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Letter to the Editor: Sequential assignments of the isolated N-terminal domain of 5-enolpyruvylshikimate-3-phosphate synthase

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Biological context

5-Enolpyruvylshikimate-3-phosphate (EPSP) synthase catalyzes the formation of EPSP from shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) in the sixth step of the shikimate pathway. This pathway is absent from mammalian systems but present in plants, bacteria, fungi and parasites, making EPSP synthase a promising model for the development of new antibiotics. One suggested approach toward inhibitor design is the prevention of domain closure, an event that is necessary for catalysis. However, what triggers domain closure is in question due to conflicting crystal structures of the enzyme • S3P complex. One shows the enzyme in the open state, with S3P bound only to the N-terminal domain, while the other has S3P in contact with residues of both domains and the enzyme in the closed state.

We have prepared the isolated domains of EPSP synthase in order to analyze the individual contributions of the domains to the events of substrate binding and catalysis. In particular, isolation of the N-terminal domain will allow us to differentiate between the chemical shift effects of S3P binding and those of domain closure, which will provide a very specific basis for designing inhibitors of domain closure. Here we report the backbone and side chain chemical shift assignments for the isolated N-terminal domain. The assignments have provided a basis for interpretation of $^{2}H_2O$ exchange experiments (Stauffer et al., 2001a) and chemical shift mapping of the S3P binding site (Stauffer et al., 2001b).

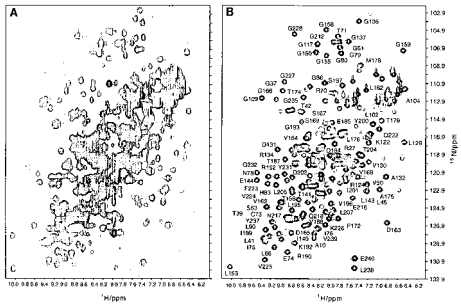

Figure 1. ^{15}N-HSQCs of the full-length EPSP synthase (A) and the N-terminal domain (B) with resolved portions labeled in (B) according to the obtained assignments. Some resonances (e.g., Ser23 and Ser25) are below the contour level.

Methods and experiments

The 240-residue N-terminal domain (M_r 26 kDa) of EPSP synthase was produced as described elsewhere (Stauffer et al., 2001a). Isotopes for labeled samples were obtained from Cambridge Isotope Labs and used as follows: $^{15}NH_4Cl$ at 0.5 g/l; 1,2-$^{13}C_2$-NaOAc at 3 g/l; and 99.9% D_2O at 70% final deuterium concentration. The triple-labeled protein, grown in 70% D_2O with ^{13}C-NaOAc and $^{15}NH_4Cl$ as carbon and nitrogen sources, was obtained at 21 mg/L yield. The same yield was achieved for the double-labeled protein grown under the same conditions, except that 100% H_2O was used. The proteins were analyzed by MALDI mass spectrometry (single-, double-, and triple-labeled samples in succession), and mass differences between isotopically labeled samples were used to estimate the percent isotope incorporation to be ∼100% for ^{15}N and ^{13}C, and ∼50% for ^{2}H. NMR samples were prepared by desalting the purified protein to <1.0 mM KCl and concentrating to ∼1.0 mM

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in 50 mM Tris, pH 7.8, 3 mM NaN_3, 1 mM DTT buffer, followed by addition of D_2O to ∼8%. All NMR experiments were performed at 22 °C on a Varian Inova 500 NMR spectrometer (499.86 MHz, ^{1}H). Backbone assignments were made using data from the following Varian Protein Pack experiments: on the U-^{15}N, ^{13}C, ∼50%-^{2}H-labeled N-terminal domain, ^{15}N-HSQC, HNCA, HN(CO)CA, HNCACB, HNCO, HN(CA)CO, and ^{15}N-NOESY-HSQC (t_m = 150 ms); on the U-^{15}N, ^{13}C-labeled N-terminal domain, ^{15}N-HSQC, HNCA, and H(CA)CO. For all experiments, 16 scans were collected with each FID consisting of 512 complex time domain points for an acquisition time of 0.064 s. Spectral widths were 8000 Hz for ^{1}H, 2000 Hz for ^{15}N, and set appropriately for ^{13}C. All experiments were sensitivity-enhanced. Spectra were processed and analyzed on a Silicon Graphics O2 workstation using NMRPipe and PIPP. ^{1}H chemical shifts were referenced to external TSP at 0 ppm, and ^{15}N and ^{13}C shifts were calculated relative to this as recommended by IUPAC. Processing included application of a 90°-shifted sinebell window function and appropriate phase corrections, as well as zero filling to 128, 512, and 2048 points in F1, F2, and F3, respectively. Linear prediction was applied in F1 (^{15}N). Side chain assignments were obtained using CCC-TOCSY, HCC-TOCSY, and HCCH-TOCSY experiments from Varian's Protein Pack. Acquisition of the TOCSY experiments was carried out with 512 (F3), 128 (F2), and 32 (F1) points, appropriately set spectral widths, and a pre-scan delay of 0.8–1.0 s. Processing these data employed zero-filling to 1024 (F3), 512 (F2), and 128 (F1) points, application of a 90°-shifted sinebell function, and appropriate phase corrections, except that the F1 (^{13}C) dimension of the HCCH-TOCSY was zero-filled to 256 to increase the resolution on crowded carbon planes. Linear prediction was used in the ^{15}N dimensions.

Predictions of secondary structure from uncorrected shifts using the chemical shift index (CSI) were made according to the guidelines provided by Wishart et al. We extracted crosspeak intensity information for $^1H^N$-$^1H^N$ and $^1H^N$-^{1}H correlations from the ^{15}N-NOESY-HSQC experiment, and patterns in the observed NOE intensities were used to predict secondary structure as outlined in Wüthrich et al. (1984).

Extent of assignments and data deposition

98% of the backbone atoms (C′, H^N, N, C, H) were assigned, including 223 non-proline residues. Residues 1–3, 170, and 171 remain unassigned. The N-terminal residues likely undergo fast exchange with water, while 170–171, which are located at the active site, may also undergo chemical exchange or be subject to restricted motion. Two outlying peaks at 5.40 and 3.99 ppm ($^1H^N$, not shown) were assigned to Gly93 and Asn147, respectively. Uncharacteristic chemical shift values indicate that these residues have a unique chemical environment. Figure 1 shows the ^{15}N-HSQC with 97 well-resolved resonances labeled according to the obtained assignments. 97% of the side chain atoms (C^β, etc.) were assigned, including prolines and excluding aromatic residues. Secondary structure predictions from CSI and NOEs for the N-terminal domain, as well as the appearance of the N-terminal peak pattern in the full-length HSQC (Figure 1), suggest that it retains the structural elements present in the intact protein.

The chemical shifts have been deposited in the BioMagResBank (http://www.bmrb.wisc.edu) under accession number 4854. $^1H^N$and ^{15}N values are from the protonated sample, while all ^{13}C and side chain ^{1}H shifts are from the 50% deuterated sample and were not corrected for the deuterium isotope effect due to the unavailability of a standard correction factor for all carbon types.

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