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Immobilization of a Bacterial Cytochrome P450 Monooxygenase System on a Solid Support

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Abstract: *Bacterial cytochrome P450s (P450s), which catalyze regio- and stereoselective oxidations of hydrocarbons with high turnover rates, are attractive biocatalysts for fine chemical production. Enzyme immobilization is needed for cost-effective industrial manufacturing. However, immobilization of P450s is difficult because electron-transfer proteins are involved in catalysis and anchoring these can prevent them from functioning as shuttle molecules for carrying electrons. We studied a heterotrimeric protein-mediated co-immobilization of a bacterial P450, and its electron-transfer protein and reductase. Fusion with subunits of a heterotrimeric Sulfolobus solfataricus proliferating cell nuclear antigen (PCNA) enabled immobilization of the three proteins on a solid support. The co-immobilized enzymes catalyzed monooxygenation because the electron-transfer protein fused to PCNA via a single peptide linker retained its electron-transport function.*

Enzymes are widely used in the food industry, detergents, organic syntheses, analysis, and research.[1] The effective use of enzymes, especially in industrial organic synthesis and in biosensors, requires immobilization techniques that enable the enzymes reuse, continuous operation, and easy separation from products.[2] Immobilization methods are classified into three groups:[3] 1) binding to a prefabricated support, 2) carrier-free insolubilization by cross-linking, and 3) entrapment (encapsulation) in a polymer network or membrane device. Support binding is most widely used, because supports insolubilize enzymes and provide additional physical and chemical properties (mechanical strength, filterability, magnetism, static electric charges, and hydrophilic/hydrophobic balance), and various supports with functional moieties that readily attach enzymes are commercially available.[4] Except for encapsulation in microcapsules, which has mass-transfer limitations, enzymes have to be tightly fixed to prevent leakage. Although multienzymatic reactions, which can produce valuable compounds with multiple chiral carbon centers, functional groups, and/or high-energy bonds, have attracted much interest in metabolic engineering and synthetic biology,[5] single enzymes have mainly been targeted for immobilization. The use of immobilization in multienzymatic reactions is not difficult[6] and has been achieved by immobilization of multiple enzyme mixtures and assembly of separately immobilized single enzymes. However, conventional immobilization methods are not always suitable for immobilizing multienzymatic systems.

Cytochrome P450 monooxygenases (P450s), which have physiologically important roles in various organisms, are generally coupled to reductases. P450s catalyze regio- and stereoselective oxidations of C–C and C–H bonds with molecular oxygen under atmospheric conditions, and are promising environmentally friendly biocatalysts for fine chemical manufacturing.[7] In fact, P450-catalyzed reactions are increasingly attractive for chemical synthesis, for example, a sequential two-step hydroxylation of vitamin D_3 to an active metabolite known as calcitriol,[8] enantioselective epoxidations of alkenes,[9] and decarboxylation of fatty acids to alkenes.[10] The heme at the active site accepts two electrons per reaction cycle to generate an active species. The heme protein scaffold cannot directly withdraw electrons from natural small reducing agents, such as NAD(P)H, but accepts electrons from an electron-transfer protein/domain, such as ferredoxin and flavodoxin, which is in turn reduced by its specific reductase/domain by NAD(P)H. Bacterial P450s, which have higher catalytic activities than eukaryotic P450s, generally require isolated electron-transfer proteins and reductases.[11] Their monooxygenation reactions are strongly coupled to reductions of electron-transfer proteins by their reductases.

The interacting regions of electron-transfer proteins with P450s also interact with reductases, requiring these proteins to shuttle between P450s and reductases even when immobilized. Tight binding of the proteins to solid supports prevents monooxygenation reactions catalyzed by bacterial P450s. In fact, covalent co-immobilization on solid supports of P450s and their electron-transfer proteins and reductases has not been reported. Adsorption of bacterial P450-containing cell-free extracts on ion-exchange supports has been reported,[12] but the enzymes leaked from the supports. To overcome these problems 1) the P450, electron-transfer protein, and reductase should be aligned close to each other and 2) the electron-transfer protein must retain its mobility to carry electrons from the reductase to the P450 in three-protein co-insolubi-

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lization. Herein, we propose a heterooligomeric protein-mediated immobilization method for multienzymatic systems linked with a carrier protein.

