Molecular BioSystems

PAPER

A photocrosslinking assay for reporting protein interactions in polyketide and fatty acid synthases†

Zhixia Ye,[a] Morgan Bair,[ab] Hemant Desai[a] and Gavin J. Williams*[a]

Understanding protein–protein interactions that occur between ACP and KS domains of polyketide synthases and fatty acid synthases is critical to improving the scope and efficiency of combinatorial biosynthesis efforts aimed at producing non-natural polyketides. Here, we report a facile strategy for rapidly reporting such ACP–KS interactions based on the incorporation of an amino acid with photocrosslinking functionality. Crucially, this photocrosslinking strategy can be applied to any polyketide or fatty acid synthase regardless of substrate specificity, and can be adapted to a high-throughput format for directed evolution studies.

Introduction

Three broad classes of biosynthetic enzymes assemble a huge variety of polyketide natural products[1] and fatty acids by the combined action of three mandatory active sites – a ketosynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP).[2] In the type I or so-called 'modular' polyketide synthases (PKSs), these domains are organized into modules, each of which is responsible for a complete elongation step in the construction of the polyketide.[3] Fungal PKSs represent another class of important type I enzymes, these are non-modular and iterative, accordingly the length and extent of modification of the polyketide product is 'cryptically' encoded in the PKS.[4,5] Conversely, type II synthases such as the bacterial fatty acid synthases,[6] employ discrete, monofunctional proteins that function more or less independently. Type III PKSs or the 'chalcone synthase' family use acyl-CoA substrates directly without the use of an ACP, and produce relatively simple aromatics with modest structural diversity.

The use of protein-bound substrates in the type I and II synthases requires that the ACP interact with every other domain within a module (Fig. 1, paths **a**/**b**).[7] Moreover, in type I PKSs, a fully processed intermediate also needs to be transferred to a downstream module, either on the same or different polypeptide (Fig. 1, path **c**), each requiring inter-modular protein–protein interactions. A growing body of evidence illustrates the critical importance of such inter-polypeptide protein interactions for the type I PKSs.[8–11] For example, the loss of interpolypeptide interactions between

ACP and KS domains is likely responsible for poor productivity of non-natural bimodular PKSs.[12,13] In general, such hybrid synthases are often inactive or display greatly diminished activity, even with the established importance of linker domains.[10,14] Cumulatively, these studies highlight the need to probe and assay protein–protein interactions in PKSs.

Chemical crosslinking with dibromopropanone or other bifunctional electrophiles, has been used to provide structural information related to polyketide and fatty acid synthase architecture, but can suffer from a lack of spatial control and crosslinking specificity.[15–18] Moreover, these chemical methods fail to provide crosslinking information when interacting partner proteins are acylated with substrate, this is important given the identity of the acyl chain substrate can influence these interactions.[19] Burkart and co-workers developed
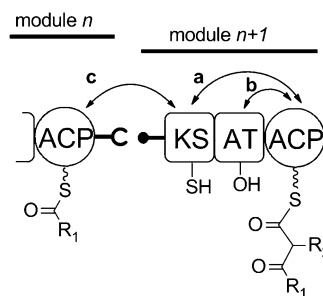
Fig. 1 ACP-based interactions that take place during each round of chain extension on modular, type I PKSs. Paths **a,b** represent interactions within the same module. Path **c** represents interactions between different modules, in this case separate polypeptides (*i.e.* interpolypeptide protein–protein interactions). Also shown at the ACP–KS interface are docking domains required for efficient inter-polypeptide recognition (ball and socket). Only those domains minimally required for chain elongation are shown. Similar interactions occur in Type II PKSs. Wavy line, phosphopantetheine; ACP, acyl carrier protein; KS, ketosynthase domain; AT, acyltransferase domain.

