

A Nuclear Magnetic Resonance Method for Probing Molecular Influences of Substrate Loading in Nonribosomal Peptide Synthetase Carrier Proteins

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S Supporting Information

ABSTRACT: Carrier proteins (CPs) play a central role in nonribosomal peptide synthetases (NRPSs) as they shuttle covalently attached substrates between active sites. Understanding how the covalent attachment of a substrate (loading) influences the molecular properties of CPs is key to determining the mechanism of NRPS synthesis. However, structural studies have been impaired by substrate hydrolysis. Here, we used nuclear magnetic resonance spectroscopy to monitor substrate loading of a CP and to overcome hydrolysis. Our results reveal the spectroscopic signature of substrate loading and provide evidence of molecular communication between an NRPS carrier protein and its covalently attached substrate.

Nonribosomal peptide synthetases (NRPSs) are bacterial and fungal enzymatic systems that produce complex secondary metabolites from simple starting materials such as amino or aryl acids, many of which have found use as antibiotics and anticancer agents.¹ NRPSs possess a remarkable assembly line architecture, in which substrates are covalently attached to contiguous modules and condensed to form the final product. Each module is comprised of a core set of conserved domains, and a long-standing goal of the field is swapping domains or modules with differing substrate specificities to generate novel pharmaceuticals.² Unfortunately, the molecular mechanisms of NRPS synthesis, and particularly domain communication, remain largely unknown, impeding progress in reprogramming NRPS assembly lines. Among NRPS domains, carrier proteins (CPs) play a central role as they tether the substrates to the assembly line and, hence, visit many catalytic domains during NRPS synthesis. CPs are first converted from an inactive apo to an active holo form via covalent attachment of a 4'-phosphopantetheine arm (4'-PP) onto a conserved serine. Next, adenylation (A) domains catalyze both substrate adenylation and thioester bond formation between the activated substrate and the 4'-PP of holo carrier proteins to generate a substrate-loaded CP. Finally, condensation domains catalyze the formation of the peptide bond between two substrates loaded on neighboring CPs to extend the peptide. NMR and crystallographic studies indicate that NRPS modules are not rigid, but their domains are subject to inter- and intradomain dynamics.^{3–7} Moreover, attachment of the 4'-PP altered the structure and dynamics of an isolated CP.⁵ Studies of structurally related fatty acid synthases (FAS)

and polyketide synthases (PKS) have implicated substrate loading in influencing large scale domain rearrangements.^{8,9} However, the lability of NRPS substrate thioester bonds has precluded similar studies of loaded NRPS carrier proteins. Understanding how CPs efficiently orchestrate sequential, transient interactions with partner domains and elucidating the role of tethered substrates in modulating these interactions are vitally important to understanding NRPS assembly line synthesis and, ultimately, rationally redesigning these systems. Here, we exploited the noninvasive nature of nuclear magnetic resonance (NMR) to overcome hydrolysis and study a loaded aryl carrier protein (ArCP) from yersiniabactin synthetase. Our results reveal that NRPS ArCPs interact either directly or indirectly with the substrates attached at the end of the 20 Å long 4'-PP.

In the yersiniabactin synthetase system, the free-standing A domain YbtE initiates synthesis by loading salicylate (Sal) onto the holo aryl carrier protein of the multidomain protein HMWP2.¹⁰ The excised ArCP was overexpressed in *Escherichia coli* BL21(DE3)ΔEntD cells (a gift of C. Chalut and C. Guilhot) and purified to yield pure, homogeneous apo ArCP. Apo ArCP was phosphopantetheinylated *in vitro* and purified to produce holo ArCP. To study the loaded form of ArCP, two major obstacles had to be addressed: hydrolysis and transthioylation from thiol-containing reducing agents,¹¹ which were necessary to prevent disulfide bond formation in holo ArCP. Transthioylation was avoided by using tris(2-carboxyethyl)-phosphine as a reducing agent. Although hydrolysis of thioesters is slower than transthioylation, it was rapid enough to preclude quantitative analysis of loaded ArCP.

Indeed, when ArCP was loaded with Sal [confirmed by matrix-assisted laser desorption ionization mass spectrometry (MALDI MS)] and freshly purified, NMR spectra featured signals of both holo ArCP and a previously unobserved form. Unfortunately, the intensities of the new signals decreased over time, raising the possibility that they were an artifact of sample preparation and not reporters of substrate loading. Purified, loaded ArCP was therefore unsuitable for NMR studies.