We previously reported heterotrimeric protein-mediated complex formation of a bacterial P450, and its specific ferredoxin and ferredoxin reductase.[13] The proliferating cell nuclear antigen (PCNA) from *Sulfolobus solfataricus* is a heterotrimeric protein composed of three distinct subunits, PCNA1, PCNA2, and PCNA3 (Figure 1 a). PCNA3 cannot interact with PCNA1 or PCNA2 individually but binds to a heterodimer of PCNA1 and PCNA2 with high affinity ($K_D = 200$ nM).[14] Consequently, a *Pseudomonas putida* P450 (P450cam) fused to PCNA3 exclusively formed a heterotrimeric protein complex with equimolar amounts of *P. putida* ferredoxin reductase (putidaredoxin reductase, PdR) and ferredoxin (putidaredoxin, PdX) fused to PCNA1 and PCNA2, respectively. The complex, in which PdX carries electrons from PdR to P450cam by transient interactions with PdR and P450cam, showed P450cam monooxygenase activity.

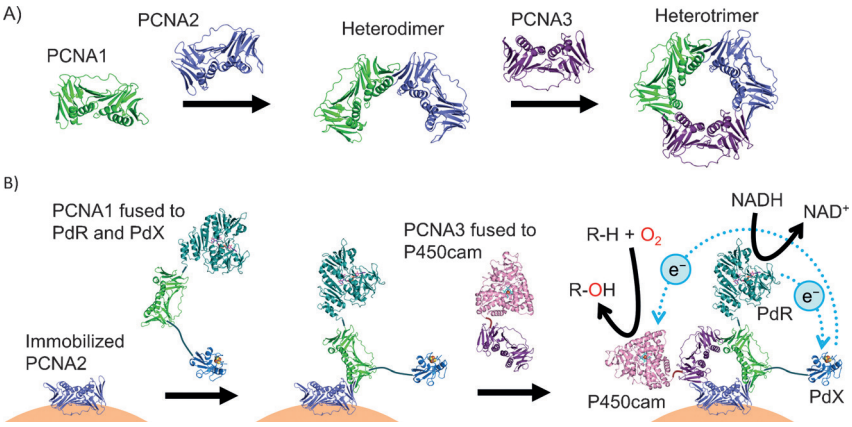

Figure 1. Co-immobilization of PdR, PdX, and P450cam using *S. solfataricus* PCNA. A) Stepwise heterotrimerization of *S. solfataricus* PCNA. B) Selective assembly of PdR, PdX, and P450cam on an immobilized PCNA subunit (PCNA2 in this study).

The PCNA-assembled protein complexes of P450 and electron-transfer-related proteins (PUPPETs) are expected to completely retain their monooxygenase activity on immobilization through PCNA. However, it is difficult to selectively conjugate the PCNA domain of a PUPPET with chemical reagents or place it on a solid support after complex formation. We therefore attempted to sequentially construct PUPPETs on solid supports; one PCNA subunit is directly immobilized on the solid support followed by binding of another subunit fused to PdX and PdR along with the other subunit fused to P450cam to the immobilized subunit (Figure 1 b). PCNA2 was directly immobilized on the solid support, because surface plasmon resonance analysis showed that among the three subunits, PCNA2 immobilized by amine coupling gave the highest accumulation yield of the heterotrimer (Figure S1 in the Supporting Information).

