Localization of the ActIII actinorhodin polyketide ketoreductase to the cell wall

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Abstract
Structurally diverse polyketides provide a rich reservoir of bioactive molecules. Actinorhodin, a model aromatic polyketide, is synthesized by minimal type II polyketide synthase and tailoring enzymes. The ActIII actinorhodin ketoreductase is a key tailoring enzyme in actinorhodin biosynthesis. With purified antibodies against actinorhodin polyketide synthase α subunit (KSα) and ketoreductase, we conducted systematic localization experiments of the two proteins in Streptomyces coelicolor subproteomes. The results support the membrane location of KSα and cell-wall location of ketoreductase. Considering previous evidence that some other tailoring enzymes of actinorhodin biosynthesis may be located outside the cytoplasm, a picture is emerging of an extensive role for extracellular biochemistry in the synthesis of type II polyketide antibiotic.

Introduction
Polyketides represent a large group of structurally diverse natural products with useful biological activities. A comprehensive understanding of their biosynthesis will help in the manipulation of their structures. In streptomycetes, aromatic polyketides are synthesized by a set of individual enzymes, collectively known as minimal type II polyketide synthase (PKS) (McDaniel et al., 1994), consisting of ketosynthase/chain length factor (KS/CLF), acyl carrier protein (ACP) and fatty acid synthase malonyl CoA : ACP transacylase (MAT). MAT is shared between polyketide and fatty acid synthesis (Carreras & Khosla, 1998). The KS/CLF component of a minimal PKS catalyzes repeated condensation of small carboxylic acids supplied by ACP until a full-length polyketide is synthesized; both KS/CLF and MAT are predicted to interact with ACP, which shuttles the pathway precursors and intermediates between minimal PKS components. In a complete polyketide biosynthetic pathway, additional tailoring enzymes, such as ketoreductase(s), cyclases and oxygenases, are often functionally associated with minimal PKS. And these tailoring enzymes are critical in generating structural diversity (Rix et al., 2002). Although it is known that KS/CLF forms a membrane-associated heterodimer in its native environment (Carreras & Khosla, 1998; Keating-Clay et al., 2004), the interactions of KS/CLF with other minimal PKS components and between minimal PKS and tailoring enzymes remain to be elucidated.

The aromatic polyketide actinorhodin, a blue isochromenequinone antibiotic produced by Streptomyces coelicolor (Carreras & Khosla, 1998), has been investigated extensively and has greatly helped in our understanding of the biosynthesis of aromatic polyketides. The complete actinorhodin biosynthetic gene cluster (act) has been cloned and the functions of many gene products have been characterized (Rudd & Hopwood, 1979; Malpartida & Hopwood, 1986; Hallam et al., 1988; Bartel et al., 1990; Fernandez-Moreno et al., 1994; Bentley et al., 2002). In the act cluster, actI-ORF1 and ORF2 encode ketosynthase α subunit (KSα) and β subunit KSβ (also known as chain length factor), respectively; actI-ORF3 encodes ACP and actIII encodes a ketoreductase.

Previously, the cellular locations of PKS-tailoring enzymes have not been investigated in detail, the prevailing assumption being that PKS-tailoring enzymes either reside in the cytoplasm or are bound to the membrane, and that biosynthetic reactions leading to polyketides occur...
intracellularly. Therefore, discoveries of PKS-tailoring enzymes, such as ActI-ORF3 dehydrase (Hesketh et al., 2002; Hesketh & Chater, 2003) as well as two other oxygenases (Widdick et al., 2006), in the cell-wall fraction of S. coelicolor were considered to be anomalies. In our efforts to detect interactions between minimal PKS and tailoring enzymes of actinorhodin biosynthesis, we tried to track minimal PKS components and cognate tailoring enzymes with their corresponding antibodies. Surprisingly, we did not detect the actinorhodin ketoreductase (ActIII, SCO5086) in the cytoplasmic protein fraction. After careful analysis of proteins in subproteomes, we were able to demonstrate that ActIII is a cell-wall-associated protein. This finding, together with earlier reports of the localization of PKS tailoring enzymes on the cell wall (Hesketh et al., 2002; Hesketh & Chater, 2003; Widdick et al., 2006), have opened a new vista on the future study of PKS functional organization and polyketide biosynthesis.

