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Engineering and Characterization of the Isolated C-Terminal Domain of 5-Enolpyruvylshikimate-3-phosphate (EPSP) Synthase

KIM, HAK JUN[1], HYUN WOO KIM[2], AND SUNG-HO KANG[1]*

[1]*Department of Applied Polar Science, Korea Polar Research Institute, Incheon 406-840, Korea*
[2]*Department of Marine Biology, Pukyong National University, Busan 608-737, Korea*

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Abstract 5-Enolpyruvylshikimate-3-phosphate (EPSP) synthase catalyzes the formation of EPSP and inorganic phosphate from shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) in the biosynthesis of aromatic amino acids. To delineate the domain-specific function, we successfully isolated the discontinuous C-terminal domain (residues 1-21, linkers, 240-427) of EPSP synthase (427 residues) by site-directed mutagenesis. The engineered C-terminal domains containing no linker (CTD), or with gly-gly (CTD^{GG}) and gly-ser-ser-gly (CTD^{GSSG}) linkers were purified and characterized as having distinct native-like secondary and tertiary structures. However, isothermal titration calorimetry (ITC), ^{15}N-HSQC, and ^{31}P-NMR revealed that neither its substrate nor inhibitor binds the isolated domain. The isolated domain maintained structural integrity, but did not function as the half of the full-length protein.

Keywords: EPSP synthase, herbicide, glyphosate, protein engineering, isothermal titration calorimetry

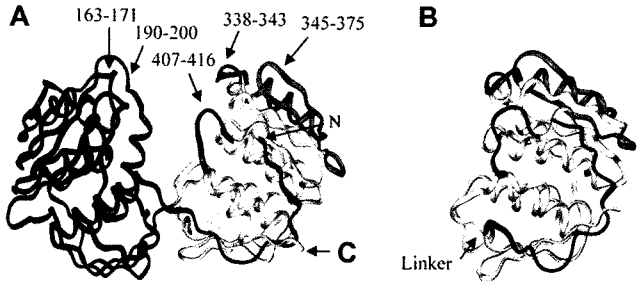

Fig. 1. Ribbon diagrams of EPSP synthase (**A**) and the engineered C-terminal domain (**B**).
The N-terminal domain is displayed in blue, and the C-terminal domain in yellow with the N-terminal tail in blue. In A, two tryptophan residues (residues 289 and 337) located in the C-terminal domain are displayed as stick. Residues that undergo a large displacement on both domains during catalysis are in red. A stretch of amino acids susceptible to proteolysis upon conformational change is in pink.

5-Enolpyruylshikimate-3-phosphate (EPSP) synthase (EC 2.5.1.19) catalyzes the formation of EPSP and inorganic phosphate from shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) in the biosynthesis of aromatic amino acids (scheme 1) [8, 13]. For more than two decades, EPSP synthase has attracted the attention of researchers from agricultural and pharmaceutical industries and academia for reasons as follows: Firstly, EPSP synthase is the target of glyphosate, the active ingredient of the broad-spectrum post-emergence herbicide Roundup that sells the most worldwide to control weeds, and its glyphosate-tolerant mutant has been used for the genetically modified organisms [5, 9, 11, 12, 15]. Secondly, since the shikimate pathway is absent in mammals, EPSP synthase is an intriguing target for the development of new antibiotics and antiparasitics in the advent of antibiotic resistant pathogens such as *Staphylococcus aureus* and *Streptococcus pneumoniae* [2, 3, 18].

The crystal structure of the unliganded protein has shown that it is a monomeric 46 kDa protein of 427 residues and consists of two distinct domains, the N- and C-terminal domains, connected by two linkers (Fig 1A) [20]. These domains are structurally similar, each formed by 3-fold replication of a protein folding unit comprising two parallel helices and four-stranded sheets ($\beta\alpha\beta\alpha\beta\beta$). Although two domains have been thought to be duplicated during the molecular evolution, the C-terminal domain is discontinuous and is likely to be responsible for catalytic function [19]. Biochemical studies using fluorescence [1], isothermal titration calorimetry [17], and proteolysis [14] suggested that ligand and inhibitor binding to the enzyme induces a significant conformational change from an open to a closed state.