First, molecular assembly of P450cam, PdX, and PdR on free PCNA2 was confirmed. We prepared a PCNA1 fusion protein, PdR-PCNA1$_{G108C}$-PdX, in which PdR and PdX are genetically linked at the N- and C-terminus of the G108C variant of PCNA1, respectively, and a PCNA3 fusion protein, P450cam-PCNA3$_{R112C/T180C}$, in which P450cam is genetically linked at the N-terminus of the R112C/T180C variant of PCNA3. Cysteine substitutions were introduced to stabilize the heterotrimeric complex by forming disulfide bonds between the subunits in the PCNA heterotrimer.[15] The UV/Vis spectra of the fusion proteins had characteristic absorption peaks from their component proteins (Figure S2), suggesting that PdR, PdX, and P450cam in the fusion proteins retained their cofactors, which play central roles in their functions. PdR-PCNA1$_{G108C}$-PdX alone showed cytochrome *c* reduction activity (Figure S3), reflecting reduction of PdX as a result of intramolecular reduction by PdR in the fusion protein. The PUPPET prepared from PdR-PCNA1$_{G108C}$-PdX, the L171C variant of PCNA2 (PCNA2$_{L171C}$), and P450cam-PCNA3$_{R112C/T180C}$, in which the PCNA subunits were covalently linked by disulfide bonds (Figure 2 A), showed much higher activity (8.8×10^2 nmol min^{-1} (nmol of P450cam)$^{-1}$) than equimolar mixtures of PdR-PCNA1$_{G108C}$-PdX and P450cam-PCNA3$_{R112C/T180C}$ (Figure 2 B). The activity had a linear relationship with the PUPPET concentration. This clearly shows that P450cam, PdX, and PdR can be assembled on PCNA2 with sufficiently close proximity for efficient electron transfer from PdR to P450cam through PdX.

Next, P450cam, PdX, and PdR were assembled on immobilized PCNA2. PCNA2$_{L171C}$ was immobilized on magnetic beads using a standard amine coupling method. PdR-PCNA1$_{G108C}$-PdX and P450cam-PCNA3$_{R112C/T180C}$ were loaded on PCNA2$_{L171C}$-immobilized beads. The beads heated in Laemmli buffer at 98 °C released the two fusion proteins (Figure 3 A), indicating that they were immobilized on the magnetic beads through PCNA2$_{L171C}$. The immobilized PUPPET hydroxylated D-camphor with 99 % coupling efficiency, the ratio of D-camphor consumption to NADH consumption (Table S1). The specific activity (7×10^2 nmol min^{-1} (nmol of P450cam)$^{-1}$), which was calculated from the initial NADH consumption rate and coupling efficiency, was similar to that of P450cam in the free complex of PdR-PCNA1$_{G108C}$-PdX, PCNA2$_{L171C}$, and P450cam-PCNA3$_{R112C/T180C}$. The PdR, PdX, and P450cam assembled on immobilized PCNA2$_{L171C}$ were not in direct contact with the beads and behaved like those assembled on free PCNA2$_{L171C}$. In contrast, P450cam directly co-immobilized with PdX and PdR on beads through amine coupling did not hydroxylate D-camphor because direct immobilization inactivated PdR (Figure S4).

Disulfide bonds between the PCNA subunits contributed significantly to retention of P450cam on the beads. PCNA1 dissociated slowly from the immobilized PCNA2, but the wild type PCNA3 dissociated rapidly from the PCNA1:PCNA2