Materials and methods

Bacterial strains and plasmids

Escherichia coli BL21 was used as the host for protein expression and E. coli DH5α was used as the host for gene cloning. Streptomyces coelicolor A3(2) and its mutant YU105 (actinorhodin gene cluster deleted) were used as sources for cellular proteins. pGEX-6P1 (Amersham Biosciences) was used to express ketoreductase and KSα in E. coli.

Construction of recombinant expression vectors

Genomic DNA of S. coelicolor A3(2) was used as template. For cloning of actIII (ketoreductase), the primer pair 5’-CCGCTCTAGTGCTGAACGAGACCCTGCA-3’ (XhoI underlined) and 5’-CGGATTCATGCCACGCAGAC TCCGAAG-3’ (EcoRI underlined) was used; and for cloning of actI-ORF1 (KSα), the primer pair 5’-GGAATTCCGTGCC GCTGGAAGCCGGCGCG-3’ (EcoRI underlined) and 5’-C CGTCGAGCGACGCGACGACTCCGGTG-3’ (XhoI underlined) was used. The amplified DNA fragments were purified, digested with corresponding restriction enzymes and ligated into pGEX-6P1 digested with the same restriction enzymes. Ligation products were transformed into E. coli DH5α. Successful cloning of these genes was confirmed by DNA sequencing (Sangon Ltd, Shanghai). The recombinant plasmids were extracted from E. coli DH5α and transformed into E. coli BL21 (DE3) for protein expression following standard protocols (Sambrook & Russel, 2000).

Protein overexpression and purification

Escherichia coli cells harboring recombinant plasmids were induced for protein expression according to standard protocols. Cells expressing the target protein were collected by centrifugation at 6000 g. To 10 g of cell paste, 50 mL lysis buffer [25 mM Tris, pH 8.0, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF)] was added and the resuspended cells were disrupted by passing through a French Press (FA-076-E1, Thermo Spectronic) three times at 68.948 kPa, the lysed cells were centrifuged and the supernatant was saved. Then, 1 mL slurry of glutathione-Sepharose 4B beads (Amersham Bioscience; equilibrated in lysis buffer) was added to the supernatant. After incubation with gentle agitation at 4 °C for 30 min, the above suspension mixture was centrifuged at 500 g for 5 min to sediment the Sepharose matrix and the supernatant was discarded. After washing the glutathione-Sepharose 4B pellet with 10 bed volumes of wash buffer (25 mM Tris, pH 8.0, 150 mM NaCl), the suspension was centrifuged at 500 g for 5 min to sediment the matrix again. The wash was repeated three times. Then, 1 mL elution buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0) was added to the matrix pellet, and the suspension was mixed gently and incubated at 4 °C for 10 min to elute the bound proteins from the matrix. The supernatant was then transferred into a fresh centrifuge tube. The elution was repeated and eluted fractions were combined for further use. Subsequently, the resulting eluted proteins were concentrated in an ultrafiltration tube (Millipore) at 4000 g for 2 h. The protein concentration was estimated by the Bradford method (Wang & Fan, 2000).

Polyclonal antibody purification

The above-purified proteins were used to inject rabbits (New Zealand white) and to raise antiserum at the Animal Experiment Center, Institute of Genetics and Development, Chinese Academy of Sciences. To purify the antibodies, ammonium sulfate was added to the antiserum to a final concentration of 40%, and the solution was centrifuged at 10000 g for 30 min. The resulting sediment was collected and resuspended in phosphate-buffered saline (PBS) and then dialyzed in 25 mM 2-(N-morpholino)-ethan sulfonic acid (MES buffer, pH 6.0). The solution was then passed through a Cation-Exchanger S (Whatman International Ltd) column equilibrated with 25 mM MES buffer. After the column was washed with three bed volumes of 25 mM MES (pH 6.0), proteins bound to the column were eluted with 25 mM MES plus 500 mM NaCl.