It was not until recently, however, that x-ray crystallographic efforts have met with success in demonstrating the binary

*Corresponding author
Phone: 82-32-260-6251; Fax: 82-32-260-6256;
E-mail: shkang@kopri.re.kr

(EPSPS•S3P) and ternary (EPSPS•S3P•GLP) complexes of this enzyme [4, 19]. The ternary complex structure of Schonbrunn *et al.* [19] clearly illustrated that the active-site residues involved in S3P and glyphosate binding are distributed almost equally in the closed conformation of the two domains (Fig. 1) [19] Therefore, the domain-specific substrate and/or inhibitor binding and involvement in domain closure during catalysis may provide insights on rational drug or inhibitor design. For this to be true, we tried to isolate the N- and C-terminal domains and characterize them using various biochemical techniques. Stauffer *et al.* [21–23] showed that the isolated N-terminal domain (residues 1-240) adopts a native-like fold and binds S3P with similar affinity as that of the full-length EPSP synthase. Surprisingly, binding of S3P also induces significant conformational changes in the isolated N-terminal domain [21]. Hence, each isolated domain study may provide useful information that cannot be obtained from the full-length protein.

Here, we describe the design, construction, and characterization of the isolated C-terminal domains of EPSP synthase. An initial attempt to characterize the C-terminal domain (residues 241-427) without the N-terminal tail (residues 1-20) was not successful (data not shown). The N-terminal tail appeared to be important for the proper folding of the protein. Prior to the construction of the engineered C-terminal domain, structures of the various C-terminal domains were modeled from the coordinate of the crystal structure of unliganded *E. coli* EPSP synthase using the InsightII software package (Accelrys, San Diego, CA, U.S.A.) operating under IRIX 6.5. The Biopolymer module of InsightII software package was used to delete residues 22-239 from the crystal structure and add linkers connecting residue Ser21 to Glu240. Backbones of Ser21 and Glu240 were 4.3 Å away from each other, and these residues were selected as cutting and linking sites. Molecular modeling studies were carried out on the C-terminal domains (1-21, linker, 240-427) with no linker, and with Gly, Gly-Gly, Gly-Ser-Gly, and Gly-Ser-Ser-Gly linkers between Ser21 and Glu240. Molecular dynamics simulations predicted that the Gly-Gly linker would result in more favorable turn and dihedral angles than the others. A Ramachandran plot from the PROCHECK has shown that about 90% of backbone dihedral angles of the C-terminal domain in full-length EPSP synthase are in the most favored region, whereas the modeled C-terminal domains have slightly different values in that region: CTD^{GG} (78%), CTD^{GSSG} (77%), CTD^{G} (76%), CTD (74%). The following three C-terminal domains were constructed upon the prediction for comparison; with no linker (CTD), Gly-Gly linker (CTD^{GG}), and Gly-Ser-Ser-Gly linker (CTD^{GSSG}). CTD and CTD^{GG} were constructed by looping out the entire gene fragment encoding the N-terminal domain (residue 22-239) from pWS250 with the primers 5'-pGAA GAT GCA TCG CCT TCG *GAA CCG GGC AGA TTA A*-3' (at the 5', G is phosphorylated to facilitate the ligation and the underlined region complements residues 240-246, whereas the italicized region complements residues 16-21) and 5'-pAGA TGC ATC GCC TTC ACC ACC GGA