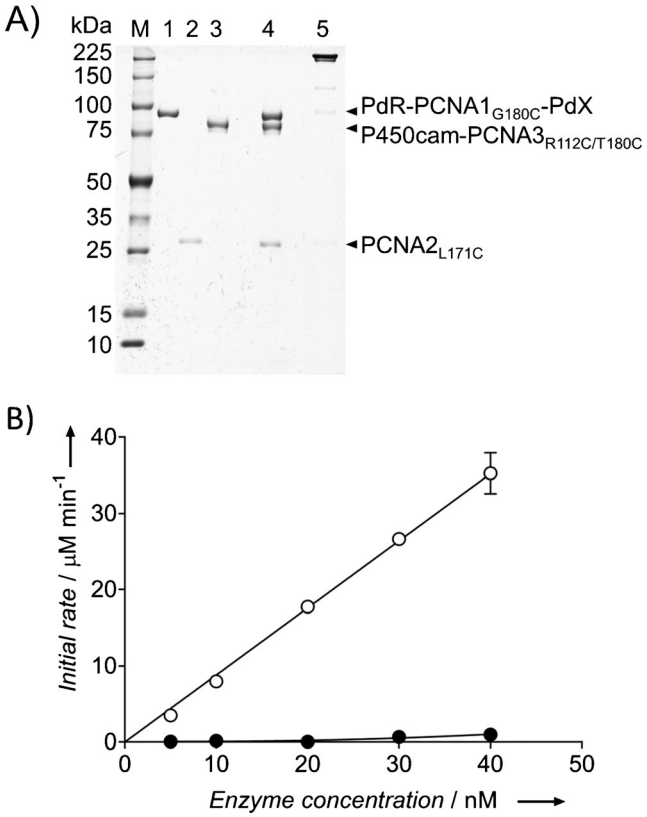

Figure 2. Molecular assembly of PdR, PdX, and P450cam on free PCNA2. A) SDS-PAGE analysis of PdR-PCNA1$_{G108C}$-PdX (lane 1), PCNA2$_{L171C}$ (lane 2), P450cam-PCNA3$_{R112C/T180C}$ (lane 3), and PUPPET (lanes 4 and 5). Proteins were denatured in Laemmli buffer with 2-mercaptoethanol (lanes 1–4). PUPPET was denatured in Laemmli buffer without 2-mercaptoethanol (lane 5). B) Initial activities of complex (open circles) and equimolar mixtures of PdR-PCNA1$_{G108C}$-PdX and P450cam-PCNA3$_{R112C/T180C}$ (closed circles). Initial reaction rates were plotted against protein concentrations. Error bars represent standard deviations of three replicates.

