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Improved Catalytic Activity of a Purified Multienzyme from a Modular Polyketide Synthase after Coexpression with *Streptomyces* Chaperonins in *Escherichia coli*.

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6-Deoxyerythronolide B (6-dEB), the aglycone of the broad-spectrum polyketide antibiotic erythromycin A, is synthesised in the filamentous bacterium *Saccharopolyspora erythraea* by a giant multienzyme synthase known as 6-deoxyerythronolide B synthase (DEBS). DEBS contains six distinct sets—or modules—of catalytic domains, each of which accomplishes one of the required six cycles of chain extension. Two extension modules are housed in each of three large polypeptides: DEBS 1 (371 kDa), DEBS 2 (374 kDa) and DEBS 3 (332 kDa) (Scheme 1).[1] Because DEBS multienzymes are homodimeric,[2] the overall size of the DEBS assembly-line multienzyme complex is predicted to be around 2 MDa. Several individual domains[3] and didomains[4] from DEBS and related polyketide synthases (PKSs) have recently been expressed in *E. coli* and their crystal structures determined, but so far an X-ray crystal structure of one or more complete modules has proved elusive. However, recent successful X-ray crystallography of mammalian[5] and fungal[6] fatty acid synthase multienzymes has pointed the way to achieving this challenging goal. Structural and functional characterisation of such intact PKS units will be essential for a proper understanding of conformational changes and interdomain interactions during catalysis. Intact DEBS multienzymes have been purified previously in small amounts from *S. erythraea*,[2] and after heterologous expression in *S. coelicolor*[7] and *E. coli*;[8] that work provided the starting point for this study, which aimed to provide an improved source of homogenous recombinant DEBS subunits.

The heterologous expression of PKS proteins in *E. coli* has been intensively studied, and with considerable success, as a platform strategy for the convenient production of high-value polyketide and polypeptide natural products.[9] Alteration of the codon usage of the actinomycete (G + C-rich) genes, together with optimisation of expression vectors and of the fermentation process, have led to very significant production levels of—for example—6-deoxyerythronolide B (20 mg L^{-1}, relative to 45 mg L^{-1} in *S. coelicolor*) and other erythromycin A biosynthetic intermediates.[10] Notably, coexpression in these systems of *E. coli* chaperones, an established tactic for improving the solubilities and yields of recombinant proteins in *E. coli*, has been demonstrated to improve the production of polyketides.[9,10] This was shown to be at least in part due to the maintenance of PKS proteins in the soluble fraction of the cell in the presence of the chaperones.[10e] However, it is not yet known whether this would be reflected in improved yields or integrity of the DEBS proteins isolated from such overproducing strains. Here we report a comparison of the expression and catalytic activity of affinity-purified, His-tagged DEBS 3 multienzyme obtained through coexpression in *E. coli* with specific GroES/GroEL chaperonins, relative to a control without such chaperonins.

For two reasons we elected to use the three *Streptomyces coelicolor* chaperonins GroEL1, GroEL2 and GroES. Firstly, we were interested in seeing whether they would also confer an advantage, because their sequence identity with their *E. coli* counterparts is only 40–60%, which might be expected to compromise their ability to interact productively with the protein synthesis and folding machinery of the *E. coli* host. Secondly, we had previously observed, when expressing other heterologous proteins in *E. coli* containing over-expressed GroEL/ES, that the chaperone expression levels often swamped those of the target protein, and that they could be tenacious contaminants of the purified protein (data not shown). In comparison, we expected the actinomycete chaperonins to be expressed at relatively modest levels. Here we report that the presence of the actinomycete chaperonins did indeed prove to have a beneficial effect on the integrity and catalytic activity of the purified recombinant DEBS 3.

