Biochimica et Biophysica Acta 1864 (2016) 268–279

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Visualizing the tunnel in tryptophan synthase with crystallography: Insights into a selective filter for accommodating indole and rejecting water

Eduardo Hilario [a], Bethany G. Caulkins [b], Yu-Ming M. Huang [b,1], Wanli You [b], Chia-En A. Chang [b], Leonard J. Mueller [b,*], Michael F. Dunn [a,*], Li Fan [a,*]

[a] Department of Biochemistry, University of California at Riverside, Riverside, CA 92521, USA
[b] Department of Chemistry, University of California at Riverside, Riverside, CA 92521, USA

ARTICLE INFO

Article history:
Received 30 September 2015
Received in revised form 20 November 2015
Accepted 1 December 2015
Available online 17 December 2015

Keywords:
Substrate channeling
Hydrophobic nanopore
Substrate diffusion
Hydrophobic barrier
Dewetted channel
Tryptophan synthase

ABSTRACT

Four new X-ray structures of tryptophan synthase (TS) crystallized with varying numbers of the amphipathic N-(4′-trifluoromethoxybenzoyl)-2-aminoethyl phosphate (F6) molecule are presented. These structures show one of the F6 ligands threaded into the tunnel from the β-site and reveal a distinct hydrophobic region. Over this expanse, the interactions between F6 and the tunnel are primarily nonpolar, while the F6 phosphoryl group fits into a polar pocket of the β-subunit active site. Further examination of TS structures reveals that one portion of the tunnel (T1) binds clusters of water molecules, whereas waters are not observed in the nonpolar F6 binding region of the tunnel (T2). MD simulation of another TS structure with an unobstructed tunnel also indicates the T2 region of the tunnel excludes water, consistent with a dewetted state that presents a significant barrier to the transfer of water into the closed β-site. We conclude that hydrophobic molecules can freely diffuse between the α- and β-sites via the tunnel, while water does not. We propose that exclusion of water serves to inhibit reaction of water with the α-aminoacrylate intermediate to form ammonium ion and pyruvate, a deleterious side reaction in the αβ-catalytic cycle. Finally, while most TS structures show βPhe280 partially blocking the tunnel between the α- and β-sites, new structures show an open tunnel, suggesting the flexibility of the βPhe280 side chain. Flexible docking studies and MD simulations confirm that the dynamic behavior of βPhe280 allows unhindered transfer of indole through the tunnel, therefore excluding a gating role for this residue.

1. Introduction

Knowledge of the mechanisms by which small molecules are transported has been dependent on the determination of structures for the macromolecular protein assemblages involved in modulating transport. For example, the substantial body of structural information now available for membrane proteins shows that transport mechanisms are fundamentally dependent on the presence of macromolecular structures which form pores with dimensions and polarity designed to provide selective transfer of the desired small molecule or ion [1–6]. These pores or "channels" are capable of considerable selectivity, *viz.* the potassium channel [1,2]. It is now apparent that similar transport processes take place within assemblages of enzymes in certain metabolic pathways. The functions of these transport processes are postulated to protect labile small molecule intermediates from deleterious side reactions, to enhance catalytic efficiency, and to prevent loss of hydrophobic molecules by adsorption into hydrophobic environments such as the membranes of the organelles comprising the cell.

Substrate channeling plays an important role in many different enzymatic reactions wherein a common metabolite must be transferred

Abbreviations: TS, tryptophan synthase from *S. typhimurium*; F6, N-(4′-trifluoromethoxybenzoyl)-2-aminoethyl phosphate; F9, N-(4′-trifluoromethoxybenzenesulfonyl)-2-aminoethyl phosphate; PLP, pyridoxal-5′-phosphate; IGP, 3-indole-D-glycerol 3′-phosphate; G3P, D-glyceraldehyde 3-phosphate; E(Ain), the internal aldimine (Schiff base) intermediate; E(GD_1), the L-Ser gem diamine species; E(Aex_1), the external aldimine intermediate formed between the PLP cofactor and L-Ser; E(Q_1), the L-Ser quinonoid intermediate; E(A-A), the α-aminoacrylate Schiff base intermediate; E(Q_3), the quinonoid intermediate that accumulates in the reaction between E(A-A) and indole; E(Aex_2), the L-Trp external aldimine intermediate; E(GD_2), the L-Trp gem diamine species; F6-1, F6-2, F6-3, the binding sites for F6 in the β- and α-subunits; MD, molecular dynamics; loop αL2, residues α53–α60; loop αL6, residues α179–α193; COMM domain, residues β102–β189; section T1, the tunnel extending from the α-site to βPhe280 in the β-subunit; section T2, the tunnel extending from βPhe280 to PLP in the β-site; B factor, indicator of the relative vibrational motion of atoms in crystal structures; MR, molecular replacement. The Worldwide Protein Data Bank accession numbers for all published structures are provided in the text.