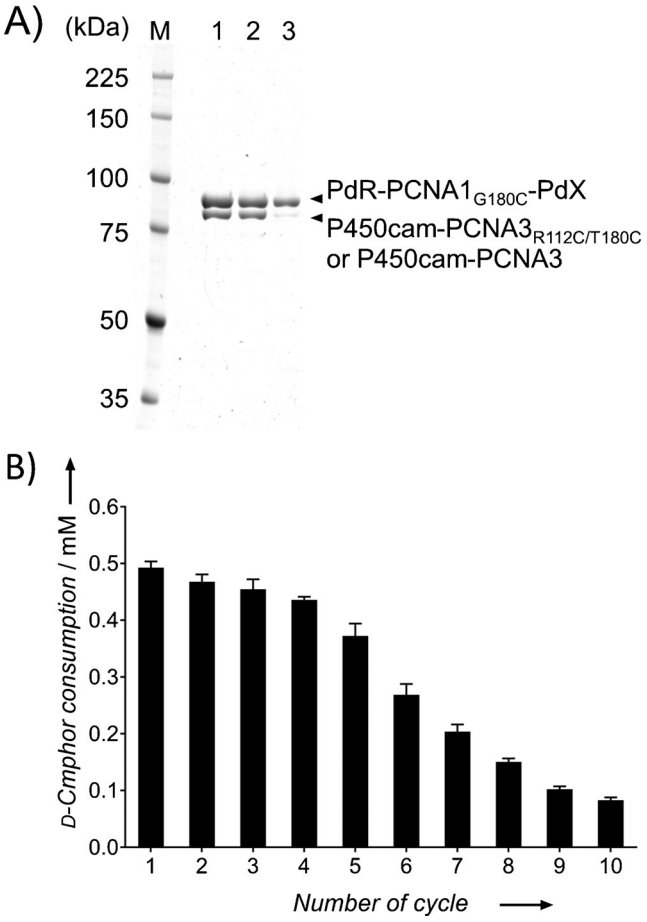

Figure 3. Reusability of immobilized PUPPET. A) PCNA2$_{L171C}$-immobilized beads loaded with PdR-PCNA1$_{G108C}$-PdX and P450cam-PCNA3$_{R112C/T180C}$ (lane 1), the beads after 10 cycles of reaction (lane 2), and PCNA2$_{L171C}$-immobilized beads treated with PdR-PCNA1$_{G108C}$-PdX and P450cam-PCNA3 (lane 3) analyzed by SDS-PAGE. B) D-Camphor consumption by immobilized PUPPET up to 10 cycles. Reaction mixture containing 1 mM D-camphor and 0.5 mM NADH was incubated at room temperature for 10 min and analyzed by GC. Error bars represent standard deviations of three replicates.

heterodimer immobilized through PCNA2 (Figure S1). PdR-PCNA1$_{G108C}$-PdX was retained on the immobilized PCNA2$_{L171C}$ though there is no disulfide bond between PCNA1$_{G108C}$ and PCNA2$_{L171C}$ (Figure 3 A, lane 3). P450cam-PCNA3, which cannot form disulfide bonds with PCNA1$_{G108C}$ and PCNA2$_{L171C}$, was washed out during immobilization, and therefore little was retained. In contrast, disulfide bond formation between PCNA1$_{G108C}$ and PCNA3$_{R112C/T180C}$, as well as PCNA2$_{L171C}$ and PCNA3$_{R112C/T180C}$, greatly improved the assembly yield of P450cam (Figure 3 A, lane 1).

Disulfide bond formation also contributed to the reusability of the immobilized PUPPET, which showed D-camphor hydroxylation activity even in the 10th cycle, though the activity decreased with cycle number (Figure 3 B). SDS-PAGE analysis showed that P450cam-PCNA3$_{R112C/T180C}$ was completely retained on the beads with PdR-PCNA1$_{G108C}$-PdX even after the 10th cycle (Figure 3 A, lane 2). The decrease in the activity is therefore probably caused by inactivation of PdR, PdX, and/or P450cam, as previously reported.[16]

Our approach was also useful for immobilization of a self-sufficient P450. CYP102A1 (P450 BM3), a cytochrome P450 from *Bacillus megaterium*, is easily handled and a target for various biotechnological applications,[17] because the enzyme has a heme protein scaffold, an electron-transfer domain, and a reductase domain in a single polypeptide chain and does not require auxiliary proteins.[18] Recently, immobilization of P450 BM3 through specific binding site introduction for cost-effective use of the enzyme has been reported.[19] We attempted to immobilize this P450 BM3 by fusion to PCNA. P450 BM3 fused to PCNA3$_{R112C/T180C}$ was immobilized on magnetic-bead-bound PCNA2$_{L171C}$ with the assistance of PCNA1$_{G108C}$. The catalytic activity of the indirectly immobilized P450 BM3 was much higher than that of the directly immobilized one, and the immobilized P450 BM3 had good reusability (Figure 4). The C-terminal half domain containing FAD and the N-terminal half domain containing heme and FMN of P450 BM3 were reassembled on the beads by fusion to PCNA1$_{G108C}$ and PCNA3$_{R112C/T180C}$, respectively. However, the reassembled P450 BM3 showed lower activity and reusability than intact P450 BM3 on the beads (Figure S5). This is

