Shikimate-3-phosphate Binds to the Isolated N-Terminal Domain of 5-Enolpyruvylshikimate-3-phosphate Synthase†

Melissa E. Stauffer,‡ John K. Young,‡ and Jeremy N. S. Evans*,‡,§

School of Molecular Biosciences and Center for NMR Spectroscopy, Washington State University, Pullman, Washington 99164-4660

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ABSTRACT: 5-Enolpyruvylshikimate-3-phosphate (EPSP) synthase catalyzes the transfer of the enolpyruvyl moiety from phosphoenolpyruvate (PEP) to shikimate-3-phosphate (S3P). Mutagenesis and X-ray crystallography data suggest that the active site of the enzyme is in the cleft between its two globular domains; however, they have not defined which residues are responsible for substrate binding and catalysis. Here we attempt to establish the binding of the substrate S3P to the isolated N-terminal domain of EPSP synthase using a combination of NMR spectroscopy and isothermal titration calorimetry. Our experimental results indicate that there is a saturable and stable conformational change in the isolated N-terminal domain upon S3P binding and that the chemical environment of the S3P phosphorus when bound to the isolated domain is very similar to that of S3P bound to EPSP synthase. We also conclude that most of the free energy of S3P binding to EPSP synthase is contributed by the N-terminal domain.

5-Enolpyruvylshikimate-3-phosphate (EPSP)[1] synthase (EC 2.5.1.9) catalyzes the formation of EPSP from shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) in plants and bacteria. This reaction (Figure 1) occurs as the penultimate step of the shikimate pathway, which is ultimately responsible for the biosynthesis of aromatic amino acids (*1*). The pathway is not present in mammals, so enzymes of the pathway are promising targets for antibiotic (*2*), antiparasitic (*3*), and herbicide (*4*) development. EPSP synthase is in fact the site of action of glyphosate (GLP) (*5*), the active ingredient of the commercially available herbicides RoundUp and Touchdown.

The crystal structure of EPSP synthase at 3 Å resolution reveals an open conformation of two globular domains whose secondary structural elements are strikingly similar (*6*). This structure shows that the six α-helices comprising each domain are oriented with their N-termini toward the cleft between the domains, suggesting the possibility that the resulting macrodipole may direct the negatively charged substrates into the cleft where the reaction occurs. Numerous mutagenesis studies (*7*−*10*) have confirmed the active site location by showing that mutation of many residues lining the cleft decreases or abolishes enzymatic activity. According to these experiments, residues from both domains contribute to the active site. However, various sources suggest that the N-terminal domain may be the primary contributor to S3P binding in EPSP synthase.

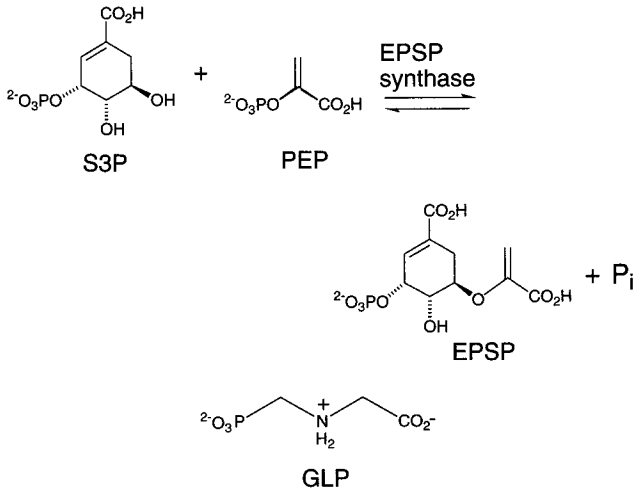

FIGURE 1: Reaction catalyzed by EPSP synthase. The inhibitor, glyphosate, is also shown.

An R27A mutation abolished S3P binding in the *E. coli* enzyme (*10*), and addition of S3P and GLP to the *Petunia hybrida* enzyme protected the equivalent R28 from chemical modification (*11*). Krekel et al. observed that addition of S3P to the *E. coli* enzyme moderately increased the stability of the enzyme toward tryptic digestion, and particularly that of two N-terminal peptide segments (*12*). Also, investigators at Monsanto have published pictures of a crystal structure of the S3P-bound EPSP synthase and reported several residues that are claimed to be directly involved in S3P

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* Correspondence should be addressed to this author at the School of Molecular Biosciences, Washington State University, Pullman, WA 99164-4660. Tel: (509) 335-6321; FAX: (509) 335-9688; Email: evansj@wsu.edu.

‡ School of Molecular Biosciences.

§ Center for NMR Spectroscopy.

