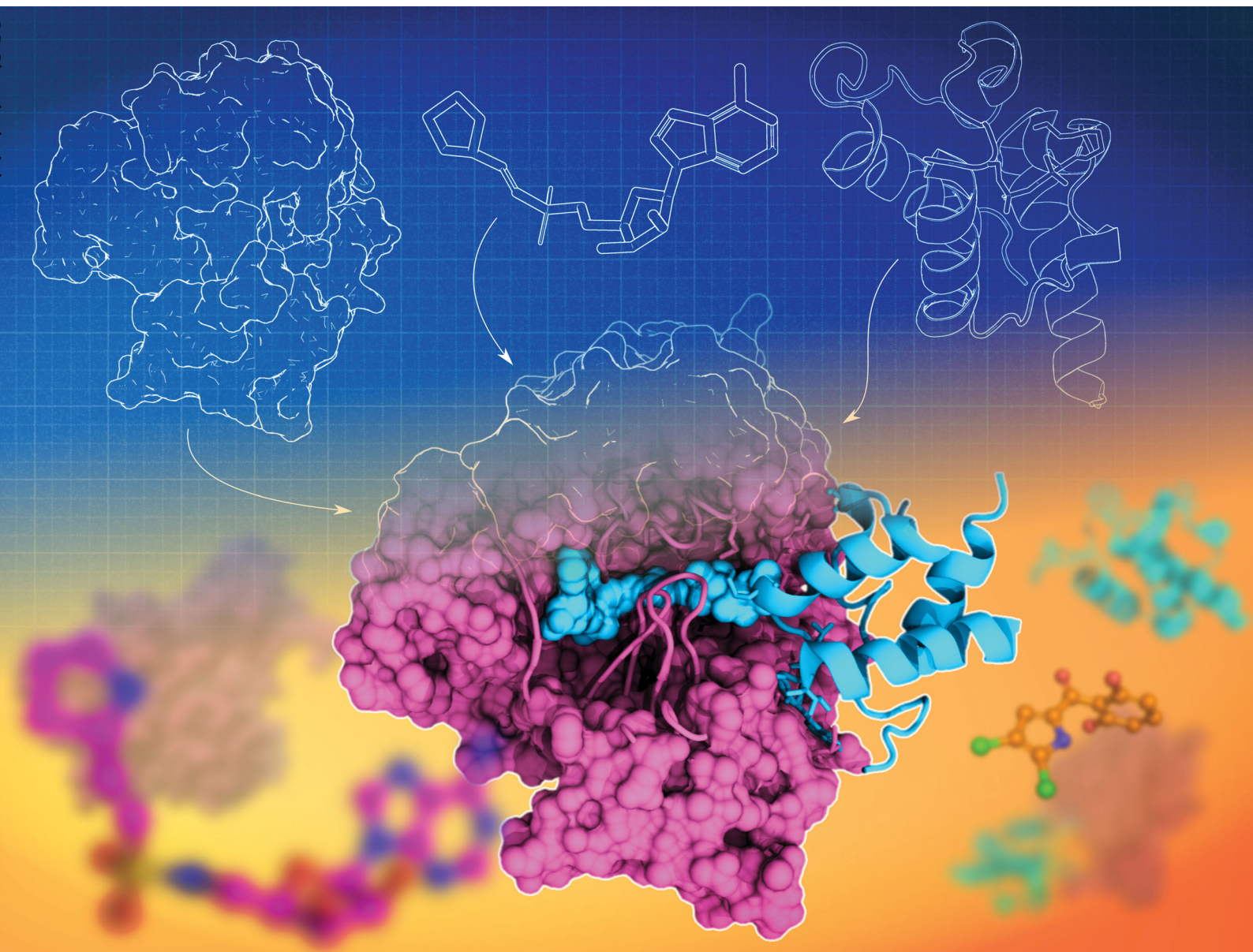


RSC Chemical Biology

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ISSN 2633-0679

COMMUNICATION

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COMMUNICATION

[View Article Online](#)
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Accepted 26th February 2020

DOI: 10.1039/c9cb00015a

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Dynamic visualization of type II peptidyl carrier protein recognition in pyoluteorin biosynthesis†

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Using a covalent chemical probe and X-ray crystallography coupled to nuclear magnetic resonance data, we elucidated the dynamic molecular basis of protein recognition between the carrier protein and adenylation domain in pyoluteorin biosynthesis. These findings reveal a unique binding mode, which contrasts previously solved carrier protein and partner protein interfaces.