* Corresponding authors.
[1] Present address: Howard Hughes Medical Institute and Department of Chemistry and Biochemistry, University of California, San Diego, USA.

http://dx.doi.org/10.1016/j.bbapap.2015.12.006
1570-9639/

from one enzyme active site to another within a multi-enzyme complex [7–9]. Channeling typically involves diffusion through a largely hydrophobic tunnel, and the phenomenon has been implicated in many multi-enzyme complexes. For example, the structurally well-described amidotransferase family of enzymes employs tunnels to channel ammonia and other substrates between the active sites of the protein complexes [9–12]. The most remarkable member of this family, the carbamoyl phosphate synthase complex, transfers NH_3, carboxyphosphate, and carbamate among three active sites through a 100 Å-long network of tunnels [9,11,12]. Gating mechanisms have been postulated to play a role in the regulation of substrate channeling in some enzymes, for example imidazole glycerol phosphate synthase. Yet, it has been demonstrated by Amaro et al. [13,14] that gating is not significant, and, interestingly, molecular dynamics studies indicate that the channel in this complex disfavors the conduct of water while optimizing ammonia transport [15].

In the pyridoxal-5′-phosphate-requiring family of enzymes, the 143 kDa tryptophan synthase complex (TS, EC 4.2.1.20) has been shown to transfer the common metabolite indole from one site to another via a 30 Å tunnel [16]. Bacterial TS [16] catalyzes the biosynthesis of L-tryptophan from substrates indole-3-glycerol phosphate (IGP) and L-serine (reactions 1–3) [17]. The enzyme consists of two α-subunits (29 kDa each) and two β-subunits (43 kDa each), arranged in a nearly linear assembly of αβ dimeric units to give an αββα structure. TS was the first enzyme system demonstrated to use substrate channeling to connect two active sites for sequential reactions [16,18,19]. The TS catalytic mechanism depicted in Scheme 1ab shows indole is produced in the α-site reaction and consumed in the β-reaction [17,20,21].

$$\text{IGP} \rightarrow \text{indole} + \text{G3P} \qquad (1, \alpha - \text{reaction})$$

$$\text{Indole} + \text{L} - \text{Ser} \rightarrow \text{H}_2\text{O} + \text{L} - \text{Trp} \qquad (2, \beta - \text{reaction})$$

$$\text{IGP} + \text{L} - \text{Ser} \rightarrow \text{L} - \text{Trp} + \text{G3P} + \text{H}_2\text{O} \qquad (3, \alpha\beta - \text{reaction})$$

Reactions 1–3

Early studies of TS showed that free indole is not found in solution during the overall αβ reaction, leading to the hypothesis that the active sites of both subunits are buried in the interior of the complex adjacent to each other [17,19,22,23]. This hypothesis provided a structural basis for the lack of any build-up of indole during the αβ reaction. In the late 1980s and 1990s, investigations of the tunnel hypothesis involving both solution and structural studies were undertaken in parallel. The 2.5 Å resolution crystal structures of the TS internal aldimine, E(Ain), and the complex of E(Ain) with indole propanol phosphate (IPP) were published in 1988 [16]. These structures indicated that rather than aligned adjacent to each other, the active sites within each αβ dimeric unit are actually separated by a distance of approximately 30 Å and are connected by a 25 Å long hydrophobic tunnel. The dimensions of the tunnel appeared sufficient to accommodate the passage of indole from the α-site to the β-site. Kinetic analyses revealed that formation of the α-aminoacrylate intermediate at the β-site increased IGP cleavage at the α-site nearly 30-fold and that indole channeling is rapid relative to the turnover of substrates in the αβ reaction. A series of kinetic studies [24–29] demonstrated that the tunnel is functional in the transfer of indole from the α-site to the β-site and that, in the αβ-reaction, the transfer occurs within αβ dimeric units wherein the α- and β-subunits have closed conformations, postulated to prevent the escape of indole [25,27,28,30]. Thus, allosteric communication between the α- and β-sites within αβ-dimeric units of the tetrameric assembly switches the subunits between open (inactive) and closed (active) conformations that synchronize the catalytic cycles of the α- and β-sites and prevent the build-up and escape of indole during the αβ-catalytic cycle [12,20,25,30–32].