[1] Abbreviations: DTT, dithiothreitol; EPSP, 5-enolpyruvylshikimate-3-phosphate; GLP, glyphosate; HSQC, heteronuclear single quantum coherence; MALDI, matrix-assisted laser desorption ionization; NMR, nuclear magnetic resonance; PEP, phosphoenolpyruvate; S3P, shikimate-3-phosphate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TAD, 50 mM Tris, pH 7.8, 3 mM sodium azide, 1 mM DTT buffer; TCEP, tris(2-carboxyethyl)phosphine.

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binding, all of which are in the N-terminal domain (*13*). Finally, comparison to UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA), an enzyme structurally and functionally analogous to EPSP synthase (*14*−*16*), also supports the idea that the enolpyruvyl acceptor molecule (S3P for EPSP synthase, UDP-*N*-acetylglucosamine for MurA) binds to the N-terminal domain. Because of the pronounced similarities between the two enzymes, their active sites are expected to have similar organizations. The crystal structure of MurA in the presence of a fluorinated intermediate reveals that residues from both domains contribute to the active site of this enzyme (*16*). However, the C-terminal domain is mainly involved in PEP binding, while mostly residues from the N-terminal domain contribute to the UDP-NAG binding site. This portion of the active site corresponds to the S3P binding site in EPSP synthase. It is thus reasonable to expect that the N-terminal domain is primarily responsible for S3P binding in EPSP synthase.

Though the structure (*6*, *10*) and mechanism (*17*−*22*) of EPSP synthase have been extensively investigated, the identity of residues that bind the substrates and inhibitor is as yet unknown. Here we attempt to establish the binding of S3P to the isolated N-terminal domain as a prelude to NMR studies that will define the binding site of S3P in atomic detail. Because the isolated N-terminal domain retains the secondary structural organization that it possesses in the full-length protein (*23*), studies of the binding of S3P to the isolated domain should be applicable to the full-length enzyme.

MATERIALS AND METHODS

Protein Expression and Purification. Wild-type EPSP synthase was expressed and purified as described previously (*24*). The N-terminal domain plasmid construct consisted of the entire *aroA* gene sequence modified by the insertion of a stop codon immediately after codon 240 (Muta-gene mutagenesis kit, Biorad). The modified gene was ligated into pET24b (Novagen) for overexpression; the new construct was confirmed by DNA sequencing. *E. coli* BL21(λDE3) was transformed with the plasmid, and cultures were incubated at 37 °C in M9 media until $OD_{600} \cong 0.6$, at which time overexpression was induced by the addition of 0.4 mM IPTG and growth continued to completion. Cells were harvested by centrifugation and stored at −70 °C. Lysis was effected by addition of lysozyme and DNase I (both from Sigma) in 50 mM Tris, pH 7.8, 3 mM NaN_3, and 1 mM DTT (TAD buffer). Protein was precipitated from the lysis supernatant by NH_4SO_4. The resolubilized pellet was loaded onto a 315 × 35 mm custom gel filtration column (Pharmacia) in TAD buffer, followed by elution from a MonoQ anion exchange column (Pharmacia) in 0−500 mM KCl, also in TAD. This process afforded the N-terminal domain at >95% purity by SDS−PAGE analysis. The domain was characterized by N-terminal amino acid sequencing and MALDI mass spectrometry (data not shown). For isotopic labeling, M9 medium was used with $^{15}NH_4Cl$ as the nitrogen source. Growth conditions and purification procedures were the same as for unlabeled protein.

NMR Spectroscopy. S3P was isolated from cultures of *Klebsiella pneumoniae* (*18*) and purified as described previously (*24*). NMR samples contained ∼1 mM ^{15}N-labeled N-terminal domain in ∼500 μL of TAD buffer. S3P was titrated into one sample by addition of small volumes of a 136 mM stock, and ^{15}N-HSQCs (*25*) were acquired after each addition to monitor the change in N-terminal domain peak positions. After acquisition of high-quality ^{15}N-HSQCs, samples of N-terminal domain alone and in saturating concentrations of S3P were lyophilized to dryness and redissolved in D_2O buffer. The pH was maintained at 7.8, and ^{15}N-HSQCs were acquired at 0.5, 2, 4, 8, 16, 27, and 42 h. Experiments were performed at 22 °C on a Varian Inova 500 MHz spectrometer. Spectra were referenced externally to the ^{1}H resonance of TSP at 0.0 ppm. The ^{15}N-HSQC pulse sequence used was from Varian's Protein Pack software. Data were processed using NMRPipe (*26*) and analyzed with PIPP (*27*). FIDs were defined by 512 and 128 complex points in F2 and F1, respectively. Processing typically employed zero filling to 2048 (F2) and 1024 (F1), application of a 90° phase-shifted sinebell-squared window function, and appropriate phase corrections.