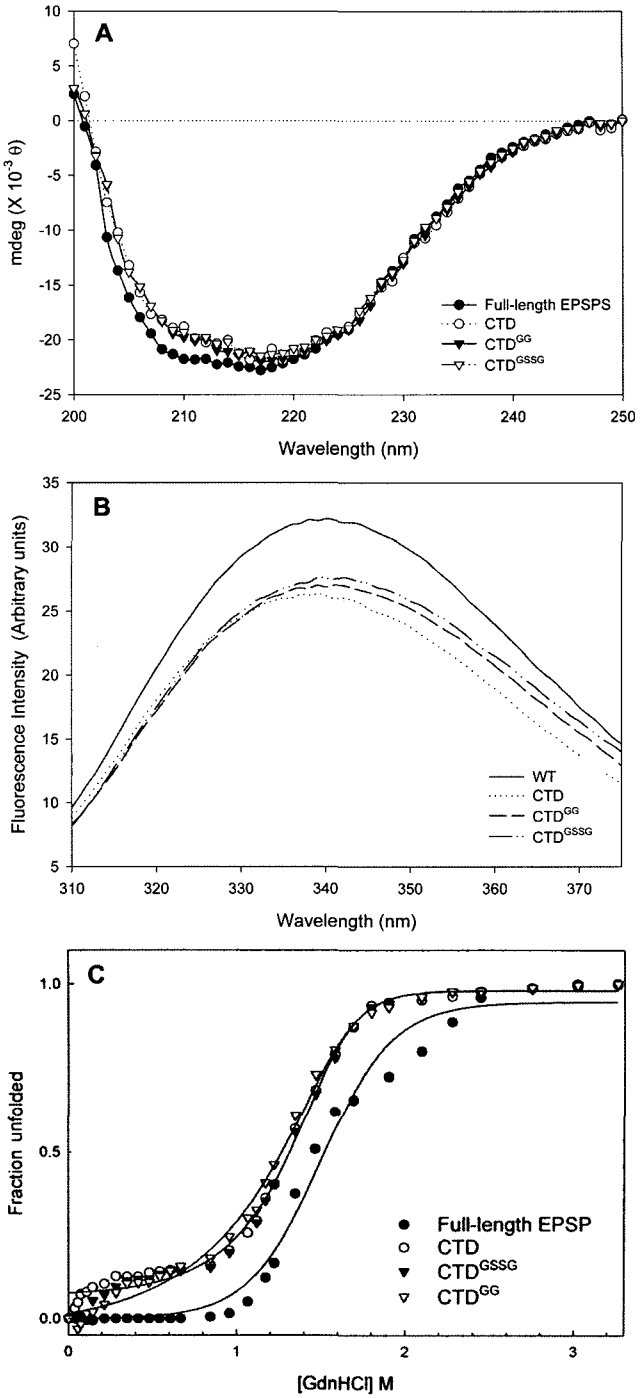

Fig. 2. Far-UV CD (**A**) and fluorescence (**B**) spectra of the full-length protein and the engineered C-terminal domains. **C.** Equilibrium unfolding curves of the full-length protein and the engineered C-terminal domains induced by GdnHCl and monitored by tryptophan fluorescence.

ACC GGG CAG-3' for CTD and CTD^{GG}, respectively, using the Muta-Gene Phagemid *In Vitro* Mutagenesis kit (BioRad). CTD^{GSSG} was directly constructed from a CTD^{GG} plasmid using the Quik Change method (Stratagene).

The isolated domains were expressed purified as described previously [21]. To rule out the possibility of contamination by the wild-type EPSP synthase in the protein samples, all the plasmids were transformed into AB2829 (DE3), an *aro*A^- strain, for overexpression. Unlike CTDΔ2-20, the engineered C-terminal domains, CTD, CTD^{GG}, and CTD^{GSSG}, were all overexpressed as soluble proteins, which indicates that the N-terminal tail folded into the C-terminal domain as expected and verified by NMR [10]. Molecular weights of all domains were determined by multiangle light scattering using Y3441 Tsk-gel HPLC size-exclusion chromatography. The data indicate that there is no evidence of dimer formation and that all of the C-terminal domains are monomers.

