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Effects of sample preparation conditions on biomolecular solid-state NMR lineshapes

David L. Jakeman, Dan J. Mitchell, Wendy A. Shuttleworth and Jeremy N.S. Evans*
Department of Biochemistry and Biophysics, Washington State University, Pullman, WA 99163-4660, U.S.A.

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Abstract

Sample preparation conditions with the 46 kDa enzyme complex of 5-enolpyruvyl-shikimate-3-phosphate (EPSP) synthase, shikimate-3-phosphate (S3P) and glyphosate (GLP) have been examined in an attempt to reduce linewidths in solid-state NMR spectra. The linewidths of ^{31}P resonances associated with enzyme bound S3P and GLP in the lyophilized ternary complex have been reduced to 150 $\pm$ 12 Hz and 125 $\pm$ 7 Hz respectively, by a variety of methods involving additives and freezing techniques.

Introduction

One of the greatest challenges facing the application of solid-state NMR to biomolecular structure determination lies in reducing the large linewidths often encountered with biological samples (Tycko, 1996). This is crucial if internuclear distances between spin-1/2 nuclei are to be measured using homonuclear or heteronuclear dipolar recoupling techniques (Griffiths and Griffin, 1993; Smith and Peersen, 1992; McDowell and Schaefer, 1996; Evans, 1996). Strategies for line-narrowing that involve the physical orientation of the sample (Opella, 1997; Glaubitz and Watts, 1998) by layering it between glass plates are exciting developments, but are limited to membrane proteins and myristoylated soluble proteins. While methods for partially orienting samples in the liquid-state have been developed (Tjandra and Bax, 1997) which will likely revolutionize liquid-state biomolecular structure determination, there still remains the need for accurate distance measurements in larger macromolecular assemblies, or in unoriented soluble proteins whose molecular weight exceeds the current liquid-state NMR limits. This paper focuses on the physical preparation of the NMR sample in an attempt to find a generally applicable method for line-narrowing in biomolecular solid-state NMR.

We have investigated a variety of sample preparation conditions for the ternary complex of 5-enolpyruvyl-shikimate-3-phosphate (EPSP) synthase, shikimate-3-phosphate (S3P) and glyphosate (GLP) in an effort to reduce linewidths in solid-state NMR spectra. EPSP synthase catalyses the synthesis of EPSP from phosphoenolpyruvate (PEP) and S3P as part of the shikimate pathway (Barlow et al., 1989; Evans, 1992; Evans, 1995; Appleyard et al., 1994). It has been the subject of intense research in the past decade because it is the primary site of action of the broad-spectrum post-emergence herbicide GLP. To date, there is no widely accepted view of how GLP binds and inhibits EPSP synthase (Sikorski and Gruys, 1997), although some limited structural information about the orientation of GLP and S3P has been reported (Christensen and Schaefer, 1993; McDowell et al., 1996a; McDowell et al., 1996b). Reduced linewidths will enable more accurate distance measurements using dipolar recoupling techniques. Therefore the effects of additives and freezing methods upon linewidths are examined here.

The effects of hydration on enzymes in the lyophilized state have been studied by ^{13}C solid-state NMR. Lysozyme showed improvements in spectral resolution upon increasing hydration (Gregory et al.,

*To whom correspondence should be addressed.

1993b), although, in contrast, the spectrum of bovine serum albumin was only improved in spectral resolution around the aliphatic carbon resonances (Gregory et al., 1993a). One of the first additives to a solid-state NMR sample was polyethylene glycol (PEG-3400) (Tomita et al., 1994), which reduced linewidths from 4–6 ppm (100 MHz ^{13}C spectra) to 2 ppm. The effects of trehalose as a protectant against dehydration were first noted by Clegg in 1965 (Clegg, 1965), who discovered that up to 20% dry weight of some anhydrobiotic organisms contain trehalose, where it stabilizes the bilayer structure of the dry membrane. Solid-state NMR research delved into the chemistry behind this stabilization (Lee et al., 1986; Lee et al., 1989), and recently it has been noted that many disaccharides, including trehalose, stabilize both protein structure and function against dehydration and freezing stresses (Butler and Falke, 1996; Crowe et al., 1996; Lin and Timasheff, 1996; Xie and Timasheff, 1997a; Xie and Timasheff, 1997b). The study of protein formulations for drug delivery has begun to address the chemistry and physics of protein lyophilization (Carpenter et al., 1997), and their practical findings have aided the preparation of superior samples for solid-state NMR analysis.