For ^{31}P NMR experiments, samples consisted of 0.6 mM EPSP synthase or 1.0 mM N-terminal domain in TAD (pH 7.8). S3P was added at 0.5×, 1.0×, and 2.0× relative molar concentration to the full-length enzyme sample, and at these same concentrations following a 0.25× addition to the N-terminal domain sample. At 2.0× S3P, GLP was added to each sample, also at a 2.0× molar ratio. Control spectra included samples of each protein alone and both S3P and GLP alone. After each addition, a 1D ^{31}P spectrum was acquired at 22 °C on a Varian Inova 500 MHz spectrometer. Spectra were referenced to external 1 M KH_2PO_4, pH 7, at 0 ppm. FIDs consisted of 24K points, and acquisition times were 1 s. Processing was accomplished in FELIX 98.0 (Molecular Simulations) with zero filling to 32K points, application of an exponential window function using 20 Hz line broadening, and appropriate phase corrections.

Calorimetry. Samples were prepared of full-length EPSP synthase and N-terminal domain by dialysis of a 0.5−1.0 mM protein stock into 50 mM Tris, pH 7.8, 1 mM TCEP (Molecular Probes, Inc.). S3P was prepared by dilution from a 136 mM stock in water into the same buffer. Isothermal microtitration calorimetry was performed at 27 °C using a VP-ITC calorimeter from Microcal Inc. Heats of dilution were measured by injecting S3P into buffer in the absence of protein and were taken into account when fitting the data. Subsequently, a series of 5−10 μL injections of 2.4−2.8 mM S3P were dispensed into a 0.1 mM solution of the full-length enzyme or the N-terminal domain. Data were fit via a least-squares algorithm to a single site model using software provided with the instrument. The same program calculated the stoichiometry (N), association constant (K_a), enthalpy (ΔH), and entropy (ΔS) for the reaction. The K_d referred to in the text is the inverse of the calculated K_a.

RESULTS

NMR Spectroscopy. It was immediately apparent from the HSQCs that a conformational change took place in the N-terminal domain upon addition of S3P. Peak positions changed gradually until saturation was observed, which occurred at 1.5× relative molar concentration of S3P. The S3P-bound state was stable for at least 7 days in a properly preserved sample. The original and final peak positions are