To allow for prolonged detection of loaded ArCP, we exploited the isotope editing ability of NMR and generated the loaded form *in situ* to bypass the need for purifying loaded

Received: November 19, 2014

Revised: January 21, 2015

Published: January 26, 2015



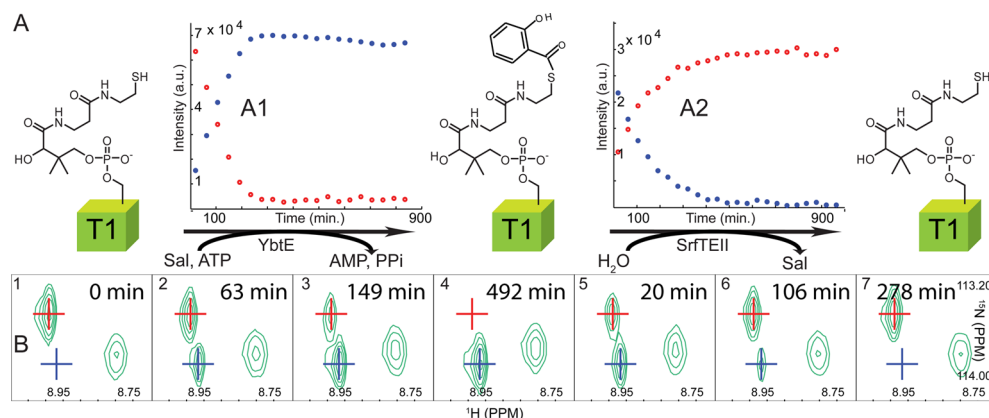


Figure 1. (A) Reactions catalyzed by YbtE and SrfTEII and their time courses (A1 and A2, respectively), here monitored by the signal of Ser52, the phosphopantetheinylation site. (B) Signal of Ser52 during the loading reaction (B1–B4) and following the addition of SrfTEII after purification to remove YbtE and substrates (B5–B7). Blue indicates loaded ArCP and red holo ArCP.

ArCP. Sal and ATP (2 mM each) were added in large excess to ¹⁵N-labeled holo ArCP (300 μM). No interaction between holo ArCP and the reagents was detected in HN-HSQC spectra (Figure S1 of the Supporting Information). We then added catalytic quantities of YbtE (100 nM) and collected a series of HN-HSQC spectra. At such concentrations, the binding of YbtE to ArCP does not induce shifts in NMR signals. During the reaction, intensities of signals of loaded ArCP increase while intensities of signals of holo ArCP decay (as shown for the phosphopantetheinylation site, Ser52, in Figure 1 A1, B1–B4). MALDI MS confirmed the conversion into ArCP loaded with Sal. To demonstrate that all signals observed by NMR report on substrate loading and not some undesired side effects, we induced substrate hydrolysis by enzymatic catalysis. We added the promiscuous thioesterase SrfTEII¹² to purified loaded ArCP, and the NMR signals of loaded ArCP disappeared as those of holo ArCP reappeared (Figure 1 A2, B5–B7). The HN-HSQC spectrum collected following the completion of SrfTEII-catalyzed hydrolysis (confirmed by MALDI MS) overlays perfectly with that of holo ArCP, demonstrating that the new peaks observed upon incubation of holo ArCP with ATP, Sal, and YbtE are indeed the spectroscopic signature of substrate loading (Figure S5 of the Supporting Information). Comparison between the kinetics of uncatalyzed hydrolysis (Figure S4 of the Supporting Information) and substrate loading suggested that hydrolysis may be compensated by continuous reloading of Sal on ArCP. Indeed, spectra of loaded ArCP recorded after 5 days displayed no significant regeneration of holo ArCP in the presence of YbtE, Sal, and ATP, whereas in the absence of YbtE, Sal, and ATP, ~57% of purified loaded ArCP hydrolyzes over that time (Figures S4 and S6 of the Supporting Information). Thus, adding catalytic amounts of the cognate A domain overcomes hydrolysis of loaded monomers for a duration amenable for NMR studies. This method complements other strategies used to circumvent hydrolysis in loaded carrier proteins (Page S6 of the Supporting Information).

