Molecular and functional characterization of the kstD2 gene of Rhodococcus erythropolis SQ1 encoding a second 3-ketosteroid Δ1-dehydrogenase isoenzyme

Robert van der Geize, Gerda I. Hessels and Lubbert Dijkhuizen

Previously, Rhodococcus erythropolis SQ1 kstD, encoding ketosteroid Δ1-dehydrogenase (KSTD1) was characterized. Surprisingly, a kstD gene deletion mutant (strain RG1) grew normally on steroids. UV mutagenesis of strain RG1 allowed isolation of strains (e.g. strain RG1-UV29) unable to perform the Δ1-dehydrogenation of 4-androstene-3,17-dione (AD) and 9α-hydroxy-4-androstene-3,17-dione (9OHAD). Functional complementation of strain RG1-UV29 with total genomic DNA of strain RG1 resulted in identification of a 1698 nt ORF (kstD2) showing clear similarity (35% identity at amino acid sequence level) with KSTD1. Expression of kstD2 in Escherichia coli resulted in high KSTD2 activity levels. Single gene deletion mutants of either kstD (strain RG1) or kstD2 (strain RG7) appeared unaffected in growth on the steroid substrates AD, 1,4-androstadiene-3,17-dione and 9OHAD. Strain RG7, but not strain RG1, showed temporary accumulation of 9OHAD during AD conversion. A kstD kstD2 double deletion mutant (strain RG8) was constructed. Strain RG8 was unable to grow on steroid substrates, had undetectable steroid Δ1-dehydrogenation activity and efficiently converted AD into 9OHAD. Strain SQ1 thus employs two KSTD isoenzymes in steroid catabolism. Analysis of two null mutants in KSTD2 showed that the semi-conserved Ser325 and the highly conserved Thr503 play a role in KSTD enzyme activity.

Keywords: 3-ketosteroid dehydrogenase, isoenzymes, unmarked gene deletion, steroid

INTRODUCTION

3-Ketosteroid Δ1-dehydrogenase (KSTD) [4-ene-3-oxosteroid:(acceptor)-1-ene-oxidoreductase; EC 1.3.99.4] activity plays an important role in the microbial degradation of the polycyclic ring structure of steroids. KSTD and 3-ketosteroid 9α-hydroxylase (KSH) together initiate opening of the steroid skeleton B-ring (Fig. 1). Limited information is available about the bacterial enzymes involved in steroid degradation. The industrial potential of KSTD enzymes in steroid biocatalysis has stimulated research on KSTD proteins and their corresponding genes from different genera and species (Choi et al., 1995; Drobnic et al., 1993; Fuji et al., 1999; Itagaki et al., 1990; Kaufmann et al., 1992; Molnár et al., 1995; Mouri et al., 1998; Plesiat et al., 1991; Sih & Bennet, 1962; Wagner et al., 1992; Wovcha et al., 1979). Functional studies of the steroid Δ1-dehydrogenation system in the micro-organism itself and analysis of the metabolic effects of their inactivation, however, are limited (van der Geize et al., 2000, 2001), probably due to the paucity of genetic tools for actinomycetes able to perform the KSTD reaction (e.g. Rhodococcus, Arthrobacter, Nocardia, Mycobacterium). Inactivation of kstD, encoding KSTD1 activity in Rhodococcus erythropolis SQ1, was shown to be ineffective to obtain mutants blocked in 3-ketosteroid Δ1-dehydrogenation (van der Geize et al., 2000, 2001): growth on 4-androstene-3,17-dione (AD), 1,4-androstadiene-3,17-dione (ADD) or 9α-hydroxy-4-androstene-3,17-dione (9OHAD).

Abbreviations: AD, 4-androstene-3,17-dione; ADD, 1,4-androstadiene-3,17-dione; DCP, 2,6-dichlorophenolindophenol; KSH, 3-ketosteroid 9α-hydroxylase; KSTD, 3-ketosteroid Δ1-dehydrogenase; 9OHAD, 9α-hydroxy-4-androstene-3,17-dione; PMS, phenazine methosulphate.