Materials and methods

Chemicals and enzymes

EPSP synthase was isolated from *E. coli* BLR (λDE3)(pLysS)(pWS230) and purified by literature methods (Shuttleworth et al., 1992). Shikimate-3-phosphate was isolated from cultures of *Klebsiella pneumoniae* according to known methods (Bondinell et al., 1971). Glyphosate (*N*-phosphonomethylglycine), buffer constituents and other reagents were purchased from Sigma-Aldrich-Fluka Chemical Co. (St. Louis). MilliQ™ water was used throughout. All enzyme manipulations were carried out at 4 °C unless indicated otherwise.

Enzyme assay and protein determination

EPSP synthase activity was routinely assayed for release of phosphate (Van Veldhoven and Mannaerts, 1987) and protein concentration determined by the method of Bradford (Bradford, 1976).

Shikimate-3-phosphate determination

EnzChek™ Phosphate assay (Molecular Probes Inc.), perchloric acid assay (Chen et al., 1956) and thiobarbituric assay (Millican, 1963) were performed on aliquots of shikimate-3-phosphate incubated with acid phosphatase.

Sample preparation

The samples were prepared according to the protocol listed in Table 1. GLP and S3P (10 and 37% excess respectively, from stock solutions of 0.1 M) were incubated with the enzyme at room temperature for approximately one hour. Prior to lyophilization the samples were clarified by centrifugation in a microcentrifuge to remove any precipitate. The freeze drier operated at 70 mTorr. For example, sample 5 was prepared by exchanging buffer (from TRIS•HCl (50 mM, pH 7.8) to MOPS (20 mM, pH 7.2)) in an Amicon concentrator. The enzyme concentration was determined, GLP and S3P were added, the sample incubated at room temperature for an hour, centrifuged in microcentrifuge tubes, transferred by pipette into a flask and shell-frozen in liquid N_2.

NMR spectroscopy

Solid-state NMR studies were completed on a Chemagnetics cmX-400 spectrometer using a Pencil® triple resonance probe and a 5 mm zirconia rotor, with 4 ± 0.01 kHz spinning speed. The temperature was unregulated (21 °C) except for Experiments 9 and 10. ^{31}P NMR data were acquired using a CP-MAS pulse sequence at a frequency of 161.958 MHz with a $\pi/2$ pulse of 10.2 μs, 1 ms contact time, 3 s recycle delay, spectral width of 30.030 kHz, and 20 000 scans. Proton decoupling was applied at 400.083 MHz at an RF field strength of 90 kHz. The data were processed off-line on a Silicon Graphics O2 computer using FELIX 97 (Molecular Simulations Inc.) by zero-filling to 1024 points and applying an exponential window function with a line broadening of 75 Hz prior to Fourier transformation. A Lorentzian lineshape analysis was performed on each peak. ^{31}P spectra were referenced indirectly to H_3PO_4 (85%) at 0 ppm.

Results and discussion

The solid-state NMR ^{31}P spectrum of EPSP synthase with bound S3P and GLP has been characterized previously and consists of two resonances (Christensen and Schaefer, 1993). The enzyme-bound GLP resonance has a chemical shift of 14 ppm and bound S3P occurs at 3 ppm. The spectra resulting from our sample preparation conditions are shown in Figure 1, and have been arranged in order of decreasing linewidth of the

Table 1. Sample preparation protocol and results of linewidth analysis

Spectrum	Buffer and pH	[Enzyme] / mM, total enzyme mass / mg	Additives	Freezing method	Lyophilization time (h)	Peak width at half-height (Hz) Enzyme-bound	
						GLP	S3P
1	a	0.59, 73	f	n	4	t	t
2	b	1.22, 84	f	o	24	t	t
3	b	0.52, 84	f	p	36	t	t
4	c	1.17, 54	f	n	24	t	t
5	b	0.65, 45	g, h	n	24	247 (u)	335 (u)
6	d	1.13, 65	f	n	24	208	317
7	b	0.65, 45	g, h	n	24	258 (v)	309 (v)
8	b	1.17, 54	g, i	n	24	222	293
9	b	0.63, 51	f	n	36	149	290
10	e	1.00, 46	j	n	24	207	290
11	b	0.65, 45	g, h	n	24	217	272
12	b	0.65, 45	g	n	24	206	271
13	e	1.00, 46	j, k	n	24	214	265
14	a	0.59, 73	f	q	4	162	258
15	e	1.41, 50	j	r	12	189	258
16	b	0.63, 51	f	n	3	160	245
17	b	0.52, 48	f	s	24	147	232
18	b	0.52, 48	l	n	24	151	217
19	b	1.17, 54	l, m	s	24	147	189
20	b	0.52, 48	m	n	24	125	150