The changes we observe in NMR spectra of loaded ArCP may reflect many molecular events. NMR signals in HN-HSQC spectra report on the local chemical environment of the amide group of each residue. Differences between spectra of holo and loaded ArCP, so-called chemical shift perturbations (CSPs), can indicate a variety of events, such as direct contact with the salicylate moiety, repositioning of the 4'-PP, structural

alterations, and modulation of dynamics. Figure 2A shows an overlay of holo and loaded ArCP spectra. Simple inspection

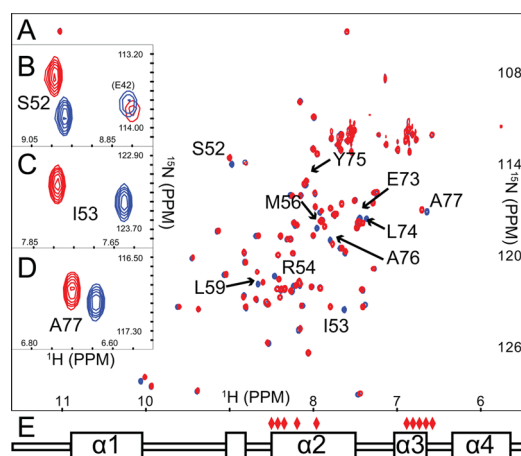


Figure 2. (A) Overlay of holo (red) and salicylate-loaded (blue) ArCP HN-HSQC spectra. Signals with CSP greater than one standard deviation above the median are indicated. Insets show close-ups of the signals of (B) Ser52, (C) Ile53, and (D) Ala77. (E) Mapping the residues with significant CSPs on the secondary structure of ArCP shows two distinct clusters of residues spanning both helices 2 and 3.

reveals that no massive structural rearrangement occurs upon loading, because most signals are the same in both spectra. However, a significant number of peaks shift markedly, indicating that loading Sal impacts the related amide groups. To gain insights into the origins of the CSPs of holo/loaded ArCP, we assigned the ¹H and ¹⁵N resonances of apo [¹⁵N,¹³C]ArCP using conventional experiments^{13,14} and transposed the assignment of apo ArCP to holo [¹⁵N]ArCP and to loaded [¹⁵N]ArCP with NOESY-HN-HSQC spectra. Panels B–D of Figure 2 highlight four peaks that show varying degrees of CSP. Mapping the largest CSPs (Figure S7 of the Supporting Information) onto the secondary structure of ArCP as determined via chemical shift indexing^{15,16} reveals distinct clusters of residues affected by loading (Figure 2E). CSPs of residues around the phosphopantetheinylation site (Ser52, N-terminus of helix 2) likely reflect a change in conformation of the 4'-PP. CSPs of residues in helix 3 together with residues in the middle of helix 2 may indicate a direct substrate interaction reminiscent of that observed for PKS and FAS acyl carrier

proteins.^{17,18} In both FAS and PKS, changing tethered substrates repositions helices 2 and 3,^{19,20} and it was suggested that these structural variations may modulate binding events.⁹ Various NMR experiments can test if salicylate alters the structure and/or dynamics of ArCP and modulates protein–protein interactions. Our newly designed conditions will permit such measurements while ArCP remains loaded.

In summary, we used NMR to directly monitor NRPS substrate loading, thereby providing the first atomic level description of this process. We found that NRPS substrates directly or indirectly interact with their cognate carrier proteins. Regardless of whether substrates bind to CPs or induce conformational fluctuations, substrate loading is expected to modulate the binding affinity of CPs for partner catalytic domains. Decades of biochemical studies have demonstrated interplay between carrier proteins and their substrates during catalytic steps involving various domains,^{21,22} and our method provides a framework for investigating the molecular determinants of this interplay.

■ ASSOCIATED CONTENT

■ Supporting Information

Supplementary figures, tables, and experimental procedures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Funding

Work supported by National Institutes of Health (NIH) Grant RO1 GM104257. A.C.G. supported by American Heart Association Mid-Atlantic Affiliate Predoctoral Fellowship 14PRE20460253.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Drs. Christian Chalut and Christophe Guilhot for providing the *E. coli* BL21(DE3)ΔEntD strain. We thank Dr. Craig Townsend for discussions on the manuscript and NRPS substrate hydrolysis and Scott Nichols for preparing SrfTEII. We thank the Mass Spectrometry and Proteomics Facility at the Johns Hopkins School of Medicine for use of their instrument.

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