Mechanism-based crosslinking as a gauge for functional interaction of modular synthases†

Andrew S. Worthington, Douglas F. Porter and Michael D. Burkart*

Received 9th December 2009, Accepted 9th February 2010
First published as an Advance Article on the web 17th February 2010
DOI: 10.1039/b925966j

Protein-protein interactions between domains within fatty acid and polyketide synthases are critical to catalysis, but their contributions remain incompletely characterized. A practical, quantitative system for establishing functional interactions between modifying enzymes and the acyl carrier protein that tethers the nascent polymer would offer a valuable tool for understanding and engineering these enzyme systems. Mechanism-based crosslinking of modular domains offers a potential diagnostic to highlight selective interactions between modular pairs. Here kinetic activity analysis and isothermal titration calorimetry are shown to correlate the efficiency of a ketosynthase-carrier protein crosslinking method to the binding affinity and transacylase activity that occurs in ketosynthase chain elongation.

Fatty acid synthases (FASs) and polyketide synthases (PKSs) are evolutionarily related enzyme systems responsible for the biosynthesis of a diverse array of compounds.[1,2] A variety of enzymatic organizations have evolved to synthesize these unique chemical structures.[3] Both fatty acid and polyketide metabolic pathways are organized into type I and type II synthases, where type I synthases are composed of large, multimodular proteins.[1,2,4] Type I systems house all of the biosynthetic machinery on a few polypeptides and contain multiple enzymatic activities that function as a molecular assembly line. Here each portion of the polyketide product is biosynthesized by a dedicated module – groups of domains that function in a coordinated fashion to build and modify the growing product.

Type II systems, on the other hand, are composed of discrete proteins that can function independently. To overcome the kinetic disadvantage of diffusion control, the type II enzymes are thought to associate in dynamic complexes, the compositions of which can change during the course of a particular biosynthetic pathway.[5] This suggests that the type II complexes function *in vivo* in a parallel fashion to the type I systems, yet rely on strong protein-protein interactions in place of covalent linkages.

Due to the structural and functional similarities between polyketide and fatty acid synthases, there have been numerous studies investigating combinatorial biosynthesis.[6] In these systems, enzymes from different synthases are exchanged in order to rationally biosynthesize novel compounds. Despite some moderate success, most of these systems have suffered from low yield.[7,8] While there are a number of potential explanations for these low yields, including disruption of overall modular architecture and incompatibility of domains' substrate specificities, protein-protein interactions are always going to be an important consideration when designing these combinatorial biosynthetic systems. It is thought that unnatural protein-protein interactions in these systems are often of insufficient strength to generate active and catalytically efficient synthases.[9] A method for assessing the interaction of proteins from different pathways would be valuable for the optimization of these combinatorial systems.

Both FAS and PKS systems rely on the acyl carrier protein (ACP), either as a domain (type I) or independent protein (type II) to shuttle building blocks and the growing product chain during biosynthesis. We recently developed a system for labeling the ACP with reactive moieties that would specifically interact with the active site cysteine residue of ketosynthase proteins of type II FAS, type II PKS, and modular type I PKS.[10–12] In this system a reactive pantetheine moiety is covalently attached to the conserved serine residue of an unmodified *apo*-carrier protein through the action of a phosphopantetheinyl transferase enzyme (Sfp) and a reactive CoA species. The resulting reactive ACP species interacts with the active site of a ketosynthase protein, and an irreversible crosslink is generated. The active site cysteine was demonstrated to be the target of these reactive ACP species through MALDI MS/MS analyses of the crosslinked products.[10,11] This approach was shown to be dependent on the natural partnerships between ACPs and keto-acylsynthase (KAS) enzymes.[10] With the chemical synthesis of a wider range of crosslinking reagents, we also demonstrated a correlation between substrate specificity of KASs and their reactivity towards analogous crosslinking reagents.[11] It was concluded that chemical crosslinking of ACP and KAS domains could serve as a valuable gauge of protein-protein interaction, however further quantitative evaluation was needed to justify this claim. In this communication, we aim to demonstrate the utility of this chemical crosslinking by directly comparing the extent of crosslinking to two separate quantification methods: the kinetics of KAS transacylation from ACP using a radioisotopic transfer assay and thermodynamics heat of binding of KAS-ACP using isothermal calorimetry (ITC).