[a] *Department of Chemistry, North Carolina State University, Raleigh, NC, 27695 USA. E-mail: gavin_williams@ncsu.edu*
[b] *Present Address: Berry College, Department of Chemistry, PO Box 495016, Mount Berry, GA 30159 USA*

† Electronic supplementary information (ESI) available: Cloning of KS/ACP domains into pET vectors, MS and CD analysis of WT and mutant proteins. See DOI: 10.1039/c1mb05270e

a system for labelling ACPs *via* the phosphopantetheinyl transferase Sfp with reactive CoA species that specifically crosslink with the active site cysteine of KSs.[20,21] Consequently, irreversibly crosslinked ACP–KS can be quantified by electrophoresis. This strategy accurately reports the known orthogonality of interactions between various ACP and KS domains,[20,21] and serves as a reliable gauge of protein–protein interactions, *i.e.* crosslinking provides an accurate estimate of the strength of protein interaction.[22] However, this strategy requires the combined action of four enzymes for installation of reactive pantetheine analogues, and each analogue requires structural tailoring to match the expected KS substrate specificity. The tedious preparation of each specialized reagent and combined use of four enzymes may hinder attempts to apply this approach to proteins with unusual substrate specificity. Further, it may be difficult to provide sufficiently modified ACPs for screening ACP–KS interactions in a high-throughput manner.

To complement the chemical crosslinking and Sfp-mediated strategies, we propose to incorporate a photocrosslinking amino acid, *via* unnatural amino acid (UAA) mutagenesis, at the interface to trap interpolypeptide interactions (Fig. 2).[23,24] Given cognate ACPs and KS approach very closely (*e.g.* < 5 Å in DEBS[19,25]) we reasoned that a suitably located photocrosslinking amino acid would provide crosslinking specificities as high as mechanism based approaches. Further, we reasoned this approach would result in efficient crosslinking, given benzophenone is reversibly excited,[26] even though its lifetime is presumably short compared to the lifetime of the ACP–KS complex. While photocrosslinking amino acids have been used to probe protein interactions in many biological systems,[27–31] to the best of our knowledge, this approach has yet to be applied to enzymes of natural product biosynthesis, including PKSs. Here, we describe the development and validation of this assay.

Results & discussion

Unnatural amino acid mutagenesis

Several ACP and KS domains were selected as model proteins to test our photocrosslinking strategy. The carrier protein AcpP from the *E. coli* type II FAS[6] was cloned from genomic DNA by PCR into pET28a. The *E. coli* type II FAS KS

proteins FabB, and FabH were cloned into pET28a, while FabF from the same system was sub-cloned into pETDuet1. The carrier protein ACP2 from the prototypical type I DEBS system was sub-cloned into pET28a. Each ACP and KS gene is appended with an N-terminal His₆-tag provided by the host vector, facilitating rapid purification. These wild-type (WT) proteins expressed well in *E. coli* BL21(DE3), and Ni-NTA purification afforded each protein in yields of 2-5 mg per L of culture (data not shown). Protein identity was confirmed by SDS-PAGE and ESI-MS analysis of each purified protein (ESI†).