Buffers: a MOPS (2 mM), EDTA (10 μM), KCl (50 μM), pH 7.2; b TRISHCl (50 mM), DTT (1 mM), pH 7.8; c TRISHCl (5 mM) DTT (1 mM) pH 7.8; d MOPS (20 mM), pH 7.2; e TRISHCl (50 mM) DTT (10 mM) pH 7.8.
Additives: f None; g PEG3350 (20 mg); h 90% GLP and S3P added; i 110% GLP and S3P added; j trehalose (equal mass with EPSP synthase); k PEG8000 (10 mg); l PEG3350 (15 mg); m trehalose (15 mg).
Freezing method: n shell frozen in liquid N_2; o complex precipitated by ammonium sulphate (70%); p complex precipitated with ethanol (70%); q frozen in 200 μl aliquots; r frozen by squirting into liquid nitrogen; s frozen rapidly at rates great than 10^5 Ks^{-1} using freeze-quench in liquid propane.
Peak width analysis: t peak too broad for analysis; u spectrum recorded at $-40\,^{\circ}C$; v spectrum recorded at $+40\,^{\circ}C$.

enzyme-bound S3P resonance. There is a good correlation between the linewidths of the enzyme-bound S3P and GLP resonances (Figure 2, regression coefficient = 0.81). There was no overall correlation between linewidth and enzyme concentration (regression coefficient = 0.10) or between linewidth and duration of lyophilization (data not shown). In general, samples that were prepared with additional additives gave narrower linewidths than those without them. Fast freezing also aids line narrowing (Evans et al., 1992; Evans et al., 1993; Lazo et al., 1992; Lazo et al., 1993; Appleyard and Evans, 1993; Appleyard et al., 1994), but in the examples presented here, a combination of additives and fast freezing failed to narrow lines further. Samples were stable if left at room temperature for up to two months with no appreciable loss of linewidth. However, samples left accumulating data for periods of two weeks (or more) gave significantly worse spectra (data not shown) at the end of the time period. We were able to alleviate this problem by increasing the concentration of dithiothreitol (DTT) from 1 mM to 10 mM in the sample.

Four different sample preparations gave spectra that were too broad for peak picking and, therefore, not amenable to linewidth analysis (Figure 1, spectra 1–4). Sample 1 (and sample 6) was obtained by repeating a literature sample preparation (Christensen and Schaefer, 1993) and in our hands gave broad resonances. Precipitation of ternary complex by either ammonium sulfate (Spectrum 2) or ethanol (Spectrum 3) gave very poor spectra. The freezing of ternary complex in dilute Tris buffer (5 mM) also failed to improve linewidths (Spectrum 4); a more concentrated Tris buffer (50 mM) gave narrower linewidths (Spectra

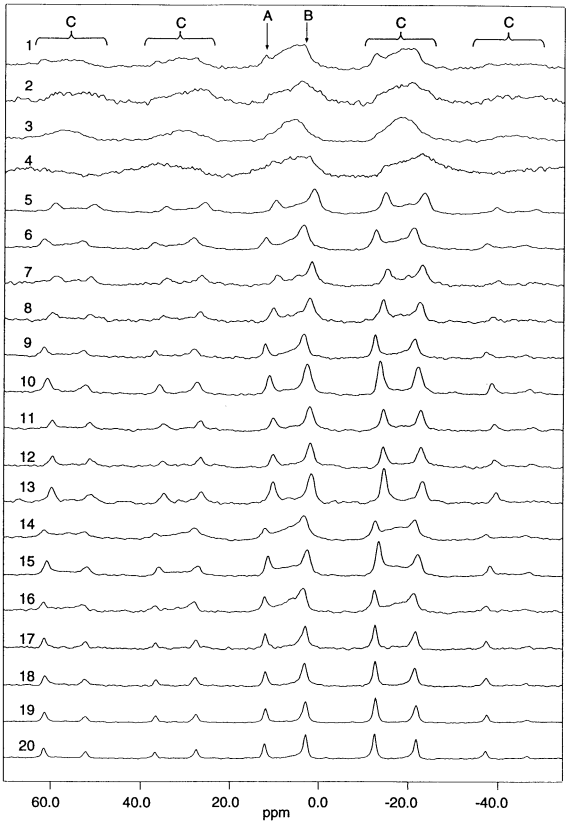

Figure 1. The 161.958 MHz CP-MAS ^{31}P NMR spectra of S3P and GLP bound to EPSP synthase. Each spectrum was obtained under conditions given in Table 1. The resonance assignments are A: enzyme-bound GLP; B: enzyme-bound S3P; and C: rotational sidebands.