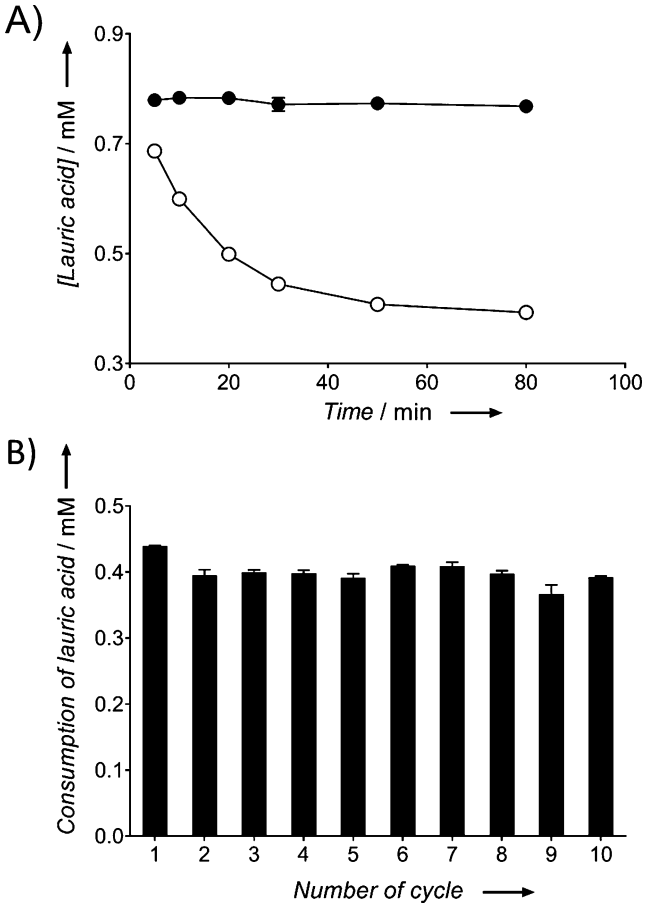

Figure 4. Consumption of lauric acid by immobilized P450 BM3. A) Time course of lauric acid consumption by P450 BM3 immobilized through PCNA (open circles) and directly immobilized P450 BM3 (closed circles). Reaction mixture containing 0.8 mM lauric acid, 0.5 mM NADPH, and 300 nM FMN was incubated at room temperature and analyzed by GC. B) Reusability of P450 BM3 immobilized on beads through PCNA. Reaction mixture containing 0.8 mM lauric acid, 0.5 mM NADPH and 300 nM FMN was incubated at room temperature for 20 min. Error bars represent standard deviations of three replicates.

probably because of inferior electron transfer between the split FAD and heme-FMN domains, and instability of the split domains.

In conclusion, we have developed a heterotrimeric protein-mediated enzyme immobilization method that can be used for a bacterial multicomponent P450 system. Fusion to *S. solfataricus* PCNA subunits enabled P450cam to form a complex with PdX and PdR on magnetic beads through the PCNA heterotrimer. The P450cam co-immobilized with PdX and PdR showed monooxygenase activity because PdX was linked via a single peptide linker and retained its function as a shuttle molecule for carrying electrons from PdR to P450cam in the complex. PCNA-mediated immobilization avoids enzyme inactivation caused by physical and/or chemical interactions with the support during immobilization, unlike direct immobilization on solid supports. Our approach was used to immobilize a self-sufficient P450 containing both heme and electron-transfer domains in one polypeptide chain. Bacterial P450s can catalyze unusual reactions including desaturation,[20] tetrahydrofuran ring formation[21] and oxidative decarboxylation of fatty acids[10] in addition to typical monooxygenase reactions, such as hydroxylation, epoxidation, and dealkylation. Protein engineering has enabled unnatural catalysis, fulfilling the demand for practical chemical synthesis.[22] For example, engineered P450cam enzymes can catalyze alkane hydroxylation,[23] aromatic hydroxylation,[24] and indole oxidation to indigo.[25] Along with the recent progress in P450 catalysis, successful immobilization of bacterial P450 systems without significant loss of catalytic activity will greatly enhance their advantages as catalysts for chemical synthesis. Furthermore, PCNA-mediated enzyme immobilization is a useful method that can be applied to other multienzymatic reaction systems requiring carrier proteins, such as non-ribosomal peptide synthetases.[26]