Next, we tested our ability to carry out unnatural amino acid mutagenesis of PKSs. We initially chose *p*-benzoyl-L-phenylalanine (pBpa, Fig. 3A) as the photocrosslinking agent given its commercial availability and success in previous protein interaction studies.[27,28] We speculated that a conserved ACP serine residue where phosphopantetheine is usually installed would constitute a good site for pBpa installation for our initial proof-of-principle studies. Site-directed mutagenesis was used to introduce the TAG amber suppression codon at positions Ser-54 and Ser-36 of ACP2 and AcpP, respectively, providing the vectors pET28a-ACP2S54TAG and pET28a-AcpPS36TAG. Each vector was co-transformed into *E. coli* BL21 (DE3) along with pEVOL-Bpf containing an engineered *M. jannaschii* aminoacyl-tRNA transferase optimized toward pBpa and the corresponding suppressor tRNA.[32] Protein expression was conducted in media containing pBpa and the inducers IPTG and arabinose. Following protein purification of ACP2-S54-pBpa and AcpP-S36-pBpa *via* Ni-NTA chromatography, the identity and purity of the modified carrier proteins was established by SDS-PAGE and MS analysis (Fig. 3 and ESI†). As an example, a yield of ∼5 mg AcpP-S36-pBpa per L of culture was obtained. The Coomassie blue stained SDS-PAGE of the purified protein (Fig. 3A) shows a single band at ∼15 kDa (ACPs are known to run anomalously by SDS-PAGE[33]). The purified protein was analyzed by ESI-MS and the deconvoluted mass spectrum revealed a molecular weight of 10968.40 Da, closely matching the expected value of 10966.93 for AcpP containing pBpa at position 36 (Fig. 3C). We could not find AcpP containing a natural amino acid at position 36, as judged by ESI-MS (ESI†). In addition,
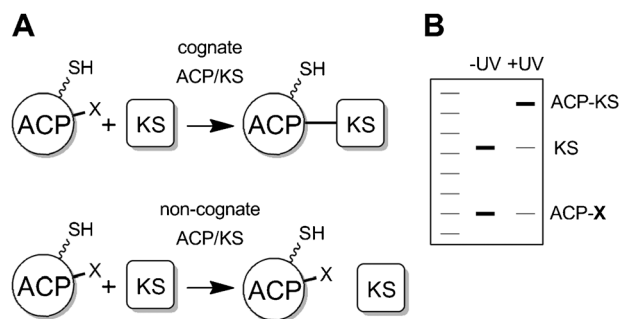
Fig. 2 Photocrosslinking assay for reporting interpolypeptide protein–protein interactions between ACP and KS. (A) Cognate *vs.* non-cognate ACP/KS pair. (B) Gel-shift assay for quantification of photocrosslinked products. X, photocrosslinking amino acid side chain. ACP and KS can be provided within intact modules, module fragments or as discrete proteins.
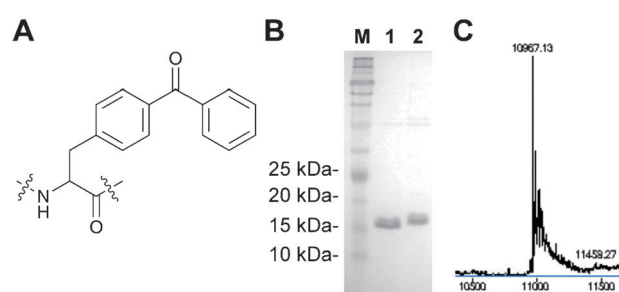
Fig. 3 Production of carrier proteins modified with unnatural amino acids. (A) Chemical structure of pBpa. (B) Coomassie stained SDS-PAGE gel of Ni-NTA purified AcpP-S36-pBpa. M, protein ladder; 1, AcpP-S36-pBpa; 2, WT AcpP. (C) Confirmation of the mass of AcpP-S36-pBpa by ESI-MS analysis. The found peak at 10967.13 agrees closely with that expected at 10966.48. AcpP containing a natural amino acid at position 36 was not detected.

the circular dichroism (CD) spectra of AcpP-pBpa closely matched that of the WT AcpP (ESI†), while the ACP2-pBpa CD spectra did not exactly match that of the WT ACP2, but was in good agreement (ESI†). Together, these results demonstrate, for the first time, the ability to introduce UAAs into carrier proteins of PKSs and fatty acid synthases.