Plasmid pLB1 was constructed to allow expression of a full-length, C-terminally His-tagged version of DEBS 3 from the T7 promoter, and also included the gene for the *S. erythraea* 4′-phosphopantetheinyl transferase SePptII, to ensure correct post-translational modification of DEBS 3.[11] A second plasmid (pL1SL2) encoding the *S. coelicolor* chaperonins GroEL1, GroEL2 and GroES was used to co-transform *E. coli* cells containing pLB1. DEBS 3 was expressed either in the presence (pL1SL2(+) strain) or in the absence (pL1SL2 (−) strain) of chaperones, and was purified in three steps (Figure 1 and Supporting Information) to yield homogenous protein as judged by SDS-PAGE (Figure 1). Size-exclusion chromatography (Figure 1) showed a single peak with an elution volume as expected for the predicted molecular weight of the DEBS 3 homodimer (664 kDa).[2,8] The identity and homogeneity of the DEBS 3 (especially the absence of contaminating chaperonin protein)

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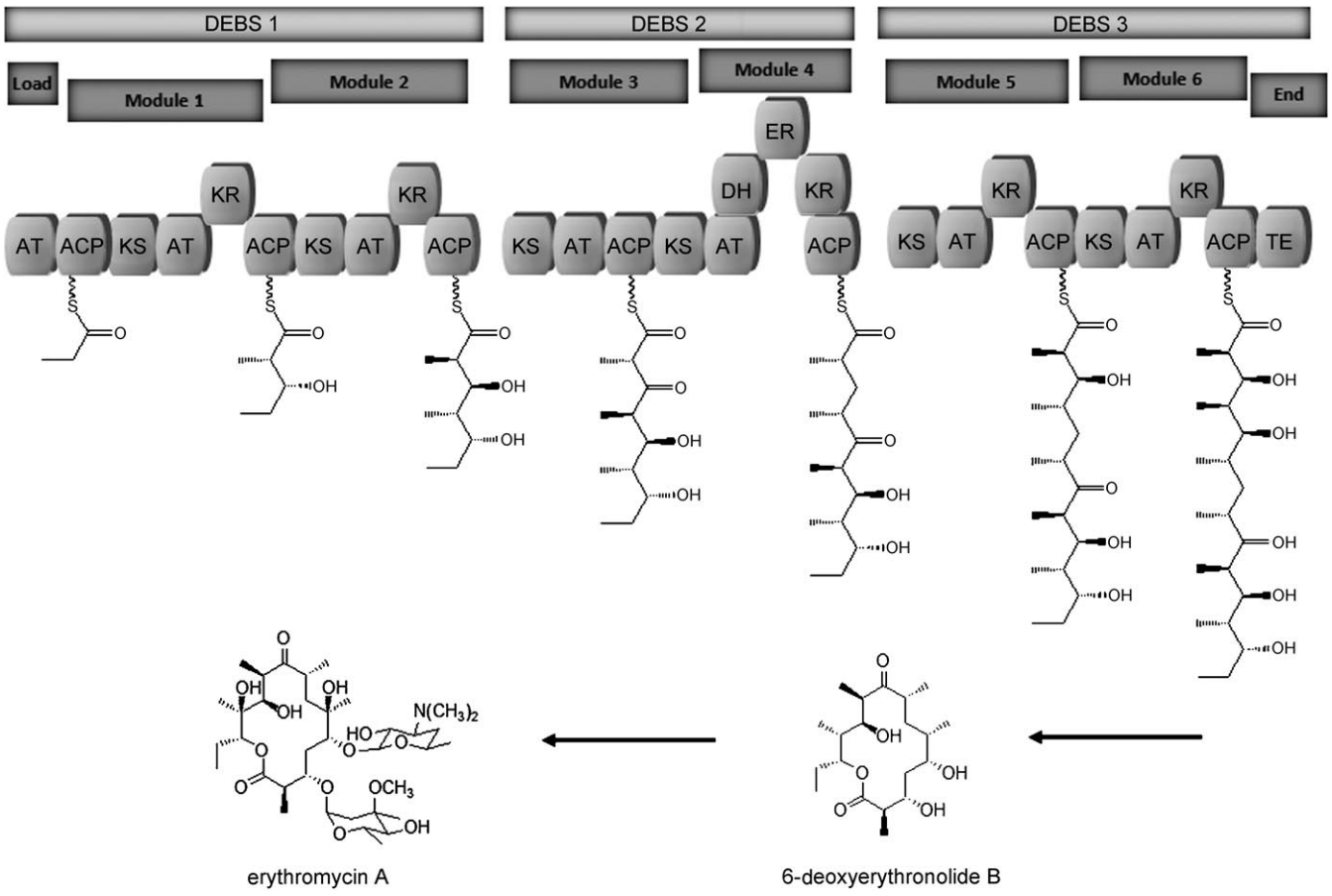