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Keywords: biocatalysis · cytochromes · enzymes · hydroxylation · immobilization

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[1] W. Aehle, *Enzymes in Industry: Production and Applications*, Wiley-VCH, Weinheim, **2007**, p. 490.
[2] A. A. Homaei, R. Sariri, F. Vianello, R. Stevanato, *J. Chem. Biol.* **2013**, *6*, 185–205.
[3] R. A. Sheldon, *Adv. Synth. Catal.* **2007**, *349*, 1289–1307.
[4] J. C. S. Dos Santos, O. Barbosa, C. Ortiz, A. Berenguer-Murcia, R. C. Rodrigues, R. Fernandez-Lafuente, *ChemCatChem* **2015**, *7*, 2413–2432.
[5] a) Z. A. King, C. J. Lloyd, A. M. Feist, B. O. Palsson, *Curr. Opin. Biotechnol.* **2015**, *35*, 23–29; b) M. J. Smanski, H. Zhou, J. Claesen, B. Shen, M. A. Fischbach, C. A. Voigt, *Nat. Rev. Microbiol.* **2016**, *14*, 135–149.
[6] F. Jia, B. Narasimhan, S. Mallapragada, *Biotechnol. Bioeng.* **2014**, *111*, 209–222.
[7] a) A. W. Munro, H. M. Girvan, A. E. Mason, A. J. Dunford, K. J. McLean, *Trends Biochem. Sci.* **2013**, *38*, 140–150; b) R. Bernhardt, V. B. Urlcher, *Appl. Microbiol. Biotechnol.* **2014**, *98*, 6185–6203.
[8] H. Sugimoto, R. Shinkyo, K. Hayashi, S. Yoneda, M. Yamada, M. Kamakura, S. Ikushiro, Y. Shiro, T. Sakaki, *Biochemistry* **2008**, *47*, 4017–4027.
[9] a) T. Kubo, M. R. Peters, P. Manihold, F. H. Arnold, *Chem. Eur. J.* **2006**, *12*, 1216–1220; b) A. Li, J. T. Liu, S. Q. Pham, Z. Li, *Chem. Commun.* **2013**, *49*, 11572–11574.
[10] A. Dennig, M. Kuhn, S. Tassoti, A. Thiessenhusen, S. Gilch, T. Bülter, T. Haas, M. Hall, K. Faber, *Angew. Chem. Int. Ed.* **2015**, *54*, 8819–8822; *Angew. Chem.* **2015**, *127*, 8943–8946.
[11] F. Hannemann, A. Bichet, K. M. Ewen, R. Bernhardt, *Biochim. Biophys. Acta Gen. Subj.* **2007**, *1770*, 330–344.
[12] M. Taylor, D. C. Lamb, R. J. Cannell, M. J. Dawson, S. L. Kelly, *Biochem. Biophys. Res. Commun.* **2000**, *279*, 708–711.
[13] H. Hirakawa, T. Nagamune, *ChemBioChem* **2010**, *11*, 1517–1520.