Photocrosslinking

With site-selectively modified ACPs and potential interacting KS partners in hand, we next demonstrated crosslinking. The Ni-NTA chromatography-purified AcpP-S36-pBpa and FabF were crosslinked for up to 120 min through irradiation with UV light of 365 nm (transilluminator, 6W) (Fig. 4). Photo-crosslinking occurred rapidly, indicated by the clear presence of a higher molecular weight protein band corresponding to the expected photocrosslinked AcpP:FabF product at ~ 60 kDa. Densitometric analysis indicated $\sim 43\%$ crosslinking efficiency after 2 h irradiation. As expected, when WT AcpP which lacks the photocrosslinking residue was used in place of AcpP-S36-pBpa, photocrosslinked AcpP:FabF was not observed (Fig. 4). Further, possible non-specific self-crosslinking of AcpP apparently did not occur, demonstrating some level of specificity is required for efficient photocrosslinking to occur. Gratifyingly, LC-MS analysis of the photocrosslinked product mixture revealed the presence of a 56760.72 Da product (ESI†), consistent with that expected from crosslinking of AcpP and FabF $(10966.48 + 45794.27 = 56760.75)$. Taken together, these results demonstrate our ability to photocrosslink a cognate ACP–KS pair using a suitably placed photocrosslinking amino acid.

Photocrosslinking specificity

Crucially, in order to accurately report interpolypeptide interactions with PKSs, photocrosslinking should occur efficiently between cognate ACP–KS pairs and poorly between non-cognate ACP–KS pairs. Accordingly, in order to test orthogonality of anticipated ACP–KS interactions, we set-up photocrosslinking experiments between different combinations of ACP and KS. Incubation and irradiation of AcpP-S36-pBpa with FabB, FabF, and FabH resulted in detectable photocrosslinking only with FabB (8%) and FabF (76%) (Fig. 5A and Table 1). Since FabB and FabF are each known to use carrier protein bound acyl donors, while FabH uses acetyl-CoA donor, complete absence of detectable crosslinking between AcpP and FabH provides some evidence that our photocrosslinking strategy faithfully reports interpolypeptide interactions.

To further test this observation, we incubated and irradiated ACP2-S54-pBpa from the type I PKS DEBS, with each of the type II fatty acid synthase domains FabB, FabF, and FabH. Subsequently, photocrosslinking between these combinations was not observed (Fig. 5B and Table 1). Gratifyingly, on the basis of sequence homology, we did not expect interactions between these type I and type II PKS proteins, although we cannot rule out the possibility that small structural perturbations of ACP2 upon pBpa installation are responsible (ESI†).

To demonstrate the expected ability of our photocrosslinking strategy to map the ACP–KS interaction interface, we next constructed a pilot seven-member panel of FabF mutants by alanine scanning mutagenesis and assayed the ability of each mutant to photocrosslink with the cognate interaction partner AcpP. The CD spectra of each FabF mutant matched very closely that of the WT protein, indicating similar secondary structures of the mutant and WT FabF (ESI†). Only alanine substitution at Arg-206 and Leu-208 significantly affected photocrosslinking with AcpP, in fact, both R206A and L208A completely failed to crosslink (Fig. 6). Although exact residues involved in the AcpP:FabF interaction are not known, Arg-206 and Leu-208 reside in a general area adjacent to the FabF active site that has been implicated in ACP recognition in other proteins from the type II FAS.[34,35] Cumulatively, this data suggests that our photocrosslinking strategy faithfully reports interpolypeptide interactions between ACP and KS domains from fatty acid biosynthesis and potentially polyketide biosynthesis.

Conclusions

Understanding interpolypeptide ACP–KS communication is crucial to improve the outcome of combinatorial biosynthesis experiments aimed at module swapping to generate polyketide
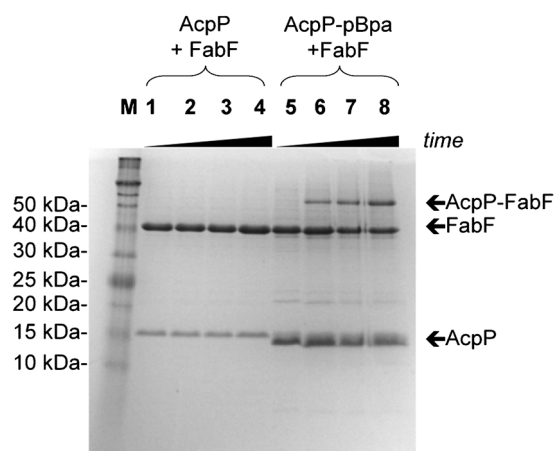
Fig. 4 Photocrosslinking between ACP and KS domains. Coomassie stained gel showing photocrosslinked products between AcpP + FabF (lanes 1-4) or AcpP-S36-pBpa + FabF (lanes 5-8). Samples were irradiated for 0, 30, 60, and 120 min.
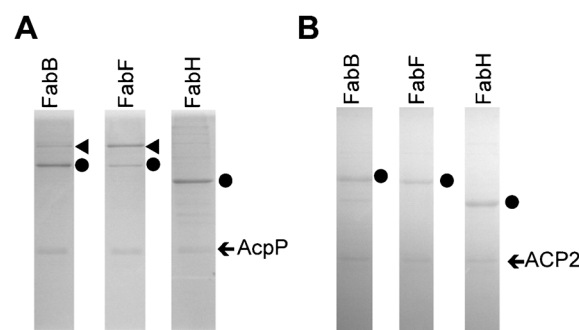