The GenBank accession number for the sequence reported in this paper is AY078169
stene-3,17-dione (9OHAD) was not affected. Biochemical data indicated the presence of a KSTD isoenzyme, named KSTD2. KSTD2 has been inactivated in R. erythropolis RG1-UV29, obtained via UV mutagenesis of a kstD mutant R. erythropolis RG1 (van der Geize et al., 2001). Here we report the molecular characterization of kstD2 in R. erythropolis SQ1, including the construction of kstD2 gene deletion mutants from parent strain SQ1 and kstD mutant strain RG1. Two apparently distinct KSTD enzyme activities have been reported in Mycobacterium fortuitum (Wochva et al., 1979), but nucleotide sequences encoding these two activities are not available. The deduced amino acid sequence of an ORF (ORF3) located downstream of ksdD (encoding KSTD1) in Arthrobacter simplex (Molnár et al., 1995) also displays clear similarities with several KSTD amino acid sequences (Dziadek et al., 1998). Expression of ORF3 in Escherichia coli or Streptomyces lividans, however, did not result in steroid biotransformation activity.

The molecular characterization of kstD2 in R. erythropolis SQ1 reported here provides the first unequivocal evidence for the functional involvement of two KSTD isoenzymes in microbial steroid catabolism. The results offer clear insights into the complexity of microbial steroid Δ1'-dehydrogenation and allow rational construction of industrial strains for steroid production.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** R. erythropolis strain SQ1 and derived mutant strains RG1, RG1-UV29, RG1-UV45, RG1-UV50, RG1-UV51 have been described previously (Quan & Dabbs, 1993; van der Geize et al., 2001). E. coli DH5α and pBlueScript (II) KS were used for general cloning steps. E. coli S17-1 (Simon et al., 1983) was used for the conjugal mobilization of pK18mobsacB (Schafer et al., 1994) derivatives to Rhodococcus recipient strains. An E. coli–Rhodococcus shuttle vector, pRESQ (van der Geize et al., 2002), was used in all complementation experiments. Rhodococcus strains were cultivated at 30 °C and 200 r.p.m. on a rotary shaker (G25; New Brunswick Scientific) in liquid medium (LBP) containing 1% (w/v) Bacto-Peptone (Difco), 0–5% (w/v) yeast extract (BBL) and 1% (w/v) NaCl. Mineral medium consisted of (g l−1): K2HPO4, 465; NaH2PO4·H2O, 1.5; NH4Cl, 3; MgSO4·7H2O, 1, supplemented with Vishniac trace elements (pH 7.2). Steroids (50 mg ml−1) were dissolved in DMSO and added to autoclaved medium. For growth on solid medium, 1.5% (w/v) Bacto-agar (Difco) was added. E. coli strains were grown in Luria–Bertani (LB) broth at 37 °C. BBL agar (1.5%, w/v) was added in the case of growth on solid medium. Sucrose sensitivity of Rhodococcus strains was tested on LBP agar supplemented with 10% (w/v) sucrose.

**General molecular techniques.** DNA modifying enzymes were purchased from Roche, New England Biolabs or Amersham Pharmacia Biotech and were used as described by the manufacturers. Isolation of DNA restriction fragments from agarose gels was done by using the QiaGen gel extraction kit.
according to the manufacturer’s protocol. All DNA manipulations were done according to standard protocols. Rhodococcus plasmid DNA was isolated using the Qiagen spin-prep kit with a slight modification: Rhodococcus cells were grown for 2–3 h in the presence of ampicillin (600 µg ml⁻¹) prior to lysozyme (2 mg ml⁻¹) treatment. PCR was performed under standard conditions using Taq polymerase (Roche) unless stated otherwise: 5 cycles of 1 min at 95 °C, 1.5 min at 60 °C and 1.5 min at 72 °C, followed by 25 cycles of 1 min at 95 °C, 1.5 min at 55 °C and 1.5 min at 72 °C.