[14] a) I. Dionne, R. K. Nookala, S. P. Jackson, A. J. Doherty, S. D. Bell, *Mol. Cell* **2003**, *11*, 275 – 282; b) F. Iwata, H. Hirakawa, T. Nagamune, *Sci. Rep.* **2016**, *6*, 26588.
[15] H. Hirakawa, A. Kakitani, T. Nagamune, *Biotechnol. Bioeng.* **2013**, *110*, 1858 – 1864.
[16] C. Y. Tan, H. Hirakawa, T. Nagamune, *Sci. Rep.* **2015**, *5*, 8648.
[17] a) C. F. Butler, C. Peet, K. J. McLean, M. T. Baynham, R. T. Blankley, K. Fisher, S. E. J. Rigby, D. Leys, M. W. Voice, A. W. Munro, *Biochem. J.* **2014**, *460*, 247 – 259; b) G.-D. Roiban, R. Agudo, M. T. Reetz, *Angew. Chem. Int. Ed.* **2014**, *53*, 8659 – 8663; *Angew. Chem.* **2014**, *126*, 8803 – 8807; c) H. Venkataraman, E. M. te Poele, K. Z. Rosłoniec, N. Vermeulen, J. N. M. Commandeur, R. van der Geize, L. Dijkhuizen, *Appl. Microbiol. Biotechnol.* **2015**, *99*, 4713 – 4721; d) P. Le-Huu, T. Heidt, B. Claasen, S. Laschat, V. B. Urlacher, *ACS Catal.* **2015**, *5*, 1772 – 1780; e) Z. Cong, O. Shoji, C. Kasai, N. Kawakami, H. Sugimoto, Y. Shiro, Y. Watanabe, *ACS Catal.* **2015**, *5*, 150 – 156; f) C. K. Prier, T. K. Hyster, C. C. Farwell, A. Huang, F. H. Arnold, *Angew. Chem. Int. Ed.* **2016**, *55*, 4711 – 4715; *Angew. Chem.* **2016**, *128*, 4789 – 4793.
[18] C. J. C. Whitehouse, S. G. Bell, L.-L. Wong, *Chem. Soc. Rev.* **2012**, *41*, 1218 – 1260.
[19] a) J. H. Lee, D. H. Nam, S. H. Lee, J. H. Park, S. J. Park, S. H. Lee, C. B. Park, K. J. Jeong, *Bioconjugate Chem.* **2014**, *25*, 2101 – 2104; b) S. Zernia, F. Ott, K. Bellmann-Sickert, R. Frank, M. Klenner, H.-G. Jahnke, A. Prager, B. Abel, A. Robitzki, A. G. Beck-Sickinger, *Bioconjugate Chem.* **2016**, *27*, 1090 – 1097; c) X. D. Wang, K. S. Rabe, I. Ahmed, C. M. Niemeyer, *Adv. Mater.* **2015**, *27*, 7945 – 7950.
[20] S. G. Bell, R. Zhou, W. Yang, A. B. H. Tan, A. S. Gentleman, L.-L. Wong, W. Zhou, *Chem. Eur. J.* **2012**, *18*, 16677 – 16688.
[21] G. Zocher, M. E. A. Richter, U. Mueller, C. Hertweck, *J. Am. Chem. Soc.* **2011**, *133*, 2292 – 2302.
[22] a) G.-D. Roiban, M. T. Reetz, *Chem. Commun.* **2015**, *51*, 2208 – 2224; b) J. B. Y, H. Behrendorff, W. Huang, E. M. J. Gillam, *Biochem. J.* **2015**, *467*, 1 – 15.
[23] a) S. G. Bell, J.-A. Stevenson, H. D. Boyd, S. Campbell, A. D. Riddle, E. L. Orton, L.-L. Wong, *Chem. Commun.* **2002**, 490 – 491; b) F. Xu, S. G. Bell, J. Lednik, A. Insley, Z. Rao, L.-L. Wong, *Angew. Chem. Int. Ed.* **2005**, *44*, 4029 – 4032; *Angew. Chem.* **2005**, *117*, 4097 – 4100.
[24] a) J. P. Jones, E. J. O'Hare, L.-L. Wong, *Eur. J. Biochem.* **2001**, *268*, 1460 – 1467; b) R. J. Sowden, S. Yasmin, N. H. Rees, S. G. Bell, L.-L. Wong, *Org. Biomol. Chem.* **2005**, *3*, 57 – 64.
[25] A. Çelik, R. E. Speight, N. J. Turner, *Chem. Commun.* **2005**, 3652 – 3654.
[26] M. A. Marahiel, *Nat. Prod. Rep.* **2016**, *33*, 136 – 140.

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