Structural biology of KRAS mutations in lung cancer.

Structural Biology

Structures of aminoarabinose transferase ArnT suggest a molecular basis for lipid A glycosylation

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Polymyxins are antibiotics used in the last line of defense to combat multidrug-resistant infections by Gram-negative bacteria. Polymyxin resistance arises through charge modification of the bacterial outer membrane with the attachment of the cationic sugar 4-amino-4-deoxy-L-arabinose to lipid A, a reaction catalyzed by the integral membrane lipid- to lipid glycosyltransferase 4-amino-4-deoxy-L-arabinose transferase (ArnT). Here, we report crystal structures of ArnT from Cupriavidus metallidurans, alone and in complex with the lipid carrier undecaprenyl phosphate, at 2.8 and 3.2 Ångstrom resolution, respectively. The structures show cavities for both lipidic substrates, which converge at the active site. A structural rearrangement occurs on undecaprenyl phosphate binding, which stabilizes the active site and likely allows lipid A binding. Functional mutagenesis experiments based on these structures suggest a mechanism for ArnT family enzymes.

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**Supplementary materials**

www.science.org/content/351/6273/608/suppl/DC1

Materials and Methods

Figs. 51 to 57

References (34–36)

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Polymyxins are last-resort antibiotics used to combat multidrug-resistant infections by Gram-negative bacteria (1, 2). They are thought to act by permeabilizing the membranes of Gram-negative bacteria, after binding to the lipopolysaccharide (LPS) of the outer membrane (1, 2). This association with the outer membrane is primarily achieved through electrostatic interactions between amino groups of polymyxins and negatively charged moieties of the backbone glucosamine and 3-deoxy-o-manno-oct-2-ulosonic acid (Kdo) sugars of lipid A, an amphotrophic saccharolipid that anchors LPS to the outer leaflet of the outer membrane (3). Resistance to polymyxins develops through active modifications of lipid A, which cap the glucosamine sugar phosphates and thus reduce negative membrane charge (4). Lipid A modification is also relevant for evasion of naturally occurring cationic antimicrobial peptides by Gram-negative bacteria (5, 6). In Escherichia coli and Salmonella enterica, the most effective modification for reduction of negative membrane charge is the attachment of the cationic sugar 4-amino-4-deoxy-L-arabinose (L-Ara4N) to lipid A phosphate groups at the 1 and 4′ positions (7). L-Ara4N is provided by the lipid carrier undecaprenyl phosphate (UndP). The reaction is catalyzed on the periplasmic side of the inner membrane by ArnT (PmrK), an integral membrane lipid-to-lipid glycosyltransferase and the last enzyme in the aminoarabinose biosynthetic pathway of Gram-negative bacteria (4, 7, 8) (Fig. 1A). The lipid A 1-phosphate group is also modified by EptA (PmrC), which adds phosphoethanolamine (pEtN), competing with ArnT for the 1-phosphate site (9, 10).

To gain insight into the structure and mechanism of ArnT function, we screened 12 structural rearrangement occurs on undecaprenyl phosphate binding, which stabilizes the active site and likely allows lipid A binding. Functional mutagenesis experiments based on these structures suggest a mechanism for ArnT family enzymes.
Fig. 1. Structure and function of ArnT from *Cupriavidus metallidurans* CH34. (A) Schematic representation of the reaction catalyzed by ArnT. The sugar L-Ara4N is transferred from the carrier UndP to lipid A. 1 and 4′ phosphate positions on lipid A are marked. (B) Analysis of 32P-labeled lipid A species by TLC showing rescue of lipid A modification with L-Ara4N when ArnT<sub>Cm</sub> is expressed in the ΔarnTΔdeptA double-knockout *E. coli* strain (dm). The background *E. coli* strain (WD101; wt, wild type) has a pmrA constitutive phenotype (pmrAC), enabling it to synthesize L-Ara4N- and pEtN-modified lipid A and to exhibit resistance to polymyxins (8). (C) Crystal structure of ArnT<sub>Cm</sub>. Two orthogonal views, perpendicular to the plane of the membrane, are presented in ribbon representation with rainbow coloring starting from the N terminus (blue) to the C terminus (purple). The approximate dimensions of the monomer are 55, 79, and 42 Å (width, height, and depth). Membrane boundaries shown were calculated using the PPM server (21). Dashed lines represent missing segments in the structure. (D) Arrangement of TM helices in ArnT<sub>Cm</sub> shown as a slice of the TM domain at the level indicated by the arrow in (C). (E) Schematic representation of the connectivity and structural elements of ArnT<sub>Cm</sub>. P, periplasm; M, membrane (inner); C, cytoplasm; TMD: TM domain; PL4 (shown in red).