Functional complementation experiments. Electrocompeptent Rhodococcus cells of mutant strain RG1-UV29 were obtained as described previously (van der Geize et al., 2000). A gene library (mean insert size 6 kb) of R. erythropolis strain RG1 was constructed by cloning Sau3A-digested chromosomal DNA into pBlueScript (II) KS (van der Geize et al., 2002). Colonies of strain RG1-UV29 obtained after introducing this gene library by electrotransformation were replica-plated onto mineral agar medium were cultivated in LBP medium for isolation of plasmid DNA that was subsequently reintroduced into strain RG1-UV29 to check for genuine complementation.

Nucleotide sequencing. Nucleotide sequencing analysis was done on an ALF-Express sequencing robot using dye-primers in the cycle sequencing method (Murray, 1989) with the thermostequenase kit RPN 2538 from Amersham Pharmacia Biotech. Protein and nucleotide sequence comparisons were performed using the facilities of the BLAST server (Altschul et al., 1990) at NCBI (National Library of Medicine, Washington, DC, USA).

Expression of kstD2 in E. coli BL21(DE3) and preparation of cell-free extracts. The kstD2 gene was isolated from chromosomal DNA of parent strain SQ1 and mutant strains RGI-UV29, RGI-UV45, RGI-UV50 and RGI-UV51 by PCR, using kstD2 forward primer 5'-GGCGGATCCGATCTAGATCCA GGCACC-3' and reverse primer 5'-GGGGAATTCCTTAC TTCTCCTGTGCGTGATG-3'. For KSTD2 expression by the T7 RNA polymerase pET3 expression system (Novagen), Ndel (forward primer) and BamHI (reverse primer) restriction sites were introduced (underlined) and used to clone kstD2 into Ndel/BamHI-digested pET3b (Novagen). The resulting plasmid, pKSD114, was introduced into E. coli BL21(DE3) (Studer & Moffatt, 1986). For expression of soluble active KSTD2, E. coli BL21(DE3)pKSD114 was grown for 40 h at 25 °C in LB broth (25 ml) supplemented with 0.5 M sorbitol. Cell pellets (7300 g, 30 min, 4 °C) were washed with phosphate buffer (1 g l⁻¹: KH₂PO₄, 2.72; K₂HPO₄, 3.48; MgSO₄.7H₂O, 2.46; pH 7.2). Cells were suspended in 5 ml phosphate buffer and disrupted in a French pressure cell at 140 MPa. Cell extracts were centrifuged (40000 g, 20 min, 4 °C) to remove cell debris. The supernatants (5–10 mg protein ml⁻¹) were used in KSTD activity assays.

Preparation of cell-free extracts of E. coli DH5α containing the KSTD1 isoenzyme was done as described previously (van der Geize et al., 2000).

Cloning of mutant kstD2 genes. Mutant kstD2 genes were isolated from four UV mutants of strain RGI (strains RGI-UV29, RGI-UV45, RGI-UV50 and RGI-UV51) by PCR using Pteo polymerase (Roche) and kstD2 expression primers. The obtained PCR products were cloned into pBlueScript (II) KS for nucleotide sequencing.

Gene deletion mutagenesis of kstD2. Unmarked kstD2 gene deletions were carried out as described previously (van der Geize et al., 2001) using plasmid pKSD201 bearing the sacB counter-selection system. Southern analysis of kstD2 gene deletion mutants was done using a digoxigenin (DIG)-labelled kstD2 probe obtained by PCR (Boehringer PCR DIG probe synthesis kit 1636090) using primers developed for heterologous kstD2 expression in E. coli.

Preparation of cell-free extracts of R. erythropolis strains. Rhodococcus strains were grown in mineral medium (100 ml) supplemented with 10 mM glucose. Cultures were induced with AD (0.5 g l⁻¹) for 10 h. Following centrifugation (7300 g, 30 min, 4 °C), pellets of non-induced and induced cultures were washed with phosphate buffer, resuspended in phosphate buffer (1 ml) and disrupted by two passages through a French pressure cell at 140 MPa. Supernatants obtained after centrifugation (40000 g, 20 min, 4 °C) were used for KSTD enzyme activity assays.