Fig. 5 Orthogonality of photo-crosslinking between ACP and KS domains. Coomassie stained gel showing photocrosslinked products between (A) AcpP-S36-pBpa and type II KS proteins, and (B) ACP2-S54-pBpa and type II KS proteins. Samples were irradiated for 120 min. Circles (●) indicate position of KS domain; triangles (◄) indicate position of photocrosslinked product, if detected.

Table 1 Photocrosslinking efficiency between ACP and KS domains from different biosynthetic systems

	Photocrosslinking efficiency[a] (expected interaction)[b]	
	AcpP-S36-pBpa	ACP2-S54-pBpa
FabF	76% (+)	N.D (−)
FabH	N.D (−)	N.D (−)
FabB	8% (+)	N.D (−)

[a] Determined by densitometric analysis of photocrosslinked products, see Experimental Procedures and ESI.† [b] Expected interaction, see references in text. N.D, non-detected.

A

B
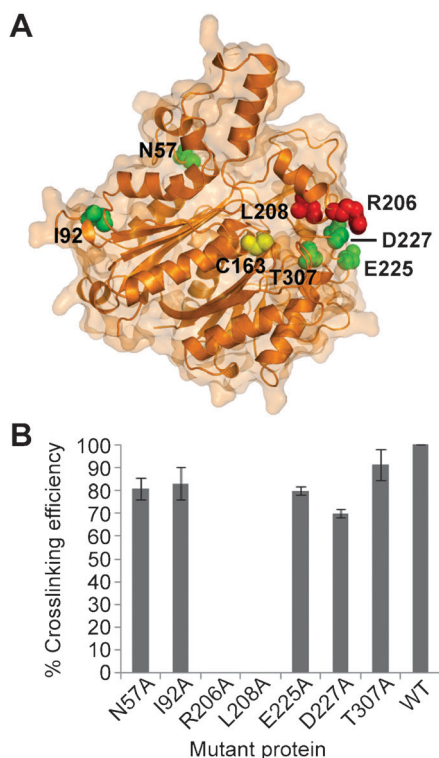
Fig. 6 Mapping the AcpP:FabF epitope by photocrosslinking. (A) Structure of FabF. *Red*, location of alanine substitutions that negatively affect crosslinking; *green*, locations of alanine substitutions that do not significantly affect crosslinking. (B) Photocrosslinking efficiency of a small panel of FabF mutants, crosslinking of WT FabF and AcpP is set to 100%. Error bars represent ± standard deviation from the average, *n* = 3.

analogues. The photocrosslinking strategy presented here is able to rapidly provide information regarding the orthogonality of protein interactions between ACPs and KSs from different sources. This strategy has several benefits for reporting protein interactions in fatty acid and polyketide synthases compared to mechanism-based approaches. For example, large quantities of mutant protein can be obtained very easily, without the requirement of complex small molecule synthesis. In addition, our photocrosslinking strategy is completely general and does not need to take into account the substrate specificity of individual domains. Currently, the crosslinking residue was installed at the site of the phosphopantetheine prosthetic arm. However, pBpa could be installed at virtually

any position in the ACP (or KS), thus allowing both phosphopantetheinylation and charging with acyl-donor *via* Sfp, which might allow probing the influence of phosphopantetheinylation and substrate loading on ACP–KS interactions. This is particularly important given the identity of the acyl chain substrate can affect these interactions.[19]