Fig. 2. Significant structural features of ArnT<sub>Cm</sub>. (A) Three views of ArnT<sub>Cm</sub> in spacefill representation showing the location of notable cavities in the structure. The middle view has the same orientation as the view in (B). The other two are obtained by 45° rotation in opposite directions around a vertical axis. Cavities are color coded as in (B), where their identity is labeled. (B) Ribbon representation of ArnT<sub>Cm</sub> showing the volumes of notable cavities in the structure. Volumes were calculated using the Voss Volume Voxelator server (22), using probes with 15 and 1.75 Å radii, corresponding to the outer and inner probe, respectively. The approximate membrane boundaries are shown as black lines. (C) Close-up of the metal coordination site in ArnT<sub>Cm</sub> showing Zn<sup>2+</sup> as a purple sphere and the coordinating residues colored by heteroatom. The dashed red line box in (B) shows the overall location of the coordination site. (D) The effect of mutations in Zn<sup>2+</sup> coordinating residues on ArnT<sub>Se</sub> expressed in a BL21(DE3)ΔarnT *E. coli* strain for rescue of polymyxin B (PMXB) resistance (13). Corresponding residue numbers in ArnT<sub>Cm</sub> are shown in parentheses here and in all subsequent figures. OD<sub>600</sub>, optical density at 600 nm. Data presented are means ± SD. N is shown for each data column.
prokaryotic putative ArnTs from diverse species to find a candidate for crystallization (1). ArnT from *Cupriavidus metallidurans* CH33 (ArnT<sub>CH33</sub>) emerged as the most promising based on expression levels (fig. S1A) and behavior in size-exclusion chromatography in detergent (fig. S1B). This protein yielded crystals in lipidic cubic phase (LCP) (12) (fig. S1, C and D) that were suitable for structure determination.

We characterized ArnT<sub>CH33</sub> to determine whether it was a true ArnT capable of transferring l-Ara4N to lipid A. Thin-layer chromatography (TLC) of 33P-labeled lipid A isolated from E. coli showed that heterologous expression of ArnT<sub>CH33</sub> in an *E. coli* strain lacking endogenous *arnT* and *epTA* (*Δ*arnTΔepTΔ) resulted in lipid A modification by l-Ara4N (fig. 1B). The identity of the transferred sugar was confirmed by mass spectrometry (fig. S2A). Unlike ArnT from *Salmonella enterica* serovar Typhimurium (ArnT<sub>Se</sub>), ArnT<sub>CH33</sub> failed to rescue resistance to polymyxin in Δ*arnT* (13, 14) (fig. S2B) and in Δ*arnTΔepTΔ* (fig. S2C) *E. coli* strains. Although it is known that ArnT<sub>Se</sub> adds l-Ara4N to both the 1 and 4′ phosphates of lipid A (7), ArnT<sub>CH33</sub> appears only to yield a single lipid A species modified at the 1-phosphate position (fig. S2D), which suggests that modification at the 1-position does not confer protection to polymyxin in *E. coli*. Consistent with this, removal or modification of the 4′-phosphate confers polymyxin resistance in other species (15, 16). Therefore, functional hypotheses derived from the structures of ArnT<sub>CH33</sub> were tested on ArnT<sub>Se</sub> by using a polymyxin growth assay previously established in *E. coli* (13, 14) and with a direct assay on ArnT<sub>CH33</sub> for some mutants. The overall sequence identity between ArnT<sub>Se</sub> and ArnT<sub>CH33</sub> is 23%, but the degree of conservation in and around the key regions for activity is substantially higher, which suggests that their structure and function are likely conserved (fig. S3).