KSTD enzyme activity assay. Enzyme activities of KSTD1 or KSTD2 were measured in triplicate spectrophotometrically at 30 °C using phenazine methosulphate (PMS) and 2,6-dichlorophenolindophenol (DCPIP). The reaction mixture (1 ml) consisted of 50 mM Tris/Tris-HCl, pH 7.0, 1.5 mM PMS, 0.4 mM DCPIP, cell-free extract and 500 µM steroid in methanol (2%). Activities are expressed as mean values ± s.d. in mU (mg protein)⁻¹. 1 mU is defined as the reduction of 1 nmol DCPIP min⁻¹ (ε₅₄₀ = 187 × 10⁻⁶ cm⁻¹ M⁻¹).

Steroid bioconversion and steroid analysis. Steroid bioconversion was done (in duplicate) in shake flasks with Rhodococcus cultures grown in 100 ml YG15 (1 g l⁻¹ yeast extract, 15; glucose, 15) medium at 28 °C (200 r.p.m. on a rotary shaker). After growth overnight till late exponential phase (OD₅₄₀ of 5–9), AD or 9OHAD (5 g l⁻¹ suspended in 0.1% v/v Tween 80) was added and bioconversion was followed for several days. Steroids were extracted from the medium (1 ml) with 3 ml methylene-chloride (GC) or 3 ml methanol-water (9:1) extraction (TLC). For HPLC analysis, samples were diluted five times with methanol/water (70:30) and filtered (0.45 µm). Steroids were analysed by HPLC (column, reversed phase Lichrosorb 10RP18, 250 × 3.0 mm (Varian Chrompack International)), UV detection (liquid phase, methanol/water (60:40), 35 °C), GC (column, J&W DB-5MS 30 m × 0.25 mm i.d. × 0.25 µm (Alltech); pre-column, 2 m × 0.25 mm i.d. [Inter-Sciences]: FID detection at 300 °C) or TLC (Kieselgel 60 F₂₅₄ 10 × 20 cm (Merck)) in toluene/ethylacetate 1:1. Retention times for HPLC (flow rate 0.8 ml min⁻¹) were t_R,9OHAD = 4.2 ± 0.2 min, t_R,ADD = 7.0 ± 0.2 min and t_R,0ADD = 10.5 ± 0.2 min, and for GC (carrier N₂₅, 100 kPa) t_R,ADD = 10.8 ± 0.2 min and t_R,0ADD = 11.1 ± 0.2 min. Retention factors for TLC were R_F,9OHAD = 0.23, R_F,ADD = 0.44 and R_F,0ADD = 0.56. Substrates used (AD, 9OHAD, ADD) were supplied by Diosynth.

In vitro conversion of AD by KSTD2. A reaction mixture (1 ml) consisting of Tris/HCl, pH 7.0 (50 mM), PMS (150 µM), AD (700 µM) and cell-free extract of E. coli BL21(DE3)pKSD114 (approx. 25 µg protein) was incubated overnight at 30 °C. Steroid analysis was done by HPLC as described above.

RESULTS AND DISCUSSION

Cloning and characterization of the kstD2 gene

R. erythropolis strain RGI-UV29, isolated during a screening for 9OHAD growth-deficient mutants of UV mutagenized cells of kstD mutant R. erythropolis strain RGI (van der Geize et al., 2001), was used for cloning of the kstD2 gene. A gene library of R. erythropolis strain RGI was introduced into strain RGI-UV29 by electro-
transformation and transformants were replica-plated onto AD mineral agar medium to screen for complementation of the AD-negative growth phenotype. Plasmid pKSD101, isolated from a transformant that showed restored growth on AD mineral medium, was transformed into E. coli DH5α for further analysis.