The purified proteins were characterized using CD, fluorescence, and Nuclear Magnetic Resonance (NMR) spectroscopy. The far-UV CD spectra were measured, as shown in Fig. 2A, using a Biologic MOS 450 spectrometer at 25°C with a 0.2-cm pathlength quartz cell. The concentration of the proteins was 22.4 μM in 20 mM potassium phosphate buffer, pH 7.5, 1 mM EDTA, and 1 mM DTT. All fluorescence spectra were collected at 22°C using an excitation wavelength of 295 nm on a Biologic MOS 250 spectrometer. The concentration of each protein was 10 μM in the same buffer as above. The engineered C-terminal domains showed that they have secondary structures similar to the full-length protein. The fluorescence emission spectra (Fig. 2B) were obtained for both full-length protein and isolated C-terminal domains since all tryptophan residues, 289 and 337, are located in the C-terminal domain. The maximal intensity at 340 nm was decreased about 20% without any shift in the wavelength. The overall environment of the two tryptophan residues in the isolated domains may be very similar to those in the full-length protein, except for Trp289, which is likely to be more exposed to the solvent that acts as a fluorescent quencher.

The stability of the C-terminal domain was determined by following changes in fluorescence of the domains by increasing the concentration of GdnHCl under equilibrium conditions. In both the C-terminal domains and in the full-length protein, the transitions appeared to be biphasic. During the unfolding transition, the proteins tended to aggregate to some degree. Hence, it is likely that there may be a folding intermediate in the protein. The values for C_M, m, $\Delta G°(H_2O)$ for all of the proteins studied here were obtained (Table 1). The removal of the entire N-terminal domain turned out to destabilize the C-terminal domain by a factor of $\Delta G°(H_2O)$ of about 1.7 kcal/mol. Although the isolated C-terminal domains showed similar $\Delta G°(H_2O)$ values to one another, CTD^{GG}, as predicted by the molecular modeling, seemed to be slightly more stable than the other two domains. Therefore, further characterization of the C-terminal domain was carried out with CTD^{GG} because of its slightly increased stability over the other domains.

Table 1. Thermodynamic properties of the domains measured by GdnHCl-induced unfolding transition.

Protein	$(GdnHCl)_{50\%}$ (M)	m (kcal/mol/M)	$\Delta G(H_2O)$ (kcal/mol)
CTD full-length	1.5 (±0.01)	3.6 (±0.1)	5.3 (±0.3)
CTD	1.29 (±0.01)	2.75 (±0.15)	3.6 (±0.2)
CTD^{GG}	1.30 (±0.02)	2.80 (±0.2)	3.7 (±0.1)
CTD^{GSSG}	1.25 (±0.01)	2.57 (±0.2)	3.6 (±0.2)

Recent crystal structures have revealed that residues involved in ligand binding are located almost evenly in the cleft formed by the two domains (Fig. 1) [19].

It has been shown that S3P can bind to the isolated N-terminal domain with a 7-fold higher dissociation constant (Kd) than that for the full-length protein [21]. However, PEP did not bind to the isolated N-terminal domain (unpublished data), even though Gruys *et al.* [7] proposed that excess amount of PEP inhibits binding of S3P by competitively binding at the S3P site [7]. Therefore, it is useful to investigate the contributions of ligand binding from the isolated domains, since these data may be useful in understanding the enzyme mechanism and motion. Substrates and/or inhibitor binding to the isolated CTD^{GG} was assessed by ITC, ^{15}N-HSQC, and ^{31}P-NMR. To get reliable data in an isothermal titration calorimetry (ITC) experiment, Ka*C=10 to 50 was used, where Ka is the binding constant and C is the molar concentration of the binding component in the sample cell. Since the Kd of the isolated C-terminal domain is unknown, a concentration of 1 mM was taken as a starting point. However, in ITC titration, neither the substrates nor the inhibitor bound to the isolated C-terminal domain, as demonstrated in Fig. 3A. NMR gives a wealth of information about the chemical environment of both a protein and a substrate, so the ^{15}N-Heteronuclear Single Quantum Coherence (HSQC) spectra provides information about conformational changes in the protein of interest upon binding of a ligand. The titrations of the ^{15}N-labeled protein with increasing concentrations of PEP or glyphosate followed by addition of S3P resulted in no change in the HSQC spectra (Fig. 3B). In EPSP synthase, the N-terminal domain alone binds S3P, whereas the C-terminal domain turned out not to bind either substrates or inhibitor. Conformational changes upon substrate binding are often required for enzyme catalysis, which accompanies significant atomic displacements in the enzyme-substrate complex (Fig. 1A) [6, 19, 24]. Interestingly, however, in the ternary complex of the EPSP synthase, a larger displacement (residues 338-343, 407-416) took place mainly in the C-terminal domain (Fig. 1A). These regions are mainly