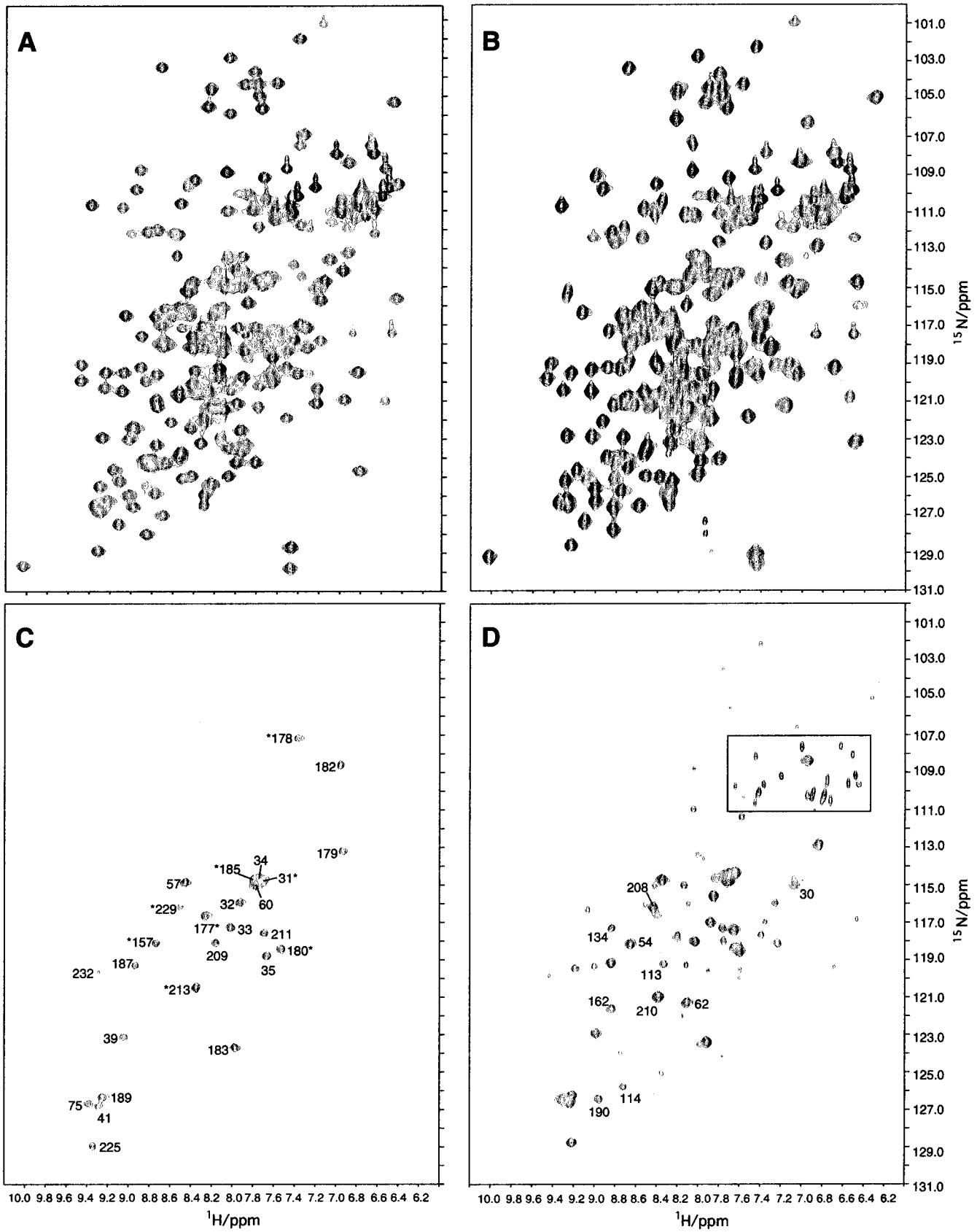

FIGURE 2: ^{15}N-HSQC spectra of (A) the N-terminal domain alone, (B) the N-terminal domain in the presence of saturating S3P, (C) the N-terminal domain alone 42 h after exchange into D_2O, and (D) the N-terminal domain plus S3P 42 h after exchange into D_2O. Peaks are labeled in (C) according to their residue number (assignments obtained as described in *23*). Most resonances in (C) are also present in (D), with the exception of those marked with an asterisk. The labels in (D) correspond to amide proton peaks that persist only when S3P is bound. The region containing side chain amine peaks is indicated by the rectangle.

compared in Figure 2, panels A and B. Peak intensities decreased with time after exchange into D_2O. After 42 h, 26 amide proton peaks remained for the sample containing the N-terminal domain alone (Figure 2C). In contrast, many

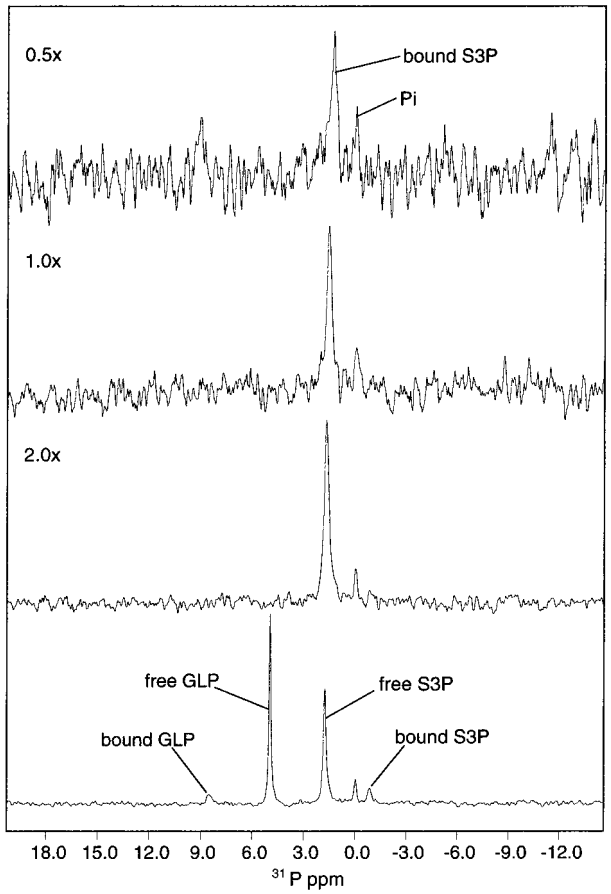

FIGURE 3: ^{31}P NMR spectra of S3P in the presence of full-length EPSP synthase. Relative molar concentrations of S3P are given in the upper left-hand corner of each spectrum. Chemical shifts are described under Results.

more peaks remained after the same amount of time in D_2O for the sample containing S3P (Figure 2D). Comparison of Figure 2C and Figure 2D revealed eight peaks (marked by an asterisk) that were present after 42 h in the sample of N-terminal domain alone but absent from the sample containing S3P. Additionally, 10 of the strong resonances that appear in Figure 2D are absent from the spectrum of N-terminal domain alone. Clearly, a conformational change is occurring in the N-terminal domain which increases the solvent exposure of some residues and decreases that of others. Although most of the peaks in Figure 2D belong to backbone amide protons, there are also many side chain amine peaks that are protected from exchange by the presence of S3P.