Scheme 1. Organisation of the erythromycin-producing polyketide synthase 6-deoxyerythronolide B synthase (DEBS). The multienzyme complex consists of six modules located in three different large homodimeric proteins (DEBS 1, DEBS 2 and DEBS 3). Abbreviations. AT: acyl transferase, ACP: acyl carrier protein, KS: ketosynthase, DH: dehydratase, ER: enoyl reductase, KR: ketoreductase, TE: thioesterase.

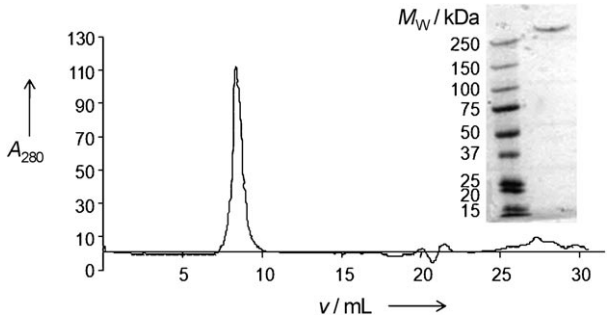

Figure 1. Analysis of purified DEBS 3. Gel filtration chromatogram of DEBS 3. Inset: an SDS-PAGE (7%) of the multienzyme obtained after gel filtration. The gel was stained with Coomassie blue. The molecular weights (M_W) of the protein standards used are indicated to the left of the gel.

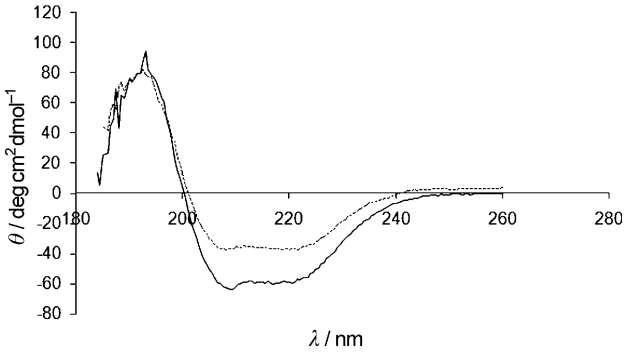

Figure 2. Comparative CD analysis of the secondary structure of purified preparations of DEBS 3. Continuous line: DEBS 3 expressed with chaperonins. Dotted line: DEBS 3 expressed without chaperonins.

was confirmed by N-terminal sequence analysis. Encouragingly, coexpression of DEBS 3 with the *S. coelicolor* chaperonins improved the overall yield of purified protein obtained from 1 L of culture approximately twofold: from 5.1 ± 0.1 mg to 8.4 ± 0.1 mg (average of three independent experiments). Further, comparison of the circular dichroism (CD) spectra of these DEBS 3 preparations (Figure 2) showed a significantly greater secondary structure content (as judged by molar ellipticity) in the DEBS 3 that had been expressed in the presence of chaperonins.

We measured the catalytic activity of the purified DEBS 3 in two ways, the first of which was by the ketoreductase-catalysed (KR-catalysed) reduction of *trans*-1-decalone (Figure 3B) as previously described.[12] The DEBS 3 purified after coexpression with GroEL1SL2 was consistently more active (k_{cat} 0.006 min^{-1}, compared to 0.0032 min^{-1} without chaperonins;