Preparation of cytoplasmic and membrane proteins

Spores of S. coelicolor A3(2) and YU105 were collected, pregerminated and inoculated as described previously (Kieser et al., 2000). Briefly, pregerminated spores were inoculated into 1 L minimal medium supplemented with
0.2% Casamino acids in a 5-L baffled flask and cultivated with shaking at 250 r.p.m. and 30 °C for 72 h until actinorhodin began to accumulate. Mycelia were harvested by centrifugation for 10 min at 8000 g. A 10-g cell pellet was resuspended in 50 mL cell lysis buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 50 mM NaF, 0.1 mM NaVO₃, 1 mM PMSF, 2.5 μg mL⁻¹ leupeptin, 2 μg mL⁻¹ aprotinin and 1 μM pepstatin A). The resuspended cells were disrupted by passing through the French Press three times at 15 000 psi. Cell debris was removed by centrifugation at 10 000 g for 30 min. The resulting supernatant was subjected to ultracentrifugation at 100 000 g for 2 h. Following ultracentrifugation, the supernatant contains cytoplasmic proteins while the sediment of the cell membrane fraction was collected and resuspended in cell lysis buffer. All operations were conducted at 4 °C. Samples of the membrane and the cytoplasmic proteins were separated via 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins on the gel were transferred to a polyvinylidene fluoride (PVDF, Millipore) membrane for Western blotting analyses according to standard procedures.

Proteins in the membrane fraction were dissolved using detergents at 1–2% in cell lysis buffer; Triton X-100, 3-[3-cholamidopropyl]-dimethylammonio]-1-propane-sulfonate (CHAPS), octyl glucoside (OG), sulfobetaine (SB3-10) and dodecyl maltoside (DDM) were used. The dissolved proteins were collected by ultracentrifugation and separated on 12% SDS-PAGE, and proteins on the gel were transferred to PVDF membrane for Western blotting.

Preparation of cell wall and associated proteins

Cell-wall and cell-wall-associated proteins were prepared according to the method of Walter et al. (1999). Briefly, the cells were washed in PBS, resuspended in PBS and then mixed with an equal volume of 8% SDS with continuous stirring at 100 °C. The resulting mixture was boiled for 10 min and then stirred overnight at room temperature. After incubation for an additional 10 min at 100 °C, the cell wall was collected by centrifugation at 20 000 g for 30 min and washed three times with PBS containing 10% 2-propanol and three times with PBS in order to remove SDS and contaminating soluble molecules.

Visualizing cell-wall-associated proteins

Cell-wall materials were resuspended in buffer (25 mM Tris-HCl, pH 8.0, 20 mM EDTA) and centrifuged again. The resulting supernatant fraction was saved for SDS-PAGE examination. Otherwise, the cell wall resuspension solution was either sonicated (15-s pulse, 250 W, 20 times) to release proteins at random, or treated with 5 mg mL⁻¹ lysozyme (Sigma) or 2 mg mL⁻¹ lysostaphin (Shanghai Hi-Tech United Biotechnological R&D Co. Ltd) at 37 °C for 16 h. After centrifugation at 15 000 g for 15 min, an aliquot of each supernatant (containing proteins released from the cell wall) was analyzed via SDS-PAGE and examined using Western blotting.

Preparation of lysozyme-released proteins from intact cells

Washed cells were resuspended in isosmotic solution (25 mM Tris–HCl, pH 8.0, 20 mM EDTA and 10.3% sucrose) with or without 5 mg mL⁻¹ lysozyme and incubated at 37 °C for 5 h. Supernatant obtained after centrifugation at 2000 g for 10 min was concentrated using ultrafiltration. The concentrated supernatants were loaded on SDS-PAGE gels and examined using Western blotting.

Results

Overexpression and purification of actinorhodin biosynthetic enzymes KSα and ketoreductase

KSα and KSβ have often been prepared as a pair of subunits from S. coelicolor CH99 (Carreras & Khosla, 1998). We expressed KSα and ketoreductase with an N-terminal GST tag in E. coli to enhance their solubility. Both GST–KSα (about 76 kDa, Fig. 1a) and GST–ketoreductase (about 55 kDa, Fig. 1b) were successfully expressed as soluble proteins. The overexpressed proteins were then purified using GST chromatography. For purification of GST–KSα, 0.3% sodium lauroyl sarcosine (SLS) was added to the binding buffer. This modification greatly reduced nonspecific binding (Fig. 1a, lane P). For purification of

![Fig. 1.](image-url)
GST–ketoreductase, one step of GST chromatography obtained relatively homogeneous protein (Fig. 1b, lane P).