Whereas the HSQCs contain information about conformational changes in the N-terminal domain, the ^{31}P NMR spectra provide insight into the chemical environment of S3P. The behavior of S3P in the presence of EPSP synthase has been reported previously (*28, 29*) and was reproduced here to ensure an accurate comparison to the results obtained for the N-terminal domain. The chemical shift of S3P bound to EPSP synthase is 1.4 ppm (Figure 3). The resonance is broad but readily observable when enzyme concentration is in excess (0.5× S3P) and only a small amount of free S3P is present. Upon addition of S3P at equivalent or higher concentrations, however, the resonance of free S3P at 1.8 ppm dominates, so that the two peaks are not resolvable at 500 MHz. The line width of bound S3P at 0.5× is 87 Hz

Table 1: Line Widths in ^{31}P Spectra[a]

	full-length EPSP synthase		N-terminal domain
concn	free S3P	bound S3P	free S3P
0.25×	nd[b]	nd	20
0.5×	−	87	33
1.0×	76	nd	35
2.0×	62	nd	36
plus GLP	33	60	31

[a] All values have units of hertz. [b] Not determined.

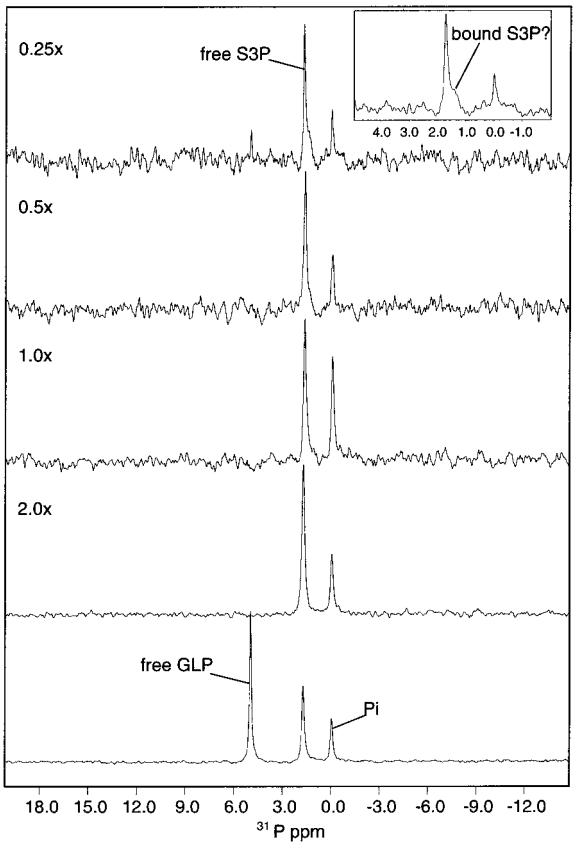

FIGURE 4: ^{31}P NMR spectra of S3P in the presence of the N-terminal domain. Relative molar concentrations of S3P are given in the upper left-hand corner of each spectrum. The inset at the top right-hand side is an expansion of the middle portion of the 0.25× spectrum, which shows the position of the putative bound S3P peak.

(see Table 1), and it contributes to the line width of the free S3P peak in the 1.0× and 2.0× spectra. Even after GLP is added to the sample, the free S3P peak remains slightly broadened (33 Hz) compared to the natural line width of S3P alone (20 Hz, data not shown) due to the exchange equilibrium between the free and bound states. When GLP is added, the bound S3P resonance shifts upfield to −0.7 ppm and exhibits a new line width of 60 Hz, while bound and free GLP peaks appear at 8.7 and 5.1 ppm, respectively. The free S3P peak remains situated at 1.8 ppm, where it also resides in the absence of protein.

The S3P phosphorus resonance behaves similarly, though not identically, in the presence of N-terminal domain. The free S3P resonance at 1.8 ppm predominates at all concentrations studied (Figure 4). However, at lower relative concentrations of S3P (0.25× and 0.5×), there appears an unresolved but reproducibly detectable shoulder upfield of the