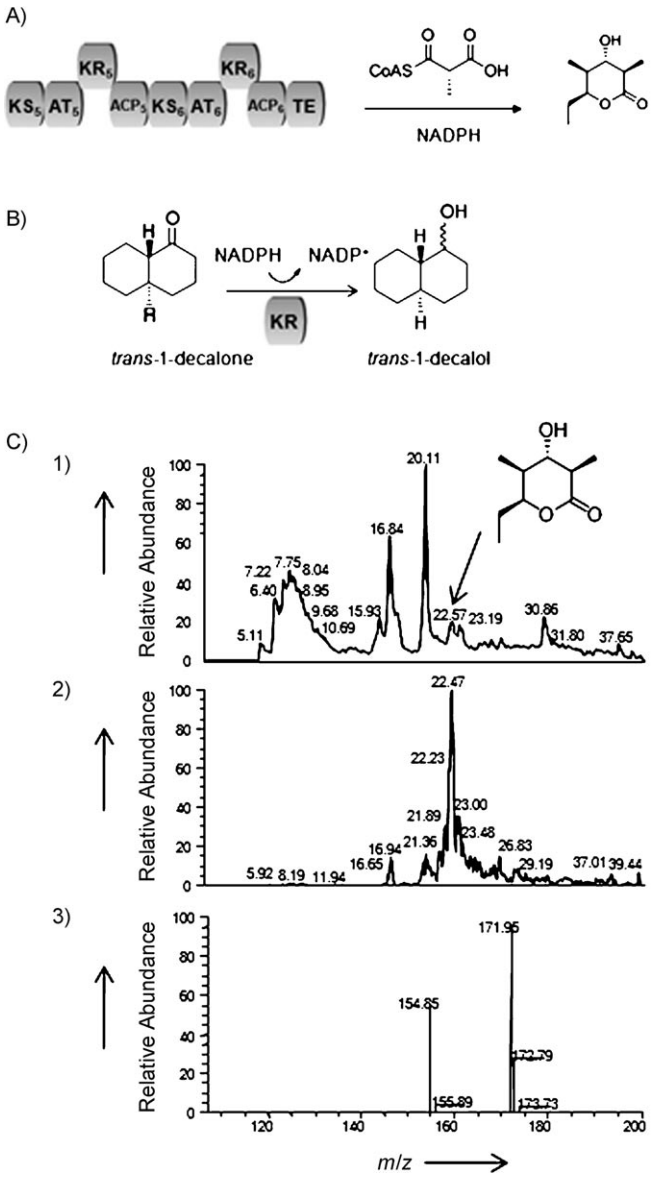

Figure 3. Assays for the activity of DEBS 3. A) The formation of triketide lactone by DEBS 3.[13] B) KR activity against a surrogate substrate.[12] C) Analysis of the triketide lactone by HPLC-MS: 1) Total ion chromatogram, 2) mass spectrum of the triketide lactone molecular ion at *m/z* 173, 3) MS/MS spectrum of the triketide lactone molecular ion at *m/z* 173.

average of three determinations). We also assayed overall triketide lactone biosynthesis, taking advantage of a previously described aberrant decarboxylation of methylmalonyl-CoA on DEBS 3 allowing priming of two rounds of polyketide chain extension (Figure 3A and C) with consumption of NADPH.[13] In the LC-MS analysis, the only polyketide detected was the expected triketide lactone, whether chaperonins were present or not. The total ion current in each case showed a number of additional peaks, but all of these were also present when control (no enzyme) incubations were analysed. The masses of these peaks were low, and did not match any plausible aberrant product of the polyketide synthase.

In this assay, too, the DEBS 3 produced in the presence of the chaperonins was consistently more active (0.17 ± 0.01 μg product per min per mg enzyme) than the control (0.1 ± 0.01 μg product per min per mg enzyme). This observed increase in the catalytic activity of a recombinant PKS multienzyme after coexpression of chaperone proteins suggests that some kinetic data previously obtained with recombinant PKS domains and proteins after expression in *E. coli*[14] may not have used fully active enzyme. More importantly, these results encourage the view that further optimisation of the quality of PKS multienzyme preparations intended for structural analysis, in which homogeneity is at a premium, is both possible and desirable. It remains to be shown whether the actinomycete chaperonins are superior in this regard to those from *E. coli*, although we have anecdotal evidence from work with other target proteins that this may be so (data not shown).