We first analyzed the crosslinking efficiency of the *E. coli* FAS AcpP, *S. roseofulvus* PKS FrnN, and *S. coelicolor* PKS OtcACP with the second ACP-dependent elongation enzyme in *E. coli* FAS, FabF (KASII). These type II carrier proteins were chosen because they had been shown to crosslink FabB (KASI) in our original study.[10] Additionally, solution and crystal structures have been published for each of these ACPs.[13–15] From evaluation of their structures it was determined that the two PKS ACPs are more closely related in structure to each other than to the FAS ACP; in

Department of Chemistry and Biochemistry, University of California-San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0358, USA. E-mail: mburkart@ucsd.edu; Fax: +1 858-822-2182

† Electronic supplementary information (ESI) available: Full experimental procedures. See DOI: 10.1039/b925966j

Table 1 Comparison of *E. coli* AcpP, *S. roseofulvus* FrnN, and *S. coelicolor* OtcACP crosslinking efficiency (using both analogs **1** and **2**) and biochemical kinetics with KASII. Initial velocities (V_0) were determined using the radioactive transacylase assay. K_D values were determined from ITC experiments

ACP	Crosslinking (**1**)	Crosslinking (**2**)	V_0 (nM ^{14}C/ mM ACP/min)	K_D (μM)
AcpP	97%	61%	210 ± 80	4.1 ± 1.8
FrnN	12%	13%	3.7 ± 0.4	19 ± 3
OtcACP	1%	< 1%	0.60 ± 0.01	31 ± 13

PKS ACPs, helix I and the loop between helix I and II are longer than in FAS. Additionally, these ACPs possess a long, flexible C-terminal loop that is absent from the FAS ACPs.[14,15]

FabF was chosen over FabB, as FabB is inhibited by *holo*-ACP (50% at 0.17 μM); FabB has been shown to form oligomeric structures larger than the functional dimers at higher concentrations (high concentrations are necessary to yield accurate binding affinity data); and the active dimeric form of FabB is less stable than that of FabF.[16,17] In addition, *E. coli* FabF has a structure more similar to the type II KAS/CLF from PKS pathways.[18] We also chose to analyze two different crosslinking reagents, a chloroacrylate-terminal pantetheine moiety (**1**) and an α-bromoamide-terminal pantetheine (**2**), since both had shown effective crosslinking in our previous studies. The results of this crosslinking study are depicted in Fig. 1B, and the quantitative results are tabulated in Table 1. We achieved an average of 80% crosslinking with the AcpP-FabF pair, 13% with the FrnN-FabF pair, and 1% with the OtcACP-FabF pair, as quantified by densitometry (Fig. 1B and Supplementary Fig. 1).

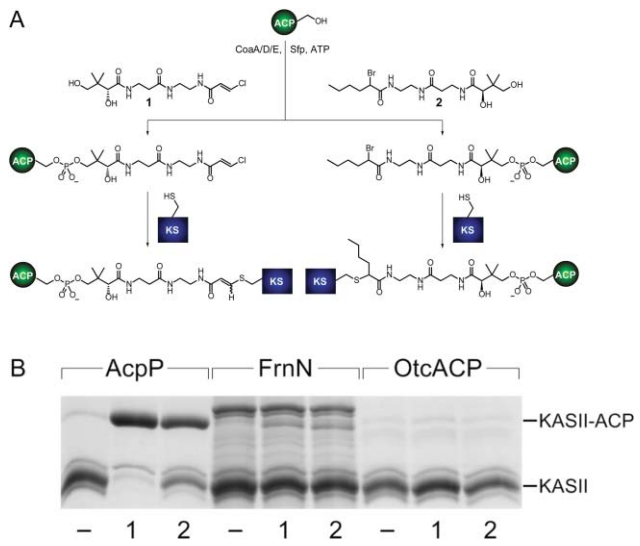

Fig. 1 Mechanism-based crosslinking of KASII (FabF) and FAS and PKS ACPs using pantetheine analogs **1** and **2**. (A) ACP is post-translationally modified through the successive activities of the ATP-dependent CoaA, CoaD, CoaE and Sfp enzymes (with analog **1** or **2**). The active site cysteine of KASII then undergoes nucleophilic attack on the reactive ACP species to covalently crosslink the two proteins. (B) Application of mechanism-based crosslinking on *E. coli* KASII (FabF) and *E. coli* AcpP, *S. roseofulvus* FrnN, and *S. coelicolor* OtcACP using analogs **1** and **2** to produce KASII-ACP; lanes marked with – are negative controls without pantetheine analog.