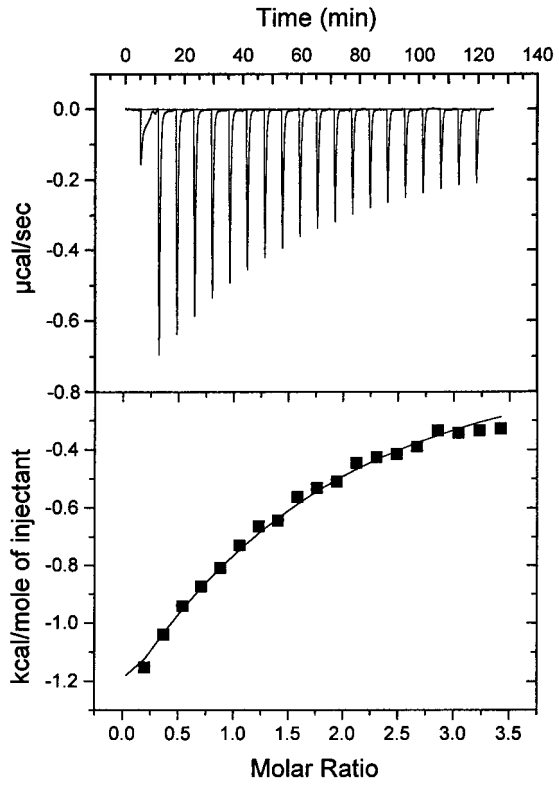

FIGURE 5: Raw (top) and fitted (bottom) calorimetry data from the titration of the N-terminal domain with S3P.

free S3P peak that likely belongs to the bound S3P. The apex of this peak occurs at 1.5 ppm. Its line width cannot be directly measured due to the overlap, but its contribution to the width of the free S3P peak is evident in the 0.5× and subsequent spectra. An average line width of 33.8 Hz in these spectra is larger than that for S3P alone (20 Hz, data not shown) and equivalent to the free S3P line width in the presence of EPSP synthase and GLP, indicating that for this sample, too, the S3P is exchanging between free and bound states. The bound state line width will be dominated by the rotational correlation time of the protein molecule, so it is significant that S3P bound to the full-length enzyme produces a peak about twice as wide (60 Hz) as that for S3P bound to the N-terminal domain (∼33 Hz). Note also that the N-terminal domain apparently does not bind GLP. This is consistent with the results of titrations monitored by ^{15}N-HSQC which indicated that GLP does not bind to the N-terminal domain alone or to the preformed N-terminal domain·S3P complex (data not shown).

Calorimetry. The ability of full-length EPSP synthase to bind S3P was tested as a control for the calorimetry conditions (data not shown). The experiments performed resulted in a calculated K_d of 9.7 μM, which agrees well with previously published data from other sources (*30*). The N-terminal domain also bound reproducibly to S3P, and the raw and fitted data from one experiment are shown in Figure 5. The Microcal data-fitting program determined a K_d of 73.5 μM for the N-terminal domain, only 7-fold higher than that for the full-length enzyme. Values for all the calculated parameters are listed in Table 2 and represent the average from three experimental data sets for which two to three fitting sessions each were performed.

Table 2: Thermodynamic and Stoichiometric Parameters Calculated from Calorimetry

	EPSP synthase		N-terminal domain	
parameter	value	error	value	error
N	0.274	±0.005	0.839	±0.162
K_a	102800 M^{-1}	±4397	13591 M^{-1}	±4002
ΔH	−4.612 kcal/mol	±0.108	−3.287 kcal/mol	±0.965
ΔS	7.57 cal/(mol·K)	nd[a]	7.69 cal/(mol·K)	nd[a]

[a] Not determined.

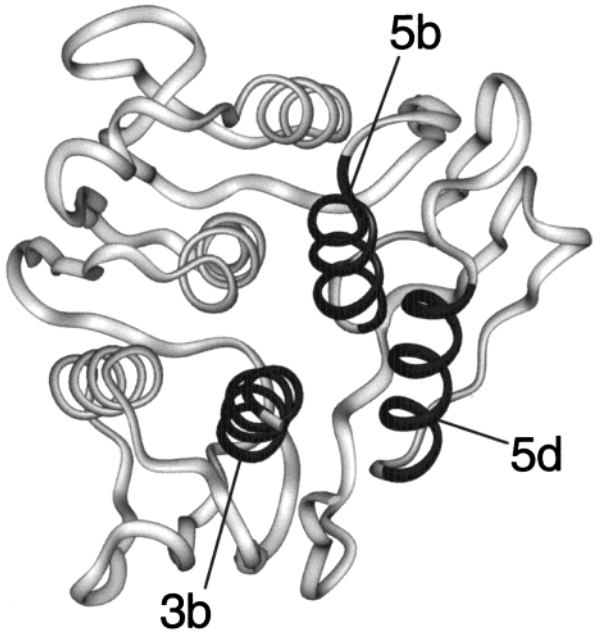

FIGURE 6: Ribbon diagram of the backbone of residues 20−240 in EPSP synthase (PDB Accession No. 1EPS). Helices 3b, 5b, and 5d are shaded to show the proposed locale of S3P binding.