Our data suggests that we will be able to rapidly identify residues that are intimately involved in ACP–KS recognition, for example by alanine scanning mutagenesis of either domain partner followed by photocrosslinking. Further, we hope to modify this strategy to a high-throughput format for screening large libraries of mutants for altered ACP–KS interactions. Given that for cognate ACP–KS combinations, the strength of the protein interaction correlates directly with catalytic activity,[22] we hope to identify mutations that rescue inactive faulty ACP–KS pairs.

Experimental procedures

Materials

Reagents were of the highest grade possible and purchased from Sigma (St. Louis, MO), unless otherwise indicated. pBpa was from PepTech Corp (Burlington, MA). Bacterial strain *E.coli* BL21(DE3) pLysS competent cells was from Promega (Madison, WI). Plasmids containing each ACP and KS domain were constructed as described in the ESI.† pEVOL-Bpf was kindly provided by the Schultz laboratory at the Scripps Research Institute.

Unnatural amino acid mutagenesis

Amber (TAG) codons were introduced *via* the site-directed mutagenesis kit from Stratagene, as described by the manufacturer. WT and mutant ACP and AcpP were expressed as previously described.[36,37] FabF, FabB and FabH were expressed following published procedures.[38,39]

Photocrosslinking

Photocrosslinking reactions were carried out on 96-well microtiter plate on ice. Reaction volume was 50 μL in PBS buffer, ACPs were five fold in excess of KASs (8.8 μM). Reaction was irradiated at 365 nm with a handheld UV lamp and then analyzed by SDS-PAGE (4-12% gradient).

Acknowledgements

Notes and references

1 K. J. Weissman and P. F. Leadlay, *Nat. Rev. Microbiol.*, 2005, **3**, 925–936.
2 M. A. Fischbach and C. T. Walsh, *Chem. Rev.*, 2006, **106**, 3468–3496.
3 J. Staunton and K. J. Weissman, *Nat. Prod. Rep.*, 2001, **18**, 380–416.
4 R. J. Cox and T. J. Simpson, *Methods Enzymol.*, 2009, **459**, 49–78.
5 R. J. Cox, *Org. Biomol. Chem.*, 2007, **5**, 2010–2026.
6 D. I. Chan and H. J. Vogel, *Biochem. J.*, 2010, **430**, 1–19.
7 K. J. Weissman and R. Muller, *ChemBioChem*, 2008, **9**, 826–848.
8 N. Wu, D. E. Cane and C. Khosla, *Biochemistry*, 2002, **41**, 5056–5066.

