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Supplementary Discussion

Analysis of the structure of AB3403

Active Site Contents. We first solved two structures of the *holo*-form of AB3403. The first is in the absence of ligands. We analyzed the amino acid adenylating activity and, among the substrates tested, AB3403 optimally activates glycine (Extended Data Fig. 2). Until the product of the pathway is identified, we cannot state with certainty that glycine is the natural substrate and glycine is used as a surrogate substrate.

Because glycine was competent as a substrate, we determined a second crystal structure after pre-incubation of the protein with Mg•ATP and glycine in an effort to obtain ligands in the adenylation domain and potentially loaded onto the pantetheine. Both structures reveal electron density for the pantetheine arm of the PCP domain that reaches into the active site of the condensation domain. The second structure shows Mg^{2+}, AMP, and glycine in the adenylation domain active site. While small panels of density are shown in Fig. 2, we present here the density in stereorepresentation for clarity (Extended Data Fig. 3).

In the structure without additional ligands, the cysteamine moiety adopts two orientations with partial occupancy. In the structure derived of protein after incubation with glycine and Mg•ATP, the pantetheine adopts only a single conformation. In this orientation, the pantetheine thiol is 4 Å from the His145, the second histidine of the conserved HHxxxD motif (Fig. 2B), equivalent to the catalytic His138 of EntF[1] and His147 of SrfA-C. The model derived from protein crystals preincubated with nucleotide and glycine showed clear density for glycine, AMP, and a Mg^{2+} ion

in the adenylation domain active site. Similar to the structure of the adenylation domain of the gramicidin synthetase[2], we interpret this density to suggest the glycyl-adenylate first formed and was subsequently hydrolyzed during crystallization (Extended Data Fig. 3B). Within the condensation domain of this liganded protein, difference density consistent with a glycine molecule was present in the condensation domain ~5Å from the pantetheine thiol; however, lacking suitable contacts to stabilize this position, a glycine was not included in the final model.

Interaction of the thioesterase and PCP domains. Two structures of the functional interaction between the PCP and thioesterase domain have been determined with didomain truncated versions of EntF by both NMR and X-ray crystallography[3,4]. Comparison of AB3403 thioesterase domain to the domains of SrfA-C and EntF identifies a 28-residue insertion near the C-terminus of AB3403 that forms three α-helices instead of the one found in SrfA-C and EntF.

Superposition of the EntF PCP-thioesterase structures on the thioesterase domain of SrfA-C shows that the thioesterase domain position in SrfA-C results in a clash between the PCP and the condensation domain. In contrast, in *holo*-AB3403, the thioesterase domain adopts a new orientation that places the active site in a position that is more accommodating for the approach of the PCP domain (Fig. 3A). Interestingly, the thioesterase domain of AB3403 binds the back face of the PCP domain opposite the pantetheine cofactor. Superposition of the thioesterases from AB3403 and the EntF functional complex with the upstream PCP domain[3,4] shows that both complexes look quite similar; in the EntF PCP-thioesterase structure, the functional face of the PCP interacts with the thioesterase domain, while in AB3403 the thioesterase engages the opposite face of the PCP (Extended Data Fig. 5). A rotation of the PCP domain by 168° degrees is needed to transfer the carrier domain from the functional interaction with the condensation domain to the thioesterase domain.

Electrophoretic analysis of EntF crystals. The crystal structure of the EntF did not show any electron density for the thioesterase domain. To determine if the protein was proteolytically cleaved during crystallization, we examined the EntF protein and crystals with native and reducing gel electrophoresis (Extended Data Fig. 7A). EntF runs on a native gel as appropriate for a protein of 142 kD (lane 1). Upon incubation of EntF with the Ser-AVS inhibitor, the protein runs more quickly in a native gel, due to the constraints on the conformation imposed by the inhibitor. The doublet observed in lane 2 can be shifted to fully inhibited upon further incubation with CoA and the phosphopantetheinyl transferase Sfp (not shown). The EntF crystals migrated at the same rate as the inhibited protein (lane 3).

To confirm that the change in mobility was a result of the conformational trapping with the inhibitor and not proteolysis, we ran both untreated and inhibited protein on a reducing SDS-PAGE gel (Extended Data Fig. 7B). Untreated (lane 1) and inhibited (lane 2) EntF co-migrated, showing that the change in mobility in the native gel results from conformational constraints and that incubation with the inhibitor does not induce proteolysis.