The structure of ArnT<sub>CH33</sub> was determined to 2.8 ˚A resolution by the single-wavelength anomalous diffraction method using SeMet-substituted protein (fig. S4 and Table 1). ArnT<sub>CH33</sub> is a monomer, consisting of a transmembrane (TM) domain and a soluble periplasmic domain (PD) positioned above it (fig. 1C). The TM domain, demarcated by a clear hydrophobic belt (fig. S5A), shows 13 TM helices, as recently predicted for ArnT from *Burkholderia cenocepacia* (17), in an intricate arrangement (fig. 1, D and E). The structure has three juxtamembrane (JM) helices (JM1 to JM3). JM1 and JM2 are both part of the first periplasmic loop and are perpendicular to each other, creating a distinctive cross-shaped structure (fig. 1C). JM3 leads into a flexible periplasmic loop between TM7 and TM8 [periplasmic loop 4 (PL4), partially disordered in the structure], previously shown to be functionally important (13, 17). TM13 leads into the PD, which has an α/β/α arrangement (fig. 1E).

ArnT is a member of the GT-C family of glycosyltransferases (18), and it has a similar fold to a bacterial oligosaccharyltransferase (OST) from *Campylobacter lari* (PglB) and to an archaeal OST from *Archaeoglobus fulgidus* (AgkB) (19, 20) (fig. S6A). The topology differs among the three (fig. S6B), but an inner core of AgkB's TM domain aligns well with that of ArnT<sub>CH33</sub> (fig. S8C) and a part of the PDs of ArnT<sub>CH33</sub> and PglB is similar (fig. S6, D and E). These similarities in fold may underscore an evolutionary relationship, but the functions of the three enzymes are markedly different. Only ArnT has a lipid as glycosyl acceptor (lipid A). Because both substrates are lipidic, ArnT must bring both from the membrane to the active site for catalysis.

The structure of ArnT<sub>CH33</sub> shows three major cavities (fig. 2, A and B), which differ in their

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<td>Unit cell: a, b, c (Å)</td>
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<td>56.65, 80.32, 150.16</td>
<td>58.91, 81.21, 151.63</td>
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<td>0.994 (0.468)</td>
<td>1.000 (0.839)</td>
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| Refinement |  |  |  |
| Relections used in refinement | 20,239 (1,962) | 12,427 (1,208) | N/A |
| Relections used for R<sub>free</sub> | 1047 (96) | 668 (59) | N/A |
| R<sub>work</sub> | 0.21 (0.41) | 0.22 (0.32) | N/A |
| R<sub>free</sub> | 0.26 (0.42) | 0.26 (0.38) | N/A |
| Number of nonhydrogen atoms | 4,562 | 4,804 | N/A |
| Macromolecules | 4,097 | 4,171 | N/A |
| Ligands | 431 | 627 | N/A |
| Protein residues | 537 | 541 | N/A |
| RMS (bonds) | 0.003 | 0.002 | N/A |
| RMS (angles) | 0.56 | 0.49 | N/A |
| Ramachandran favored (%) | 97 | 96 | N/A |
| Ramachandran allowed (%) | 2.8 | 3.7 | N/A |
| Ramachandran outliers (%) | 0 | 0.2 | N/A |
| Rotamer outliers (%) | 0.5 | 0.5 | N/A |
| Clashscore | 4.25 | 4.96 | N/A |
| Average B-factor | 75.8 | 50.6 | N/A |
| Macromolecules | 74.4 | 49.5 | N/A |
| Ligands | 89.6 | 57.4 | N/A |
| Solvent | 65.1 | 44.2 | N/A |
| Number of TLS groups | 2 | 2 | N/A |
electrostatic nature. The largest (>3000 Å³ within the membrane), cavity 1, is amphiphatic with a lower, primarily hydrophobic portion located below the level of the membrane and an upper hydrophilic one (fig. S5B). We hypothesize that cavity 1 is where lipid A binds to ArnT. Note that the hydrophobic portion of cavity 1 is directly accessible from the outer leaflet of the inner membrane, and it has a volume compatible with the acyl chains and the glucosamine sugar backbone of lipid A. This suggests a simple mechanism for lipophilic substrate recruitment, although entrance of lipid A. This suggests a simple mechanism for lipophilic substrate recruitment, although entrance

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with conserved positively charged residues (R58 and K203, respectively), play a critical role in orienting the lipid A phosphate for a nucleophilic attack on the l-Ara4N donor (Fig. 4C). Indeed, mutations of D55 and D158 in ArnT, and of any of the equivalent residues involved in these two ion pairs in ArnT<sub>cm</sub>, led to loss of activity (Fig. 4D and fig. S14). This work provides a structural framework for understanding the function of the ArnT family of enzymes, which may inform the design of compounds targeted at reversing resistance to polymyxin-class antibiotics.

REFERENCES AND NOTES


ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIALS

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