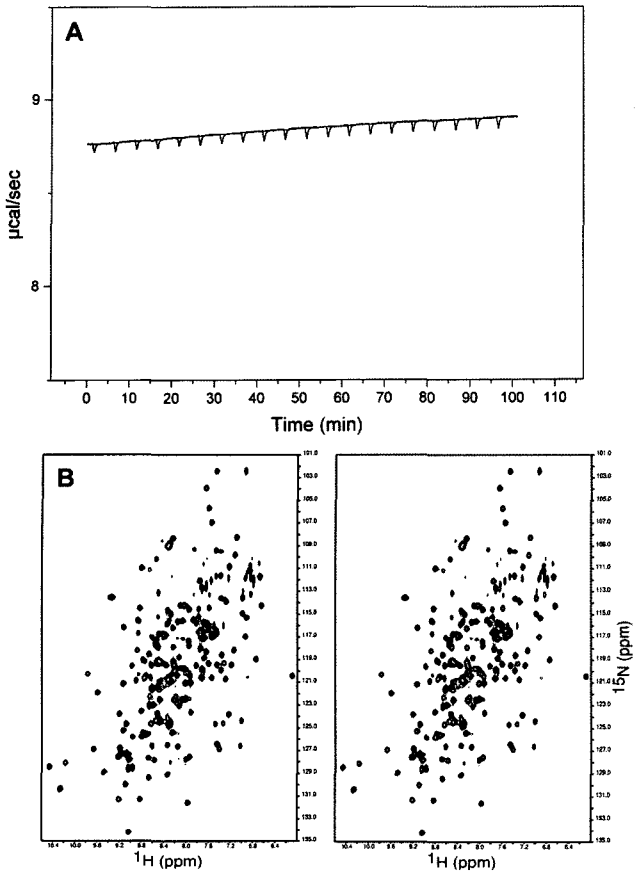

Fig. 3. Substrate binding of the engineered C-terminal domain. **A.** Representative graph of isothermal titration calorimetry titrated with glyphosate. **B.** HSQC spectra of the C-terminal domain titrated with 0× and 4× PEP added.

involved in PEP/glyphosate binding and catalysis [16, 19]. In particular, glyphosate binding causes conformational changes of the flexible loop (residues 407-416) to one turn of the 3_{10} helix (residues 407-411). In the middle of the residues 338-343, E341 may function as a proton donor during catalysis. Krekel *et al.* [14] also demonstrated that the "closed" conformation of EPSP synthase increased the stability toward proteolytic degradation, suggesting a conformational change upon substrate binding [14].

Attempts were made to functionally reconstitute the isolated N- and C-terminal domains by addition of substrates and/or glyphosate. Since two substrates bind almost equally to the N- and C-terminal domains, binding of S3P to the isolated N-terminal domain may attract the isolated C-terminal domain in the presence of PEP or glyphosate. The functional reconstitution of NTDΔ2-20/CTD^{GG} was followed using the enzyme activity assay and ^{13}C-NMR of (2-^{13}C) PEP, which gives an unambiguous chemical shift change when it is converted into EPSP [21]. The two domains appeared not to reconstitute even transiently in the presence of the substrates. Contrary to the N-terminal domain, the isolated C-terminal domain alone could bind neither substrates nor inhibitor, meaning that the presence of the N-terminal domain is required for both the substrate binding and conformational changes of the C-terminal domain.

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