DISCUSSION

The results presented here correlate well with previous data from our lab and others. Chemical shift perturbations described elsewhere (*23*) localize the conformational change upon S3P binding to subdomains 3 and 5 [nomenclature from Stallings et al. (*6*)]. A great majority of the hydrogen bonding differences between the free and liganded N-terminal domain also occur in these subdomains. According to the D_2O exchange experiments, residues 177, 178, and 180 become more accessible to solvent upon S3P binding. These residues are located in α-helix 5b just below Tyr200 and Ser197, which are purportedly involved in S3P binding (*13*, *23*). Several additional residues (30, 31, 208, and 210) that experience a change in solvent accessibility are located in adjacent α-helices. These findings support the localization of the S3P binding site to helices 3b, 5b, and 5d of the N-terminal domain (Figure 6).

The S3P phosphorus peak is known to undergo a small change in chemical shift upon binding to EPSP synthase (*28*, *29*). The change observed here, 0.4 ppm, compares favorably with the results of Castellino et al. (*28*) and previous findings in our lab (*29*). A difference of −2.1 ppm upon addition of GLP is also in agreement with these studies, as are the chemical shifts of free (5.1 ppm) and bound (8.7 ppm) GLP. Analysis of the chemical shifts of the N-terminal domain

both in the presence and in the absence of S3P indicates that the isolated domain retains the secondary structural organization that it possesses in the full-length enzyme (*23*). Based on this finding, we would predict that the S3P binding sites on each protein species are similar. This is supported by the close chemical shift values for bound S3P in the presence of EPSP synthase (1.4 ppm) and the N-terminal domain (1.5 ppm). The line widths are consistent with fast exchange between free and bound states where the bound state is dominated by the rotational correlation time of either the isolated N-terminal domain (~33 Hz) or the full-length protein (60 Hz).

Ream et al. (*30*) determined a K_d of 10 μM for the binding of S3P to full-length EPSP synthase in 50 mM Hepes, pH 6.8, 50 mM KCl. Enthalpic and entropic contributions to the free energy vary widely between buffers because of differences in heat capacities and ionization enthalpies (*31*), but the order of magnitude of the overall free energy change (ΔG) may be assumed to be similar for a given reaction in different buffers (see, for example, Chapter 7 of *32*). This is confirmed by the fact that the K_d determined for EPSP synthase in our buffer (9.7 μM) is equal within error to that of Ream et al. (*30*). The determined stoichiometry (*N*) for the full-length enzyme's binding reaction is much less than expected for a 1:1 binding ratio. This is most likely due to slight precipitation in the protein sample, which according to Doyle (*31*) does not affect the reliability of the binding constant and the enthalpy.

Since the ΔG for binding is calculated from the measured K_a (by $\Delta G = -RT \ln K$), we can compare the ΔG values for the EPSP synthase and N-terminal domain binding reactions. These are, respectively, −6.8 kcal/mol (from $K_a = 102\,800\ M^{-1}$) and −5.6 kcal/mol (from $K_a = 13\,591\ M^{-1}$). Note that a 7-fold difference in the equilibrium constant yields only a small difference in the free energies. Based on the similarity of the calculated ΔG's, we propose that most of the energy involved in the binding of S3P to EPSP synthase is contributed by the N-terminal domain. The C-terminal domain may contribute either minimally or significantly to the binding, but this cannot be predicted with any certainty from the current results. This proposal agrees well with the Monsanto picture (*13*), which may be described as an open conformation with no contacts between the bound S3P and the C-terminal domain.

The ability of the isolated N-terminal domain to bind S3P is essential for ongoing studies of the structure of the binary complex. We have presented evidence for the binding of S3P to the isolated N-terminal domain of EPSP synthase using both NMR spectroscopy and isothermal titration calorimetry. Our experimental results indicate that there is a saturable and stable conformational change in the isolated domain upon S3P binding and that the chemical environment of the S3P phosphorus when bound to the isolated domain is very similar to that of S3P bound to EPSP synthase. From free energy calculations, it appears that the N-terminal domain contributes most of the energy for binding the substrate S3P. We anticipate that further solution structural data will provide complementary information about the various conformational states assumed by EPSP synthase and the contributions of the respective domains to these changes.