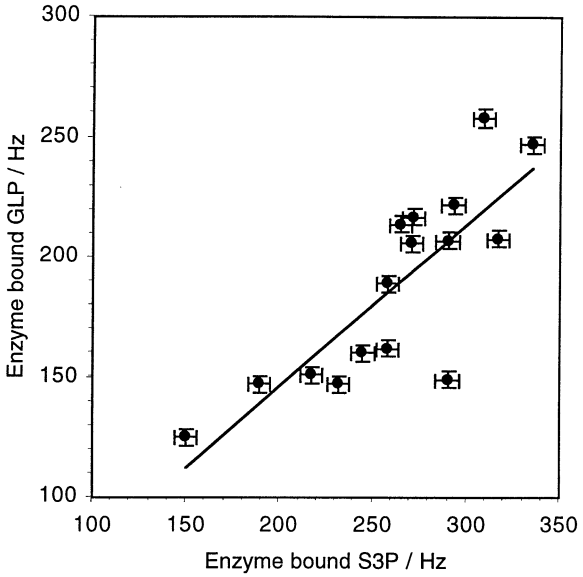

Figure 2. The correlation between EPSP synthase enzyme-bound S3P and GLP linewidths. The data (•) are for each spectrum in Figure 1. Error bars are 7 Hz (x axis) and 12 Hz (y axis). The solid line represents the linear regression fit through the data ($y = 11.1 + 0.68x$; $r = 0.81$)

9 and 16). The same correlation was observed between linewidths of spectra in dilute MOPS (2 mM) (Spectrum 6) and MOPS (20 mM) (Spectrum 1).

To assess the dependence of linewidth on S3P and GLP concentration, three samples were prepared (Samples 8, 11 and 12) that had 110%, 90% and 100% substrates respectively. The narrowest linewidths were obtained for sample 12, which had the standard quantities of S3P and GLP but all measurements were close to the experimentally determined error. Sample 11 was then run at two temperatures, $-40\,^{\circ}$C and $+40\,^{\circ}$C (Samples 5 and 7), to determine temperature susceptibility; surprisingly both spectra had linewidths worse than the room temperature sample (Sample 11, 21 °C).

The freezing rate of the sample has also been shown to be important (Christensen and Schaefer, 1993) and spectrum 14 was obtained by repeating their so-called 'fast' freezing protocol. We were able to get comparable linewidths by squirting the sample (by syringe) directly into liquid nitrogen (Spectrum 15). Significant improvements were observed by rapid freezing of the sample in liquid propane with a rapid freeze-quench apparatus (Spectrum 17), confirming what has been observed previously (Evans et al., 1992, 1993; Lazo et al., 1992, 1993; Appleyard and Evans, 1993; Appleyard et al., 1994). This is not surprising, since the thermal heat capacity of liquid nitrogen is very poor due to the *leidenfrost* phenomenon, and freezing rates on the order of only 10^2 Ks^{-1} can be achieved, whereas liquid propane has a high thermal heat capacity, and if the sample is injected into the cryogen at subsonic speeds (5–10 ms^{-1}) then freezing rates $> 10^5$ Ks^{-1} can be achieved.

Additives such as trehalose and polyethyleneglycol (PEG) have been very beneficial in preparing drug formulations (Carpenter et al., 1997) through their ability to act as cryoprotectants and prevent protein denaturation during freezing. Addition of trehalose in many of our samples (Spectra 10, 13, 15, 19 and 20) gave narrow linewidths. Indeed, just trehalose (Spectrum 20) gave the best linewidths of any sample. PEG addition (Spectra 5, 7, 8, 11, 12, 13, 18 and 19) also improved linewidths. Unfortunately, a combination of both trehalose and PEG together with fast freezing (Spectrum 19) did not give cumulative linewidth reduction.

Data accumulation over a period of weeks (data not shown) for many of the samples resulted in a broadening of the lines in the spectrum. This was overcome by preparing EPSP synthase in the presence of DTT (10 mM) (Spectra 10, 13 and 15) where spectra of similar linewidth were observed initially, and after data accumulation for two weeks. A duplicate sample 13 was prepared and its spectrum recorded to calculate errors in the data. The enzyme-bound S3P resonance had an error of $\pm$12 Hz, and enzyme-bound GLP an error of $\pm$7 Hz.

Conclusions

We have shown that the ^{31}P NMR spectrum of the ternary complex of S3P and GLP bound to EPSP synthase can be improved by a variety of simple preparation protocols. The addition of cryoprotectants like trehalose and PEG do reduce the linewidths of resonances. An alternative protocol of rapidly freezing the sample is equally effective in reducing linewidths. We have preliminary evidence that the same approaches are applicable to reducing ^{13}C NMR lineshapes, and appear to be generally applicable to biological samples. These findings are being applied towards pre-steady state kinetic solid-state NMR analyses of PEP-utilizing enzymes in our laboratory.

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