Despite intensive study, the mechanisms by which GroES/GroEL promote the correct folding of proteins in *E. coli* are not yet fully understood. DEBS 3 is too large to be encapsulated within the GroEL/GroES folding cavity, which is normally capped by GroES bound in *cis* fashion (that is, bound to the same hepatameric ring of GroEL subunits). The upper size limit of unfolded polypeptides that can be accommodated in the GroEL cavity is approximately 57 kDa, but assistance by GroEL/GroES in folding of larger proteins has been reported previously.[15] It is proposed that in such cases the polypeptide undergoes successive cycles of partial folding and release from GroEL, until its final conformation is reached. The key to this model is that GroES binds GroEL in *trans* fashion instead of in *cis*, removing the requirement for complete encapsulation. Portions of the structure of newly translated DEBS 3 could thus enter into the GroEL cavity in order to attain their native conformations, while the rest of the protein could protrude from the chaperone. Additional rounds of partial folding as proposed[15a] could promote a well-folded and fully active form of DEBS 3. *S. coelicolor*[16] and other actinomycetes,[17] as well as cyanobacteria, are unusual in that they encode two *groEL* genes with about 50–60% mutual sequence identity: *groEL1*, found adjacent to *groES*, and *groEL2*. It is intriguing that GroEL1, unlike GroEL2, has been shown not to be an essential gene, either for *Mycobacterium smegmatis*[18] or for *Clostridium glutamicum*.[19] In *M. smegmatis,* GroEL1 appears to be a dedicated chaperone involved in fatty acid biosynthesis required for biofilm formation.[18] Further work will be required to establish whether folding of other FAS and PKS enzymes in actinomycetes also benefits from such assistance.

In summary, the coexpression of GroEL1, GroES and GroEL2 chaperonins derived from actinomycetes has led to purified recombinant DEBS 3 forms displaying significant differences in secondary structure, as judged by CD measurements, in relation to controls without coexpressed chaperonins. These structural changes translated both into an almost twofold increase in the yield of the recombinant protein, and also into a 30% rise in the specific activity for triketide lactone biosynthesis. These results strongly suggest the utility of chaperones derived from polyketide-producing actinomycete bacteria in optimising

the recombinant production of PKS proteins in *E. coli* for detailed studies of structure and function.

Experimental Section

Construction of an expression plasmid for holo-DEBS 3: An expression construct that produces a C-terminally 6-His-tagged DEBS 3 in holo form by virtue of coexpression with a phosphopantetheinyl transferase (SePptII) that catalyses the essential post-translational activation of DEBS 3 was created.[11] SePptII (along with the T7 promoter, lac operator and T7 terminator sequences) was excised from plasmid pKJW191R with SphI and ligated into pYADE (DEBS 3 in pET29(+) obtained from Novagen, Madison, WI) to yield plasmid pLB1, in which the genes for SePptII and DEBS 3 are in opposite orientations.

Protein methods: Protein concentration was determined by the Bradford dye binding assay (Sigma–Aldrich) with bovine serum albumin as a standard. Protein samples were analysed by SDS-PAGE or Native-PAGE by using Phast Gels gradient 4–15% (GE Healthcare) and stained with Coomassie Phast Gel Blue R (GE Healthcare).