The type II non-ribosomal peptide synthetase (NRPS) system consists of stand-alone enzymes that commonly participate in hybrid pathways along with fatty acid synthase (FAS) and polyketide synthase (PKS) enzymes.¹ The stand-alone architecture of type II NRPS proteins makes them promising candidates for metabolic engineering, as they commonly serve to install unique chemical functionality into growing metabolites. These hybrid pathways afford complex natural products that include antibiotic, antitumor, and antifungal agents.² Examples of functionalized natural products include those that utilize dehydrogenated prolines as a pharmacophore, including prodigiosin, pyoluteorin, and chlorizidine A, all of which require type II NRPS proteins to functionalize and incorporate a pyrrole into the natural product (Fig. 1).^{3–5}

All NRPS systems include an adenylation (A) and peptidyl carrier protein (PCP) domain to activate and load substrates. The A domain activates a specific amino acid substrate and subsequently installs the substrate onto the thiol of the 4'-phosphopantetheine (PPant) arm of the PCP (Fig. 2A).⁶ The PCP is a 10 kDa protein that consists of a 4-helix bundle.⁷ The PPant modification is attached onto an invariant serine *via* a phosphopantetheinyl transferase to activate the PCP to the *holo*-form. The PCP can transport acyl or aminoacyl cargo to a variety of enzymatic domains for functionalization and incorporation of the substrate into the nascent natural product.

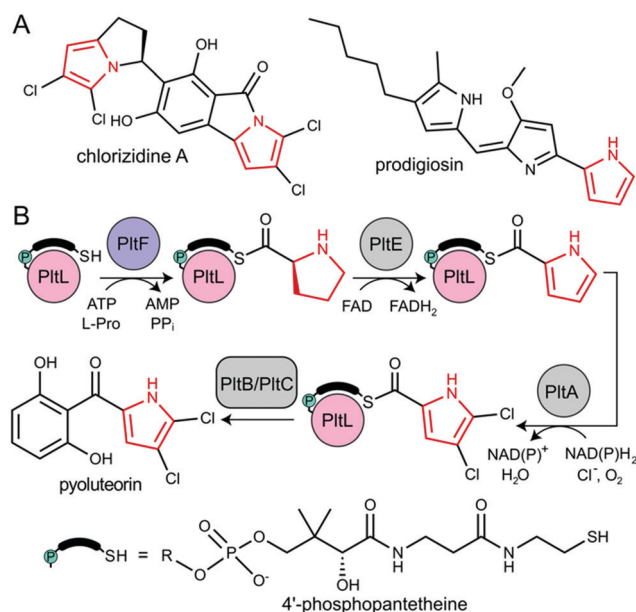


Fig. 1 (A) Natural product examples that incorporate pyrrole (red) *via* type II NRPS proteins. (B) Pyrrole functionalization and installation in pyoluteorin *via* type II NRPS proteins. PltB and PltC is a type I PKS.

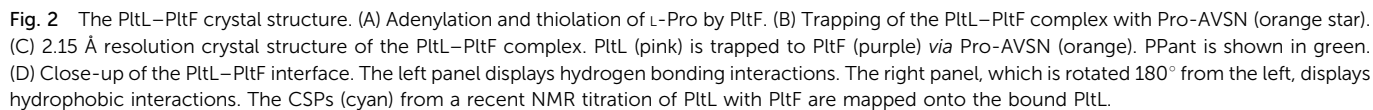
During pyoluteorin biosynthesis in *Pseudomonas fluorescens*, the type II NRPS PCP, *holo*-PltL, is loaded with L-Pro by the A domain, PltF (Fig. 1). While protecting its substrate in a hydrophobic cleft,⁸ prolyl-PltL transports the L-Pro for dehydrogenation and dichlorination before being off-loaded onto a type I PKS for the incorporation of the dichloropyrrolyl substrate into pyoluteorin.⁹

PltL has been shown to exhibit specificity towards PltF and no interactivity towards homologous A domains.^{10,11} This suggested the requirement of a specific protein–protein interaction motif for A domain activity. Studies have attempted to control the partner protein specificity of PltL; solution-phase nuclear magnetic resonance (NMR) titration experiments revealed a region of PltL, loop 1 (residues 19–41), that was postulated to