**Polyclonal antibody purification**

Individual rabbits were immunized with KSα or ketoreductase, polyclonal antisera against KSα or ketoreductase were obtained and the corresponding antibodies were purified from antiserum by a combination of ammonium sulfate fractionation and cationic ion exchange chromatography. A saturation concentration of 40% ammonium sulfate effectively precipitated the antibodies, while a further cationic ion exchange step purified the antibody to near homogeneity (data not shown).

**KSα and ketoreductase were detected in the total resuspended membrane protein fraction**

Total membrane fractions of *S. coelicolor* prepared by ultracentrifugation were resuspended, resolved on SDS-PAGE and examined using Western blotting. As shown in Fig. 2a and b (lanes 1 and 2), the two purified antibodies recognized KSα and ketoreductase specifically in several protein fractions, as indicated by the hybridizing signals from *S. coelicolor* A3(2) but not from YU105 (act gene cluster deleted). The estimated sizes of the signals also agree with the theoretical molecular weights of KSα (49 kDa) and ketoreductase (27 kDa); other weak bands common to YU105 and *S. coelicolor* samples can be attributed to nonspecific hybridizations. Ketoreductase was detected only in the total membrane fraction, while KSα was mainly detected in the total membrane fraction but also weakly in the cytoplasmic fraction (data not shown). The absence of ketoreductase in the cytoplasmic fraction implies alternative locations of ketoreductase (Fig. 2a and b, lanes 3 and 4).

**KSα but not ketoreductase could be dissolved from ultracentrifugation-sedimented membrane by detergents**

Proteins in the postultracentrifugation-sedimented total membrane fraction of *S. coelicolor* were dissolved using various detergents: Triton X-100, CHAPS, OG, SB3-10 and DDM. The samples were again ultracentrifuged and the supernatants from this step were examined using Western blotting. KSα could be dissolved out by 2% DDM and detected in the detergent supernatant fraction (Fig. 2a, lane 5), but ketoreductase could not be dissolved from the membrane with any of the above detergents. These results
suggest that the ketoreductase signals detected in the ultracentrifugation-sedimented total membrane fractions are due to proteins, which could be dissolved by SDS and boiling treatment but not extracted into detergents.

**Ketoreductase but not KSα was detected in the cell-wall fraction**

In the procedure to prepare cell wall and cell-wall-associated proteins, cells were treated in 4% SDS at 100 °C and the supernatant samples after such treatment were examined using SDS-PAGE and Western blotting (data not shown). Ketoreductase was detected in the above supernatant samples, but these signals were much weaker than the signals that remained in the cell-wall fraction. This is consistent with previous results indicating that ketoreductase could be released from total insoluble membrane fraction (suspected to contain cell wall contamination) by SDS and boiling treatment before sample loading. Subsequently, the proteins associated with insoluble cell wall were released either randomly from the cell wall by sonication or nonrandomly through enzyme treatments (lysozyme or lysostaphin). The released proteins were examined using SDS-PAGE (Fig. 3a) and Western blotting (Fig. 3b). Ketoreductase was detected in the randomly released proteins and also in lysozyme-released proteins (Fig. 3b, lanes 1 and 2) but not in cell-wall resuspension supernatant (Fig. 3b, lane 3) or lysostaphin-released proteins (Fig. 3b, lane 4). KSα was not detected in any of the cell-wall samples (data not shown).

**Ketoreductase could be released from intact cells by lysozyme treatment**

Intact cells of *S. coelicolor* were resuspended in an isosmotic solution and incubated with lysozyme, and the supernatants from cell resuspensions were concentrated and examined by SDS-PAGE (Fig. 4a) and by Western blotting (Fig. 4b). Ketoreductase was detected in the supernatant prepared from the cell resuspension with lysozyme (Fig. 4b, lane 2) but not in the supernatant from duplicate cell resuspension without lysozyme (Fig. 4b, lane 3), although the proteins in the two supernatants looked almost identical (Fig. 4a, lanes 2 and 3).