9 N. Wu, S. Y. Tsuji, D. E. Cane and C. Khosla, *J. Am. Chem. Soc.*, 2001, **123**, 6465–6474.
10 A. Y. Chen, N. A. Schnarr, C. Y. Kim, D. E. Cane and C. Khosla, *J. Am. Chem. Soc.*, 2006, **128**, 3067–3074.
11 S. Kapur, A. Y. Chen, D. E. Cane and C. Khosla, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **107**, 22066–22071.
12 S. S. Chandran, H. G. Menzella, J. R. Carney and D. V. Santi, *Chem. Biol.*, 2006, **13**, 469–474.
13 H. G. Menzella, R. Reid, J. R. Carney, S. S. Chandran, S. J. Reisinger, K. G. Patel, D. A. Hopwood and D. V. Santi, *Nat. Biotechnol.*, 2005, **23**, 1171–1176.
14 R. McDaniel, A. Thamchaipenet, C. Gustafsson, H. Fu, M. Betlach and G. Ashley, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 1846–1851.
15 J. K. Stoops and S. J. Wakil, *J. Biol. Chem.*, 1981, **256**, 5128–5133.
16 F. T. Wong, X. Jin, Mathews, II, D. E. Cane and C. Khosla, *Biochemistry*, 2011, **50**, 6539–6548.
17 R. Pieper, R. S. Gokhale, G. Luo, D. E. Cane and C. Khosla, *Biochemistry*, 1997, **36**, 1846–1851.
18 P. Kumar, C. Khosla and Y. Tang, *Methods Enzymol.*, 2004, **388**, 269–293.
19 L. K. Charkoudian, C. W. Liu, S. Capone, S. Kapur, D. E. Cane, A. Togni, D. Seebach and C. Khosla, *Protein Sci.*, 2011, **20**, 1244–1255.
20 A. S. Worthington, G. H. Hur, J. L. Meier, Q. Cheng, B. S. Moore and M. D. Burkart, *ChemBioChem*, 2008, **9**, 2096–2103.
21 A. S. Worthington, H. Rivera, J. W. Torpey, M. D. Alexander and M. D. Burkart, *ACS Chem. Biol.*, 2006, **1**, 687–691.
22 A. S. Worthington, D. F. Porter and M. D. Burkart, *Org. Biomol. Chem.*, 2010, **8**, 1769–1772.
23 J. W. Chin and P. G. Schultz, *ChemBioChem*, 2002, **3**, 1135–1137.
24 J. W. Chin, A. B. Martin, D. S. King, L. Wang and P. G. Schultz, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 11020–11024.
25 A. Y. Chen, D. E. Cane and C. Khosla, *Chem. Biol.*, 2007, **14**, 784–792.
26 G. Dorman and G. D. Prestwich, *Biochemistry*, 1994, **33**, 5661–5673.
27 H. Mori and K. Ito, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 16159–16164.
28 C. Y. Majmudar, L. W. Lee, J. K. Lancia, A. Nwokoye, Q. Wang, A. M. Wands, L. Wang and A. K. Mapp, *J. Am. Chem. Soc.*, 2009, **131**, 14240–14242.
29 K. A. Daggett, M. Layer and T. A. Cropp, *ACS Chem. Biol.*, 2009, **4**, 109–113.
30 C. Liu, A. L. Young, A. Starling-Windhof, A. Bracher, S. Saschenbrecker, B. V. Rao, K. V. Rao, O. Berninghausen, T. Mielke, F. U. Hartl, R. Beckmann and M. Hayer-Hartl, *Nature*, 2010, **463**, 197–202.
31 D. Braig, C. Bar, J. O. Thumfart and H. G. Koch, *J. Mol. Biol.*, 2009, **390**, 401–413.
32 T. S. Young, I. Ahmad, J. A. Yin and P. G. Schultz, *J. Mol. Biol.*, 2010, **395**, 361–374.
33 J. Crosby, D. H. Sherman, M. J. Bibb, W. P. Revill, D. A. Hopwood and T. J. Simpson, *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol.*, 1995, **1251**, 32–42.
34 K. Karmodiya, R. Modak, N. Sahoo, S. Sajad and N. Surolia, *FEBS J.*, 2008, **275**, 4756–4766.
35 Y. M. Zhang, B. Wu, J. Zheng and C. O. Rock, *J. Biol. Chem.*, 2003, **278**, 52935–52943.
36 V. Y. Alekseyev, C. W. Liu, D. E. Cane, J. D. Puglisi and C. Khosla, *Protein Sci.*, 2007, **16**, 2093–2107.
37 R. B. Hill, K. R. MacKenzie, J. M. Flanagan, J. E. Cronan, Jr. and J. H. Prestegard, *Protein Expression Purif.*, 1995, **6**, 394–400.
38 P. Edwards, J. S. Nelsen, J. G. Metz and K. Dehesh, *FEBS Lett.*, 1997, **402**, 62–66.
39 C. Davies, R. J. Heath, S. W. White and C. O. Rock, *Structure*, 2000, **8**, 185–195.