Expression and purification of DEBS 3: *E. coli* BL21 Codon Plus RP cells were transformed with plasmid pLB1. An overnight pre-culture from a single colony (1:1000, *v/v*) of *E. coli* pLB1 Codon Plus RP cells was inoculated into Luria broth (LB) containing kanamycin (50 $\mu g\,mL^{-1}$) and chloramphenicol (34 $\mu g\,mL^{-1}$). Cultures were grown at 30 °C to an A_{600} value of 0.7–0.9. Isopropyl β-D-1-thiogalactoside (IPTG, 0.1 mM) was added, and the cells were incubated at 22 °C for an additional 16 h to induce the expression of the recombinant DEBS 3. The cells were harvested by centrifugation (7000 *g*) for 10 min at 4 °C and resuspended in lysis buffer [sodium phosphate (100 mM), NaCl (300 mM), imidazole (1 mM), Triton X-100 (0.05%), glycerol (10%), pH 7.0]. Cell lysis was carried out by sonication in the same buffer supplemented with RNAse I, DNAse A, a dissolved tablet of EDTA-free proteinase inhibitors (Roche) and lysozyme (1 $mg\,mL^{-1}$). Cell debris and unbroken cells were removed by centrifugation (34000 *g* for 60 min at 4 °C). The supernatant was loaded onto a HiTrap Ni Chelating HP™ column (5 mL, GE Healthcare, Piscataway, NJ, USA) equilibrated with lysis buffer. Proteins were eluted with lysis buffer containing imidazole (250 mM). Fractions containing DEBS 3 were pooled and applied to a HiTrapQ HP (5 mL, GE Healthcare) previously equilibrated in buffer A [sodium phosphate buffer (50 mM) containing dithiothreitol (DTT; 1 mM), EDTA (1 mM), glycerol (10%) and Triton X-100 (0.05%), pH 7.0]. Proteins were eluted with a linear gradient from buffer A to buffer A containing NaCl (1 M). For further analysis by gel filtration the fractions containing DEBS 3 were concentrated to 200 μL (Vivascience, 100 M_W cut-off) and applied to a Superdex 200 10/300 GL column (GE Healthcare) pre-equilibrated in buffer B [sodium phosphate (50 mM), pH 7.0, containing NaCl (300 mM)].

Coexpression of DEBS 3 and chaperonins: A plasmid (pL1SL2) for the coexpression of *S. coelicolor* chaperonins (GroEL1, GroEL2 and GroES) was constructed. GroEL1, GroEL2 and GroES genes were placed under the same promoter by cloning them sequentially as XbaI-SpeI cassettes into pET-29a(+). In the resulting plasmid, L1SL2/pET29, each gene is preceded by a Shine–Dalgarno sequence and a ribosome binding site. The resulting cassette was transferred in a single cloning step to the NheI restriction site of pETcoco-2 expression vector, to generate plasmid pL1SL2. *E. coli* BL21 Codon Plus RP cells containing pL1SL2 were co-transformed with pLB1. Cell cultures were grown in LB containing kanamycin (50 $\mu g\,mL^{-1}$), chloramphenicol (34 $\mu g\,mL^{-1}$), ampicillin (100 $\mu g\,mL^{-1}$) and glucose (0.2%, *w/v*) to maintain a low copy number of the pL1SL2 plasmid. Other conditions for the expression and purification of DEBS 3 were as described above for the cells not supplemented with chaperonin genes.

DEBS 3 activity: Assays for the determination of DEBS 3 activity were carried out by monitoring the formation of triketolactone by LC-MS. The reaction mixture contained DEBS 3 (30 $\mu g\,mL^{-1}$), (*RS*)-methylmalonyl CoA (0.7 mM), NADPH (1 mM) and DTT (2 mM) in sodium phosphate buffer (400 mM, pH 7.5). Incubations were performed at 30 °C, for 6 h.

Ketoreductase activity: The reductase activity was determined spectrophotometrically (Cary 50 spectrophotometer, Varian Sunnyvale, CA, USA) by monitoring the decrease in absorbance at 340 nm due to the oxidation of NADPH. The reaction mixture contained *trans*-1-decalone (30 $\mu g\,mL^{-1}$, 50 mM), NADPH (1.2 mM) and NaCl (150 mM) in sodium phosphate buffer (400 mM, pH 7.5). Incubations were performed at 25 °C for 20 min.

Circular dichroism experiments: DEBS 3 was diluted in phosphate buffer (0.05 M, pH 7.5) to a final concentration of 0.1 $mg\,mL^{-1}$. Far-UV CD spectra (from 260 to 184 nm) were recorded at 4 °C on an AVIV 215 CD Spectrometer (AVIV Instruments, Inc., Lakewood, NJ) in 0.1 cm quartz cells (Hellma, Essex, UK). Three scanning acquisitions in 0.5 nm steps were accumulated and averaged, yielding the final spectrum after blank subtraction. CD signals are expressed as mean residue molar ellipticity.

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