**Discussion**

Our analyses reconfirmed the membrane location of the act KSα subunit (Gramajo *et al.*, 1991; Keatinge-Clay *et al.*, 2004). However, as we did not identify apparent membrane spanning segments in KSα after careful examination of its sequence and structure (Keatinge-Clay *et al.*, 2004), we deduce that KSα is attached to the membrane by unknown interactions. Previous proteomic studies (Hesketh *et al.*, 2002; Hesketh & Chater, 2003) of *S. coelicolor* identified ketoreductase in cell-free supernatant (mainly contains cytoplasmic proteins) following cell disruption by sonication, suggesting a cytoplasmic or loose membrane association of ketoreductase; however, our results clearly demonstrate the absence of ketoreductase in the cytoplasmic fraction. We believe these contradictory results are due to differences in sample preparation: we used a less disruptive method to break the cell (French Press) and to obtain cytoplasmic protein samples, whereas previous studies used sonication to disrupt cells, which we have revealed could release ketoreductase from the cell wall.

Although ketoreductase was initially detected in the total membrane fraction, we later showed that these ketoreductase signals were probably due to insoluble proteins (cell-wall fragments) present in the total membrane fraction; ketoreductase in the ‘membrane’ could not be dissolved into solution by any of the detergents that we utilized, not even with DDM and others that were able to dissolve KSα from the same sample. Therefore, we suspect the presence of ketoreductase signals in the total resuspended membrane was due to contamination of cell-wall fragments that did not sediment under 10 000 g centrifugation but did sediment at higher centrifugation force, and these contaminating proteins could be detected by the very sensitive Western blotting method. To test the last and only plausible location of ketoreductase, namely the cell wall, we prepared cell wall employing a very stringent SDS-boil method, sufficient to denature proteins and to dissolve membranes. This treatment should release those proteins that are weakly associated with the cell wall. As anticipated, these cell-wall samples gave strong ketoreductase signals but not of KSα (a membrane-associated protein); however, some ketoreductases were extracted into 4% SDS solution, suggesting that not all ketoreductases were bound to the cell wall.

It is important to show that the ketoreductase was released from the cell wall by lysozyme (cleaves the β-1,4 glycoside bond between N-acetylglucosamine and N-acetylmuramic acid of peptidoglycan) but not by lysostaphin (cleaves the pentaglycine cross bridges in some gram-positive bacteria), thus supporting the cell-wall association of ketoreductase. The results with lysostaphin should be interpreted with caution: lysostaphin is a glycyglycine endopeptidase, and is not expected to cleave the peptidoglycan of streptomycetes, which lacks glycine-rich bridges. However, several proteins (but not ketoreductase) appeared to be released by lysostaphin from the cell wall, but whether these proteins are indeed released by lysostaphin needs further investigation. Finally, we detected ketoreductase in the supernatant of intact cell resuspension incubated with lysozyme, but not in the supernatant of duplicated resuspension incubated without lysozyme, a result we believe provides convincing evidence that ketoreductase is a cell-wall-associated protein.
Because ACP is the protein that relays the full-length polyketide intermediate from KSβ/KSβ to ketoreductase, it is not surprising for ketoreductase to have an ACP interaction region (Hadfield et al., 2004); after reducing the ACP-delivered intermediate, ketoreductase is expected to pass the reduced intermediate to an act cyclase; therefore, it is likely that ACP, cyclases and other downstream tailoring enzymes are also localized extracellularly on the cell wall. In fact, we have unpublished proteomic results in support of these predictions. To allow these PKS tailoring reactions to occur extracellularly, cofactors such as NADP(H) for ketoreductase are needed; although not firmly established, it is very likely that bacteria maintain an extracellular pool of reducing power (Ju et al., 2005; Wos & Pollard, 2006).

Unlike ActVI-ORF3 dehydrase, which was predicted to contain an N-terminal signal peptide (Hesketh & Chater, 2003), ketoreductase does not contain an obvious N-terminal signal sequence when analyzed using SignalP 3.0 (Bendtsen et al., 2004) and PSORT v2.0 (Gardy et al., 2005); nor does it contain a typical C-terminal cell-wall-anchoring motif, such as the LPXTG or LAXTG motifs (Pallen et al., 2001). Also, because ketoreductase, unlike ActVI-ORF3, was detected at an unprocessed molecular weight, we suspect that it is secreted from the cell and attached to the cell wall by as yet unknown mechanisms, such as sortases that might recognize different sequences in a target protein (Pallen et al., 2001). Finally, the many ketoreductase hybridization signals appearing at higher molecular weights in SDS-PAGE (Fig. 4b) may reflect the heterogeneous cell-wall fragments attached to ketoreductase.

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Authors’ contribution
X.-P.W. and Z.-J.W. contributed equally to this work.

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