A radioactive transacylase assay has been previously reported to characterize the kinetics of substrate loading from ACP onto KAS enzymes; this is the first step in the two-step mechanism of ketosynthase activity.[19] Using similar experimental conditions, we measured the transfer of radioactive octanoate from various loaded *holo*-ACPs to the active site cysteine of FabF. We used the acyl-CoA ligase enzyme from *Pseudomonas putida*, which has been shown to have a high degree of activity with octanoic acid.[20] In our assay, radioactive octanoic acid was incubated with CoA and acyl-CoA ligase for 2 h, after which time *apo*-ACP and the phosphopantetheinyl transferase Sfp were added to generate radiolabeled acyl-ACP.[21] The transacylase assay was initiated with the addition of FabF. Time points were taken until no further increase in signal could be detected (within 30 min). Samples were boiled to terminate the reaction and run on SDS-PAGE. Radioactivity from the ketosynthase proteins was determined by phosphorimaging and quantified by densitometry. The maximum amount of radioactivity in a given experiment corresponds to saturation of the active site cysteine with radiolabeled octanoate. This value was correlated to the starting concentration of KAS. With this conversion, the increase in C8 transfer to KAS can be plotted over time, and the initial linear portion of the plot was used to determine the initial velocity of the transacylase reaction (Fig. 2). As expected, KASII shows a significantly faster (60- or 350-fold) rate of transacylation when C8-AcpP is the partner protein, as compared to C8-FrnN and C8-OtcACP (Table 1). While it is has been presumed that the transacylation step is not rate-limiting in the KAS mechanism, this data clearly demonstrates that the identity of the acyl-donating ACP plays a major role in the kinetics of the overall KAS elongation reaction (as in the native situation, only a single ACP would act as donor).

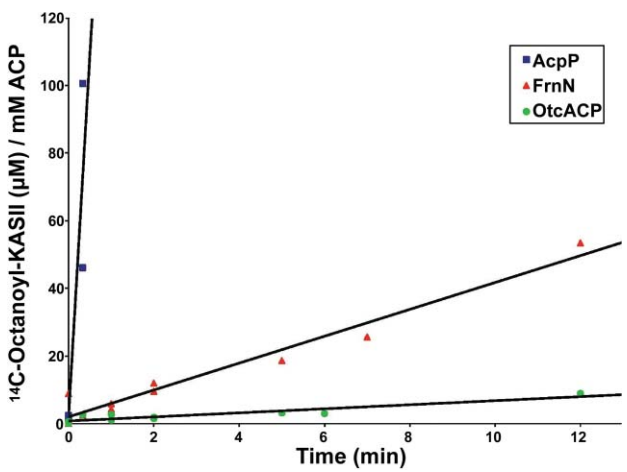

Fig. 2 Initial velocity experiments to determine transacylase activity of KASII with the three ^{14}C-acyl-ACP substrates: data points for the experiment with ^{14}C-acyl-AcpP represented as squares, ^{14}C-acyl-FrnN as triangles, and ^{14}C-acyl-OtcACP as circles.

Isothermal titration calorimetry (ITC) is the most quantitative means available for measuring the thermodynamic properties of a protein-protein interaction, enabling a direct measure of the enthalpy change when two species interact, allowing the facile determination of heat of association, stoichiometry, and binding affinity from a single experiment.[22–24] In these experiments, FabF was incubated at 37 °C within a MicroCal VP-ITC calorimeter.