An insert of approximately 6.5 kb was identified in pKSD101 and subjected to restriction mapping analysis. A 3.6 kb EcoRI DNA fragment of pKSD101 was cloned into pBlueScript (II) KS (pKSD105; Fig. 2) and into Rhodococcus–E. coli shuttle vector pRESQ (pKSD106) for complementation. Plasmid pKSD106 was still able to restore the strain RG1-UV29 phenotype, whereas a 2–4 kb Asp718 DNA fragment (located on this 3.6 kb fragment) in pRESQ could not. Nucleotide sequence analysis of the 3.6 kb EcoRI fragment revealed the presence of a large ORF (ORF1) of 1698 nt (GC content 63.9 mol%), encoding a putative protein of 565 aa (calculated mass 60.2 kDa). The deduced amino acid sequence of ORF1 showed clear similarity with KSTD1 (35% identity), encoded by kstD in R. erythropolis SQ1, and other known KSTD amino acid sequences present in databases (Fig. 3) and was thus designated kstD2. Highest similarity was found with the ORF3-encoded protein (63% identity) from A. simplex (Dziadek et al., 1998) (GenBank accession no. Z93338). For alignment purposes, the ORF3 nucleotide sequence was edited to obtain a deduced amino acid sequence matching the KSTD consensus, since ORF3 obviously contained several frame-shift errors. The alignment strongly suggests that A. simplex also contains at least two KSTD isoenzymes, encoded by ksdD (Molnár et al., 1995) and ORF3 (suggested name ksdD2), respectively. Contrary to kstD2 in R. erythropolis SQ1, ORF3 was found in close proximity to ksdD and ksdI, encoding KSTD1 and steroid 4,5-isomerase, respectively (Dziadek et al., 1998; Molnár et al., 1995). Analysis of nucleotide sequences directly upstream and downstream of the kstD2 gene showed no direct evidence for the presence of additional (co-transcribed) nucleotide sequences involved in steroid metabolism. No overlap in nucleotide sequences upstream or downstream of the kstD2 gene was found with sequences flanking kstD (van der Geize et al., 2000).

The putative N-terminal FAD-binding motif of the kstD2-encoded protein (KSTD2) was similar to the previously suggested consensus amino acid sequence for FAD-binding in KSTD, GSG(A/G)(A/G)(A/G)X5E (van der Geize et al., 2000), although not completely conserved (Fig. 3).

Expression of kstD2 in E. coli

Attempts to express kstD2 from its own promoter in E. coli were unsuccessful. Alternatively, kstD2 was cloned into the pET3b expression vector (pKSD114) for expression via the T7 RNA polymerase expression system in E. coli BL21(DE3). Highly active soluble KSTD2
enzyme was obtained in extracts of E. coli cells grown at 25 °C. Incubation of AD with cell-free extracts (25 µg protein) of E. coli BL21(DE3)(pKSD114) resulted in its conversion into ADD, confirming that in vivo KSTD2 indeed performs the 3-ketosteroid Δ1-dehydrogenation reaction. ADD formation was not observed in controls with cell-free extracts without AD. Contrary to KSTD1, KSTD2 does not show any activity in the presence of nitro blue tetrazolium. KSTD2 thus could not be visualized by activity staining on native PAGE (van der Geize et al., 2000). In vitro activity of KSTD2 was detected, however, in the presence of DCPIP and activity was further enhanced by addition of PMS. Optimal KSTD2 activity (60 ± 8 µM g M) was found at pH 7–8 at 30 °C using AD and the DCPIP/PMS system. A fivefold higher KSTD2 activity was observed with AD compared to 9OHAD. Substrate affinity of KSTD2 for AD (Km,app = 17 µM) was 10-fold higher than for 9OHAD (Km,app = 165 µM). In contrast, KSTD1 displays similar activities and affinities with AD (Km,app = 82 µM) and 9OHAD (Km,app = 87 µM).