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c9cb00015a



The PltL–PltF crystal structure contains PltL trapped with PltF in the thiolation state *via* addition to Pro-AVS_N (Fig. 2C). Successful trapping of the complex is shown by electron density of the PPant extended into the active site of PltF, with its terminal thiol covalently linked to the sulfonamide β-carbon of Pro-AVS_N (Fig. S3, ESI[†]). The putative K486 responsible for adenylation is 25 Å away from the active site, consistent with the domain alternation hypothesis proposed by Gulick and coworkers (Fig. S4A, ESI[†]).²⁰ The N-terminal domain (NTD) contains an AMP binding pocket and L-Pro binding pocket that

To gain insight into the highest relative CSPs found in PltL Trp37 and Gly38, the solution NMR structure of *holo*-PltL was

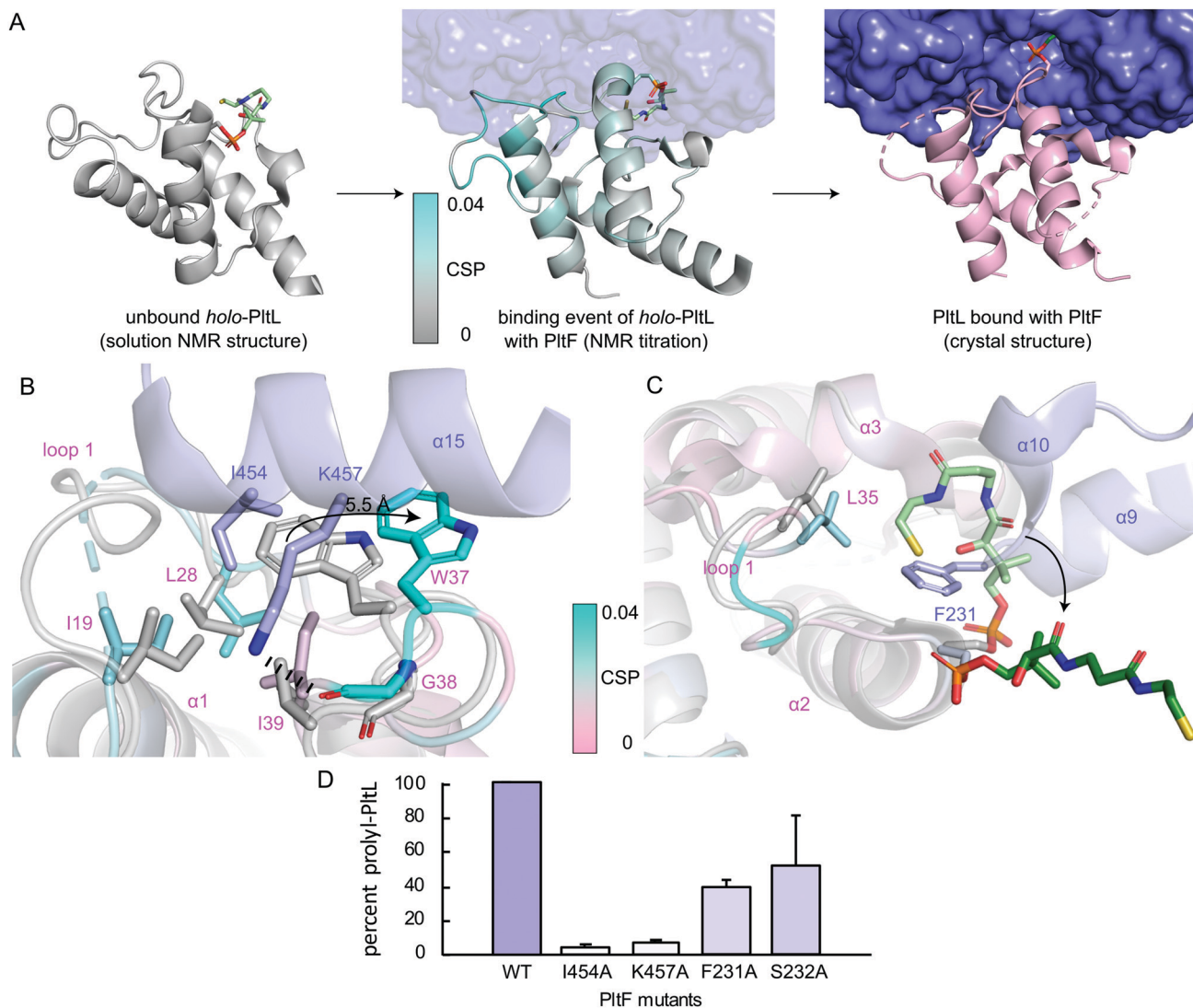


Fig. 3 Visualization of the PltL–PltF binding event. (A) Linking of the solution NMR structure of *holo*-PltL (gray, PDB ID 2N5H), the CSPs (cyan) from the NMR titration, and the PltL–PltF (pink, purple) complex. (B) Superposition of the bound (pink, cyan) and unbound (gray) PltL with PltF Ile454 (purple) and (C) Phe231 (purple). Pro-AVS_N was omitted for clarity. (D) PltF mutant aminoacylation assay with PltL.