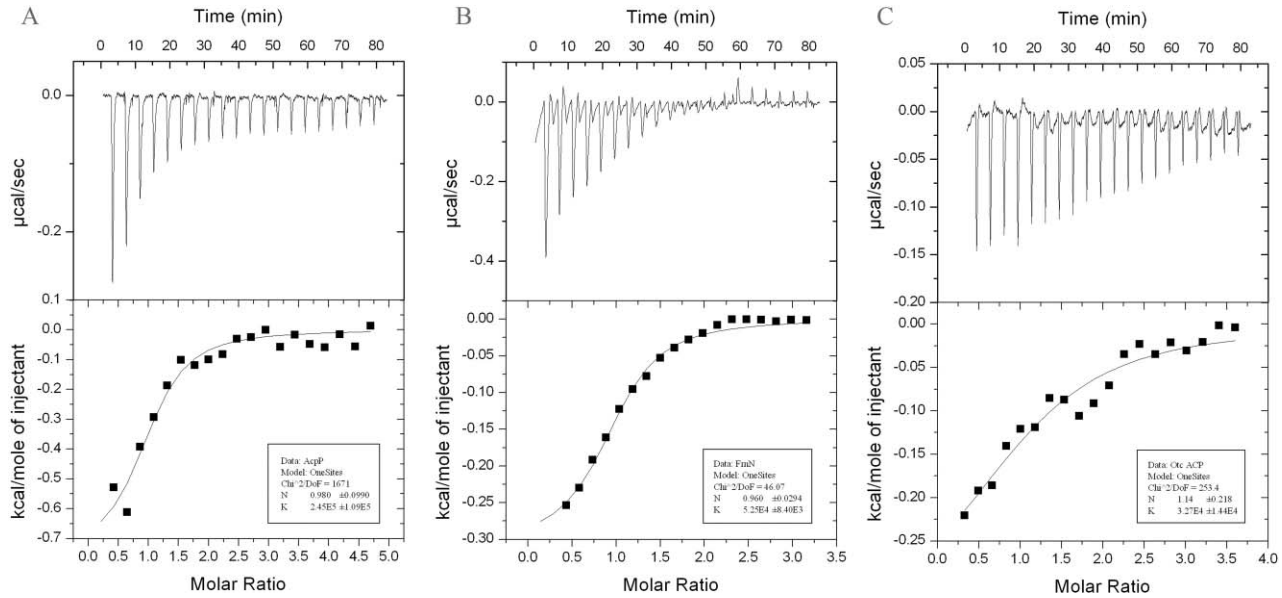

Fig. 3 Isothermal titration calorimetry (ITC) analysis of KASII and the three *holo*-ACP proteins: (A) *E. coli* AcpP, (B) *S. roseofulvus* FrnN, and (C) *S. rimosus* OtcACP. Data were analyzed using Origin 7.0 to determine stoichiometry (n) and binding affinity ($1/K$) of the interaction between KASII and ACP.

holo-ACPs were produced in *E. coli* through the coexpression of carrier protein with the phosphopantetheinyl transferase Sfp. Purified *holo*-ACP was titrated into the solution of FabF, and the resulting heat changes were monitored. These values were plotted and the thermodynamics of the FabF-ACP interaction were determined (Fig. 3). From the ITC data of FabF with *holo*-ACPs, we obtained a 1 : 1 stoichiometry in the binding of ACP to each KAS monomer. The K_D of the interaction between the two species increased from AcpP to FrnN to OtcACP, confirming the tighter association and reactivity between FabF and its endogenous AcpP (Table 1). This ITC procedure was repeated with structurally related carrier proteins from nonribosomal peptide synthetase (NRPS) systems: *E. coli* EntB (enterobactin synthetase) and *V. cholerae* VibB (vibriobactin synthetase). These experiments demonstrated no significant binding interaction occurs between FabF and NRPS carrier proteins (data not shown).

From a comparison of the crosslinking efficiency to the transacylase activity and binding affinity of KASII, there are clear trends within the three ACP proteins. AcpP has a much higher degree of crosslinking (80%), is a > 60-fold more favorable substrate in the transacylase reaction of KASII, and binds KASII most strongly. At the other end of the spectrum, OtcACP is the worst in all categories (although no significant difference in the dissociation constants with FabF between FrnN and OtcACP can be established with these data). In generating an unnatural biosynthetic system that utilized *E. coli* KASII, an ACP similar to AcpP would be the best partner protein while an ACP more similar to FrnN would be preferred over one resembling OtcACP.

The most important proteins in both FAS and PKS biosynthetic pathways are the ketosynthase and ACP. By comparing the interaction between unnatural protein partners, we can begin to assess which residues are the most important for producing favorable protein-protein interactions. Optimal activity in type II systems is dependent upon strong protein-protein interactions. While these interactions are weaker in type I systems, they are equally as important. Small conformational changes within type I domains of FAS account for much of the partner protein recognition by ACP in these systems, and these conformational changes are dependent upon the surface residues at the site of interaction.[25] Coupled with mutant libraries, this quick assay would provide valuable information on residues necessary for maintaining functional protein-protein interactions.

Conclusions

Our mechanism-based crosslinking technique is a useful, quantitative measure of the strength of functional binding interactions between ACPs and KASs. We have seen natural interactions favored in all of our studies that have compared endogenous to unnatural ACP partners in the crosslinking reaction.[10–12] In complex organisms expressing a number of ACP proteins, this technique can be useful for guiding the determination of a given ACP's binding partners.[26] More importantly, this crosslinking method would be useful for screening unnatural biosynthetic partners in the development of combinatorial biosynthetic pathways.