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REFERENCES

1. Pittard, J., and Wallace, B. J. (1966) *J. Bacteriol. 91*, 1494−1508.
2. Du, W., Wallis, N. G., Mazzulla, M. J., Chalker, A. F., Zhang, L., Liu, W. S., Kallender, H., and Payne, D. J. (2000) *Eur. J. Biochem. 267*, 222−227.
3. Roberts, F., Roberts, C. W., Johnson, J. J., Kyle, D. E., Krell, T., Coggins, J. R., Coombs, G. H., Milhous, W. K., Tzipori, S., Ferguson, D. J. P., Chakrabarti, D., and McLeod, R. (1998) *Nature 393*, 801−805.
4. Kishore, G. M., and Shah, D. M. (1988) *Annu. Rev. Biochem. 57*, 627−663.
5. Steinrucken, H. C., and Amrhein, N. (1984) *Eur. J. Biochem. 143*, 351−357.
6. Stallings, W. C., Abdelmeguid, S. S., Lim, L. W., Shieh, H. S., Dayringer, H. E., Leimgruber, N. K., Stegeman, R. A., Anderson, K. S., Sikorski, J. A., Padgette, S. R., and Kishore, G. M. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 5046−5050.
7. Padgette, S. R., Re, D. B., Gasser, C. S., Eichholtz, D. A., Frazier, R. B., Hironaka, C. M., Levine, E. B., Shah, D. M., Fraley, R. T., and Kishore, G. M. (1991) *J. Biol. Chem. 266*, 22364−22369.
8. Shuttleworth, W. A., and Evans, J. N. S. (1994) *Biochemistry 33*, 7062−7068.
9. Shuttleworth, W. A., and Evans, J. N. S. (1996) *Arch. Biochem. Biophys. 334*, 37−42.
10. Shuttleworth, W. A., Pohl, M. E., Helms, G. L., Jakeman, D. L., and Evans, J. N. S. (1999) *Biochemistry 38*, 296−302.
11. Padgette, S. R., Smith, C. E., Huynh, Q. K., and Kishore, G. M. (1988) *Arch. Biochem. Biophys. 266*, 254−262.
12. Krekel, F., Oecking, C., Amrhein, N., and Macheroux, P. (1999) *Biochemistry 38*, 8864−8878.
13. Franz, J. E., Mao, M. K., and Sikorski, J. A. (1997) *Glyphosate: A Unique Global Herbicide*, American Chemical Society, Washington, DC.
14. Schonbrunn, E., Sack, S., Eschenburg, S., Perrakis, A., Krekel, F., Amrhein, N., and Mandelkow, E. (1996) *Structure 4*, 1065−1075.
15. Skarzynski, T., Mistry, A., Wonacott, A., Hutchinson, S. E., Kelly, V. A., and Duncan, K. (1996) *Structure 4*, 1465−1474.
16. Skarzynski, T., Kim, D. H., Lees, W. J., Walsh, C. T., and Duncan, K. (1998) *Biochemistry 37*, 2572−2577.
17. Anderson, K. S., Sikorski, J. A., and Johnson, K. A. (1988b) *Biochemistry 27*, 1604−1610.
18. Bondinell, W. E., Vnek, J., Knowles, P. F., Sprecher, M., and Sprinson, D. B. (1971) *J. Biol. Chem. 246*, 6191−6196.
19. Grimshaw, C. E., Sogo, S. G., and Knowles, J. R. (1982) *J. Biol. Chem. 257*, 596−598.
20. Barlow, P. N., Appleyard, R. J., Wilson, B. J. O., and Evans, J. N. S. (1989) *Biochemistry 28*, 7985−7991, and correction p 10093.
21. Anderson, K. S., and Johnson, K. A. (1990) *Chem. Rev. 90*, 1131−1149.
22. Sikorski, J. A., and Gruys, K. J. (1997) *Acc. Chem. Res. 30*, 2−8.
23. Stauffer, M. E., Young, J. K., Helms, G. L., and Evans, J. N. S. (2001) *Biochemistry* (submitted for publication).
24. Shuttleworth, W. A., Hough, C. D., Bertrand, K. P., and Evans, J. N. S. (1992) *Protein Eng. 5*, 461−466.

25. Kay, L. E., Keifer, P., and Saarinen, T. (1992) *J. Am. Chem. Soc. 114*, 10663.
26. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) *J. Biomol. NMR 6*, 277−293.
27. Garrett, D. S., Powers, R., Gronenborn, A. M., and Clore, G. M. (1991) *J. Magn. Reson., Ser. B 95*, 214−220.
28. Castellino, S., Leo, G. C., Sammons, R. D., and Sikorski, J. A. (1989) *Biochemistry 28*, 3856−3868.
29. Evans, J. N. S. (1992) in *Pulsed Magnetic Resonance: NMR, ESR and Optics (A Recognition of E. L. Hahn)* (Bagguley, D., Ed.) pp 123−173, Oxford University Press, Oxford and New York.
30. Ream, J. E., Yuen, H. K., Frazier, R. B., and Sikorski, J. A. (1992) *Biochemistry 31*, 5528−5534.
31. Doyle, M. L. (1999) in *Current Protocols in Protein Science*, pp 20.4.1−20.4.24, John Wiley & Sons, Inc., New York.
32. Hinz, H.-J. (1986) *Thermodynamic Data for Biochemistry and Biotechnology*, Springer-Verlag, Heidelberg, Germany.

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