aligned and superposed to PltL in the bound state with PltF (Fig. 3B). Comparison of the different states reveal the lack of the loop 1 hydrophobic pocket in the unbound PltL that contacts PltF Ile454 (Fig. 3A and Fig. S8, ESI†). The superposition shows the pocket formation *via* a 5.5 Å displacement of the indole ring of PltL Trp37 by PltF Ile454. Adjacent to Trp37 is Gly38, which forms a H-bond *via* its backbone carbonyl with PltF Lys457 that may stabilize the displacement. The relevance of the hydrophobic pocket formation and H-bonding was demonstrated by mutagenesis of Ile454Ala and Lys457Ala, respectively, where individual mutations both stunted aminoacylation activity (Fig. 3D). Furthermore, previous molecular dynamic simulations revealed the relative flexibility of PltL loop 1, which supports loop 1 reorganization upon binding.¹⁰ These data provide evidence that specific H-bonding and hydrophobic interactions allowed by the conformational flexibility of PltL loop 1 is responsible for its recognition towards PltF.

Superposition of bound and unbound PltL also reveals PltF Phe231 residing between helices 2 and 3 of PltL, which occludes the hydrophobic cleft that protects the substrate (Fig. 3C).⁸ This suggests that the hydrophobic cleft is not only involved in substrate protection but also molecular recognition. Mutagenesis of PltF Phe231Ala and Ser232Ala results in decreased PltL aminoacylation, which supports its relevance in PltL–PltF recognition (Fig. 3D). Recent structural analysis of the type II PCP of pentabromopseudilin biosynthesis, Bmp1, in complex with the oxidase, Bmp2, yields similar conclusions.²²

Next, we compared the PltL–PltF interface against other PCP-A domain interfaces from the crystal structures of LgrA, EntF, EntE–EntB, and PA1221.^{14,15,23,24} The most outstanding difference is the location of the interface interactions. The PCP interfaces of LgrA, EntF, PA1221, and EntE–EntB involve the loop 1 and, to a larger extent, helix 2 of the PCP (Fig. 4). In contrast, the PltL–PltF structure reveals that only the first