Acknowledgements

This work was funded by the University of California, San Diego, Department of Chemistry and Biochemistry, NIH R01GM075797, NSF CAREER 0347681, and ACS RSG-01-011-01-CDD. We thank G. Ghosh, E.A. Komives, and J. Yang (UCSD) for the use of facilities in conducting these experiments. In addition, we would especially like to thank Mike Guttman (UCSD) for helpful assistance.

Notes and references

1 H. Jenke-Kodama, A. Sandmann, R. Müller and E. Dittmann, *Mol. Biol. Evol.*, 2005, **22**, 2027.

2 C. P. Ridley, H. Y. Lee and C. Khosla, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 4595.
3 B. Shen, *Curr. Opin. Chem. Biol.*, 2003, **7**, 285.
4 K. Watanabe, A. P. Praseuth and C. C. Wang, *Curr. Opin. Chem. Biol.*, 2007, **11**, 279.
5 R. Veyron-Churlet, S. Bigot, O. Guerrini, S. Verdoux, W. Malaga, M. Daffé and D. Zerbib, *J. Mol. Biol.*, 2005, **353**, 847.
6 K. J. Weissman and P. F. Leadlay, *Nat. Rev. Microbiol.*, 2005, **3**, 925.
7 R. McDaniel, A. Thamchaipenet, C. Gustafsson, H. Fu, M. Betlach and G. Ashley, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 1846.
8 J. Staunton and B. Wilkinson, *Curr. Opin. Chem. Biol.*, 2001, **5**, 159.
9 A. Y. Chen, N. A. Schnarr, C. Y. Kim, D. E. Cane and C. Khosla, *J. Am. Chem. Soc.*, 2006, **128**, 3067.
10 A. S. Worthington, H. Rivera, J. W. Torpey, M. D. Alexander and M. D. Burkart, *ACS Chem. Biol.*, 2006, **1**, 687.
11 A. S. Worthington, G. H. Hur, J. L. Meier, Q. Cheng, B. S. Moore and M. D. Burkart, *ChemBioChem*, 2008, **9**, 2096.
12 S. Kapur, A. Worthington, Y. Tang, D. E. Cane, M. D. Burkart and C. Khosla, *Bioorg. Med. Chem. Lett.*, 2008, **18**, 3034.
13 X. Qiu and C. A. Janson, *Acta Crystallogr., Sect. D: Biol. Crystallogr.*, 2004, **60**, 1545.
14 Q. Li, C. Khosla, J. D. Puglisi and C. W. Liu, *Biochemistry*, 2003, **42**, 4648.
15 S. C. Findlow, C. Winsor, T. J. Simpson, J. Crosby and M. P. Crump, *Biochemistry*, 2003, **42**, 8423.
16 K. A. McGuire, J. N. McGuire and P. von Wettstein-Knowles, *Biochem. Soc. Trans.*, 2000, **28**, 607.
17 P. Edwards, J. S. Nelsen, J. G. Metz and K. Dehesh, *FEBS Lett.*, 1997, **402**, 62–66.
18 K. K. Burson and C. Khosla, *Tetrahedron*, 2000, **56**, 9401.
19 P. von Wettstein-Knowles, J. Olsen, K. Arnvig Mcguire and S. Larsen, *Biochem. Soc. Trans.*, 2000, **28**, 601.
20 M. Fernández-Valverde, A. Reglero, H. Martinez-Blanco and J. M. Luengo, *Appl. Environ. Microbiol.*, 1993, **59**, 1149.
21 R. H. Lambalot, A. M. Gehring, R. S. Flugel, P. Zuber, M. LaCella, M. A. Marahiel, R. Reid, C. Khosla and C. T. Walsh, *Chem. Biol.*, 1996, **3**, 923.
22 A. Velázquez Campoy and E. Freire, *Biophys. Chem.*, 2005, **115**, 115.
23 B. A. Shoemaker and A. R. Panchenko, *PLoS Comput. Biol.*, 2007, **3**, 337.
24 M. Fivash, E. M. Towler and R. J. Fisher, *Curr. Opin. Biotechnol.*, 1998, **9**, 97.
25 M. Leibundgut, S. Jenni, C. Frick and N. Ban, *Science*, 2007, **316**, 288.
26 H. D. Mootz, R. Finking and M. A. Marahiel, *J. Biol. Chem.*, 2001, **276**, 37289.