double resonance probe using a 5-mm sapphire rotor with spinning speed regulation at 4 kHz (±10 Hz) and temperature regulation at 233 K using a custom design. Data were acquired with 12000 scans, 50-kHz spin lock fields, and a 3-s recycle time and processed off-line on a Silicon Graphics 4D25TG computer using FELIX (Hare Research) with 100-Hz line broadening and base line correction. Spectra are referenced indirectly to hexamethylbenzene, δ_C(methyl) = 17.17 ppm, which results in slight differences from solution chemical shifts for the E·I and E·EPSP complexes previously reported.[11]

Time-resolved solid-state NMR spectroscopy will be important for the study of enzymatic reaction mechanisms. Furthermore, when coupled with the elegant solid-state NMR distance measurements that have been introduced recently,[21] this technique will be uniquely able to "map out" molecular conformations of intermediates and enzyme active site–intermediate distances as an enzymatic reaction proceeds. This will provide the crucial missing structural details which Laue X-ray diffraction and allied techniques cannot provide and enable the complete definition of the molecular events of enzyme catalysis.

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Extracting Accurate Distances and Bounds from 2D NOE Exchangeable Proton Peaks

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Distance constraints derived from multidimensional NMR are used with distance geometry and molecular dynamics calculations to generate high-resolution structures of molecules in solution.[1,2] More accurate structures can be determined with more structural constraints,[1] with more accurate structural constraints, and with tighter but realistic constraint boundaries.[3] More numerous and more accurate distances between nonexchangeable protons can be determined from two-dimensional nuclear Overhauser effect (2D NOE) spectra using a complete relaxation matrix analysis, which accounts for all proton dipole–dipole interactions.[4-6] Determination of accurate distances to exchanging protons can present problems beyond that of the multispin effects noted already. Chemical exchange has been incorporated into the complete relaxation matrix formalism and demonstrated to influence 2D NOE peak intensities in the case of multiple conformations.[7-9] For the case of proton exchange from a dilute macromolecule to bulk solvent water, the total relaxation rate matrix can be modified so that chemical exchange is considered in addition to dipole–dipole relaxation:[10,11] $\mathbf{R}_T = \mathbf{R}_{DD} + \mathbf{K}$. For our case, the chemical exchange matrix **K** is diagonal; the diagonal terms correspond to off rates for exchangeable protons and are 0 for nonexchangeable protons. Another way of thinking about this is that exchange with bulk water contributes to direct relaxation, i.e., magnetization is lost via the physical "leakage" of the exchangeable proton into the pool of water. We have examined the influence of exchange on determination of distances and bounds for protons which can exchange with bulk water. The influence of solvent exchange rate and mixing time on proton cross-peak intensities for the simple case of two interacting protons is shown in Figure 1; it can be shown that the ordinate is independent of interproton distance and correlation time if the mixing time is short (<1 s). The mixing times and exchange rates shown suggest that, while exchange with water may be pertinent for some protons on proteins or other molecules, it is especially important for imino and amino protons on nucleic acids.

In spite of the paucity of base protons to provide structural constraints, studies with double-stranded DNA generally have not utilized distances to imino protons because they exchange with

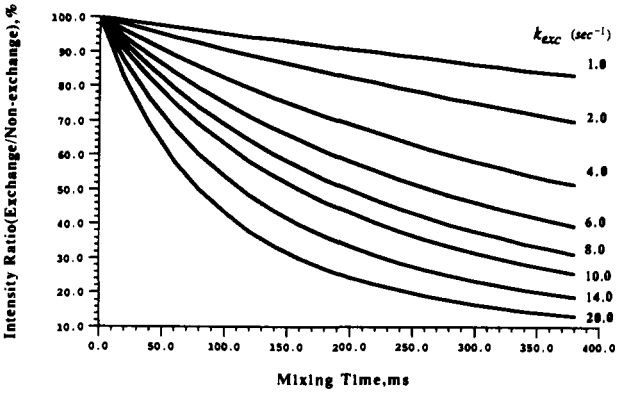

Figure 1. Diminution of 2D NOE cross-peak intensity for a system of two spins due to exchange of one proton of the pair with bulk water as a function of mixing time. The ordinate is the ratio of the cross-peak intensity in the presence to that in the absence of exchange. That ratio can be shown to be independent of interproton distance, number of exchangeable protons, and correlation time as long as the mixing time is short. The different curves are generated for protons at 500 MHz with a correlation time of 1 ns and exchange rates of (a) 1, (b) 2, (c) 4, (d) 6, (e) 8, (f) 10, (g) 14, and (h) 20 s^{-1}.

Table I. Comparison of Interproton Distances (Å) Calculated Using MARDIGRAS without and with Incorporation of Exchange from a Simulated 2D NOE Data Set for d(G1-T2-A3-T4-A5-A6-T7-G8)·d(C9-A10-T11-T12-A13-T14-A15-C16)[a]

proton interaction	calcd distance[b] without exchange	calcd distance[b] with exchange
T2-H3–T14-H3	4.69	4.01
T4-H3–T12-H3	4.59	3.92
T4-H3–T14-H3	4.36	3.71
T7-H3–T11-H3	4.24	3.61
T11-H3–T12-H3	3.63	3.07
T7-H3–G8-H1	4.31	3.99
T14-H3–A15-H2	4.99	4.68
T12-H3–A13-H2	4.77	4.47
T2-H3–A3-H2	4.61	4.32
T4-H3–A5-H2	4.54	4.25
G1-H1–T2-H3	3.58	3.30
A3-H2–T4-H3	4.03	3.76
A6-H2–T7-H3	4.05	3.78
A5-H2–T11-H3	4.96	4.70
A6-H2–T12-H3	3.89	3.63
A10-H2–T11-H3	3.77	3.52
A13-H2–T14-H3	3.62	3.37
A6-H2–T11-H3	2.98	2.77
T4-H3–A13-H2	2.97	2.76
A5-H2–T12-H3	2.97	2.77
T2-H3–A15-H2	2.96	2.76

[a] The simulated spectrum was generated for a mixing time of 100 ms using CORMA,[4,24] assuming (i) that the real structure is that previously determined for d(GTATAATG)·d(CATTATAC) at 15 °C,[18] (ii) an overall isotropic motion correlation time of 2.8 ns and fast three-state methyl jumps, and (iii) exchange rates of 10 s^{-1} for exchangeable AT protons and 1 s^{-1} for exchangeable GC protons (both amino and imino). [b] Using the simulated spectral intensities as "experimental" data, distances were calculated incorporating multispin effects via MARDIGRAS,[20,21] either incorporating or ignoring effects of exchange. Although several hundred distances are calculated, only those entailing imino or nonexchangeable protons which differ by more than 0.2 Å are listed. Of course, the distances calculated via MARDIGRAS agree within three significant figures to the model structure distance.

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