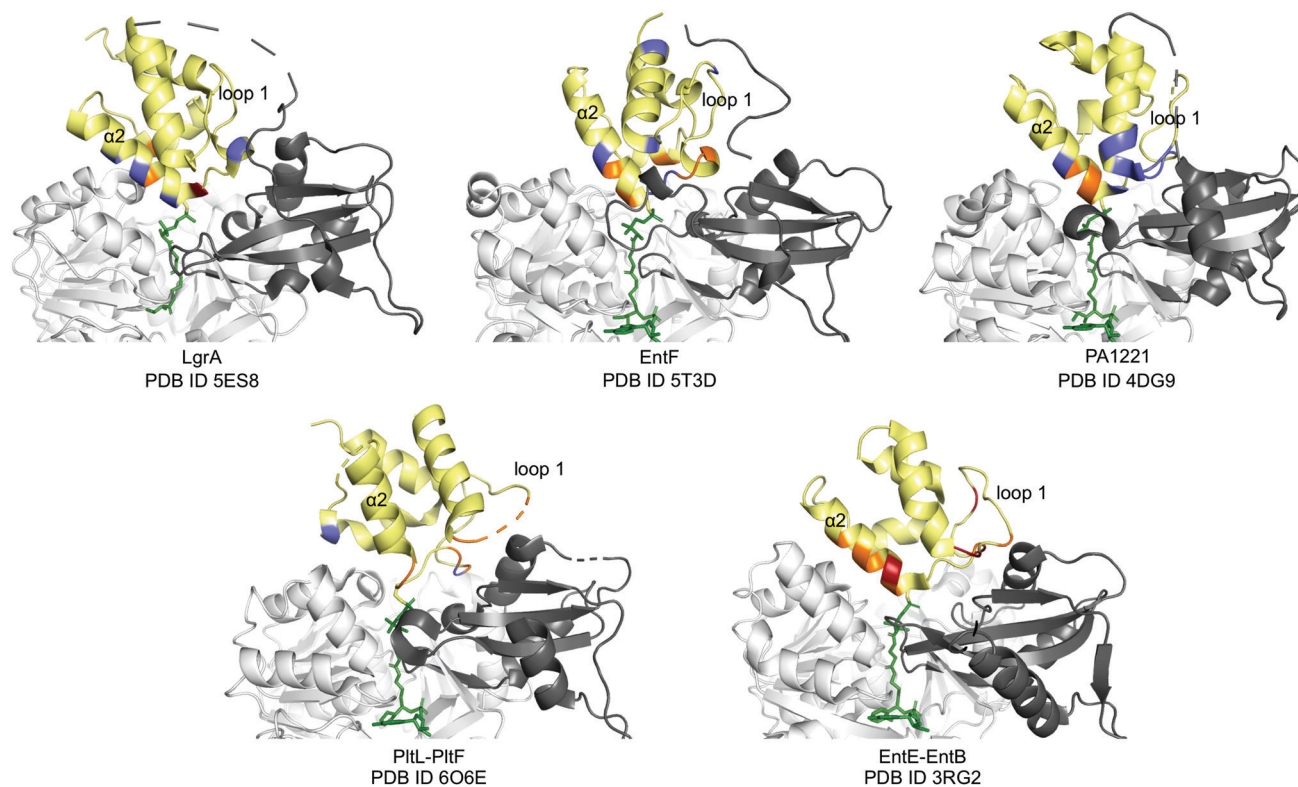


Fig. 4 PCP-A domain interface comparison. Yellow, PCP; dark grey, A domain C-terminal domain; white, A domain N-terminal domain; blue, H-bonding; red, salt bridge; orange, hydrophobic.

residue of PltL helix 2, Met43, is partially buried at the interface. The loop 1 region of PltL is instead the main contributor to the interface with PltF. This interface independent of helix 2 is surprising considering the established prevalence of helix 2 at the interface of CP-dependent pathways, such as the *E. coli* type II FAS, where the CP-enzyme interface primarily relies on helix 2 for binding (Fig. S11, ESI†).^{25–27}

While PCP-A domain interfaces consist of hydrophobic interactions, each pathway differs in number of H-bonds and salt bridges for partner protein recognition (Fig. 4). The PltL-PltF structure reveals two H-bonds at the interface, whereas EntE-EntB employs multiple salt bridges. In contrast, the LgrA, EntF, and PA1221 structures contain four or more H-bonds. The type of interaction in addition to the location of each interaction presents a challenge towards the combinatorial biosynthesis of CP-dependent pathways.

Conclusions

The current structural investigation enhances our understanding of the molecular basis of PltL-PltF interactions. While our previous NMR titrations identified potential interface residues on PltL, mutagenesis studies did not afford noncognate activity. Here, combining NMR titrations, chemical trapping, X-ray crystallography, and mutagenesis assays allows us to more precisely identify the interface to understand specificity and predict functional mutations.

This structure of the trapped PltL-PltF complex, complemented by prior NMR studies, has revealed new insights into a distinct mechanism of recognition used by A domains and PCPs. Resolving structural features of the protein-protein interface revealed the differences in the type, location, and dynamics of interfacial interactions that govern CP and partner protein recognition. These differences may serve a fundamental reason behind the limited success of prior combinatorial biosynthetic efforts in NRPSs. With our discoveries on the type II PCP and A domain from pyoluteorin biosynthesis, a layer of complexity has been revealed that will inform the future combinatorial biosynthetic efforts and engineering of CP-dependent pathways.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

We thank Jefferey Mindrebo and Dr Ashay Patel for providing feedback on the manuscript, Dr Yongxuan Su for MS analyses, Dr Anthony Mrse and Dr Xuimei Huang for NMR assistance, and George Meigs, Dr James Holton, and Jane Tanamachi for X-ray data collection assistance. JCC was funded by the NIH Molecular Biophysics Training Grant T32GM008326. Funding was provided by NIH R01GM095970 to MDB and T32CA009523 to MJJ. TDD is a San Diego IRACDA postdoctoral fellow funded



by NIH K12GM068524 and NIH K99GM129454. LMP was supported by the UC San Diego start-up funds. Beamline 8.3.1 of the Advanced Light Source, a DOE Office of Science User Facility under Contract No. DE-AC02-05CH11231, is supported in part by the ALS-ENABLE program funded by the NIH, NIGMS, grant P30GM124169-01.

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