Computational simulations have provided important contributions to understanding both the flexibility of the tunnel and the conformational allostery that play a large role in regulation of channeling in TS. Bahar and Jernigan [33] explored allosteric communication between subunits in both wild type and mutant forms of the enzyme and identified specific flexible residues integral to structural changes necessary for catalysis. Spyrakis et al. [34] undertook MD studies to focus on the conformational transition between the open and closed conformations of the α-subunit, the changes this conformational transition brings about in the αβ-subunit interface, and the pathway for the entry of substrate into the α-site from solution. Fatmi and Chang [35] employed MD and Brownian dynamics simulations to probe the effect of conformational changes in the protein on the channeling of indole. These simulations suggested that indole can freely pass through the tunnel with little steric clashing.

Efforts have been made to prepare TS variants with site-directed mutations designed to block the tunnel [36–39]. The results of these efforts were ambiguous in that the βA169L/βC170W mutant exhibited altered substrate reactivities and specificities as well as a perturbed three dimensional structure. This mutant may block the transfer of indole, but the crystal structure shows a ~2 Å shift in the position of the COMM domain relative to wild-type enzyme that likely destroys the allosteric interactions between the sites [38] and perturbs the subunit interface in the vicinity of the tunnel. Further experiments with Nile Red led to the proposal that it was sequestered within the nonpolar region of the tunnel between βC170 and βF280, yet no crystal structure was reported [39].

The evidence for the switching of αβ dimeric units between open and closed states during the αβ catalytic cycle [24–29] was strongly reinforced by the determination of the crystal structure of the complex of G3P with the E(A–A) complex [40]. This structure showed that both the α- and β-subunits have conformations wherein the α- and β-active sites are closed; closing of the α-subunit is associated with motions in α-loop L6 (αL6, residues α176–196) that switch the subunit from a disordered (open) to an ordered (closed) state, while closing of the β-subunit involves motion of the communication (COMM) domain (residues β102–189) and concomitant formation of a salt-bridge between βArg141 and βAsp305. Evidence that small molecules with dimensions similar to indole could be sequestered within the confines of the tunnel and the α- and β-sites when the αβ-dimeric unit is in the closed state was provided by the demonstration that the indole analogue, indoline, could be trapped within the complex by stabilizing the closed conformations of the αβ-dimeric unit via binding of high affinity ligands to the α-site of the α-aminoacrylate form [41,42]. The studies of Brzovic et al. [27,28] provided strong evidence that the switching from the open to the closed conformation occurs when L-Ser reacts with the PLP cofactor to form E(A–A), and Leja et al. [43] demonstrated that conversion of the L-Trp quinonoid, $E(Q_3)$, to the L-Trp external aldimine, $E(Aex_2)$, triggers both the conformational switch back to the open form and deactivation of the α-site. These conclusions have been further extended using ^{19}F NMR to investigate the binding of the ligand N-(4′-trifluoromethoxybenzoyl)-2-aminoethyl phosphate (F6, an inhibitive analogue of IGP) to probe conformational switching and catalysis in the structure and function of tryptophan synthase [44]. This switching between open and closed states likely brings about synchronization of the α- and β-reactions and prevents the loss of indole during the αβ catalytic cycle [12,20,25,30–32,43,44].

Although the existence of an interconnecting tunnel between the α- and β-sites has been proposed based on the X-ray structures of TS, only one structure has been published showing a ligand bound within this tunnel. In 2007 [45], Ngo et al. reported that at relatively low concentrations one F6 molecule binds to the α-site of TS (PDB ID: 2CLE). When present in high concentrations, F6 gave an internal aldimine crystal structure wherein F6 was bound both to the α-site and to the tunnel within the β-subunit (PDB ID: 2CLF). The structural studies presented herein show that depending on the concentrations of F6 present, crystals containing one, two or three molecules of F6 per αβ-dimeric unit can be formed wherein one F6 molecule is threaded into the tunnel from the β-site. Thus, F6 can bind to three well-defined sites: the α-