**Construction of single and double gene deletion mutants**

For kstD2 unmarked gene deletion mutagenesis, plasmid pKSD201, with a kstD2 remnant (ΔkstD2) of 605 bp, was obtained after deletion of a 1093 bp MluI internal fragment of kstD2 (Fig. 2). Plasmid pKSD201 was introduced into E. coli S17-1 and mobilized by conjugation to both parent strain R. erythropolis SQ1 and mutant strain RG1. Using the sacB counter-selection system we were able to isolate mutant strains RG7 (kstD2 mutant) and RG8 (kstD2 kstD2 mutant). Southern analysis, using DIG-labelled kstD2 gene as probe, on Aep718-digested chromosomal DNA of parent strain SQ1, strain RG7 and strain RG8, confirmed deletion of kstD2 in the latter two strains: a 2.4 kb Aep718 DNA fragment of strain SQ1 hybridizing with the kstD2 probe was reduced to a 1.3 kb DNA fragment in both mutant strains (data not shown). PCR performed on chromosomal DNA of strains RG7 and RG8 with the kstD2 primers resulted in PCR products with sizes of 0.6 kb, further demonstrating kstD2 gene deletion.

**Characterization of mutant strains RG1, RG7 and RG8**

Growth on AD, ADD or 9OHAD mineral agar medium was unaffected in both single gene deletion mutant strains RG1 and RG7. Growth on steroid substrates AD and 9OHAD was blocked in the kstD kstD2 double gene deletion strain RG8, while growth on ADD was not affected, confirming inactivation of the steroid Δ1-dehydrogenation reaction. Thus, both KSTD1 and KSTD2 are involved in steroid degradation by R. erythropolis SQ1 and the presence of only one of these enzymes is sufficient to allow degradation of the steroid skeleton to proceed. KSTD enzyme activities could not be detected in extracts of cells of parent strain SQ1 and mutant strains RG1, RG7 and RG8 grown on glucose without steroid induction. Upon steroid (AD) induction, KSTD enzyme activities (using AD as substrate) were found in cell-free extracts of parent strain SQ1 (280 ± 40 mU mg⁻¹), strain RG1 (80 ± 5 mU mg⁻¹) and strain RG7 (285 ± 30 mU mg⁻¹), indicating that both KSTD1 and KSTD2 are inducible proteins. No KSTD 3289
activity was detected in cell-free extracts of the double gene deletion mutant strain RG8.

**Bioconversion of AD**

Incubations of parent strain SQ1 and three mutants, i.e. RG1, RG7 and RG8, with 5 g AD l\(^{-1}\) (17.5 mM) or 5 g 9OHAD l\(^{-1}\) (16.6 mM) were performed (Fig. 4). Comparable rates of AD degradation (via KSTD and/or KSH activities; Fig. 1) were observed for all strains (1.0–1.7 mmol AD l\(^{-1}\) h\(^{-1}\)). No 9OHAD accumulation from AD was observed with strains SQ1 and RG1 (Fig. 4A). Strain RG7 initially accumulated 9OHAD (1.8±0.1 mmol 9OHAD formed l\(^{-1}\) h\(^{-1}\)), stoichiometrically derived from AD (1.7±0.1 mmol AD degraded l\(^{-1}\) h\(^{-1}\)), suggesting low, or even absence, of KSTD activity during the early phase of bio-transformation. With strain RG7, maximal 9OHAD accumulation was found within 20 h after addition of AD; 9OHAD was subsequently slowly degraded again (0.23±0.03 mmol 9OHAD l\(^{-1}\) h\(^{-1}\)). Similar rates of 9OHAD degradation (0.27±0.04 mmol 9OHAD l\(^{-1}\) h\(^{-1}\)) were found during incubation of strain RG7 with 9OHAD. Much higher rates of 9OHAD degradation were observed for parent strain SQ1 and kstD mutant strain RG1 (1.3±0.2 mmol 9OHAD l\(^{-1}\) h\(^{-1}\)). These experiments show that 9OHAD degradation proceeds differently in an environment with only KSTD2 present, compared to a situation in which KSTD1 is the only available KSTD enzyme. With solely KSTD2 present (strain RG1), steroid degradation of 9OHAD proceeds at the same rate as in parent strain SQ1. In contrast, the absence of KSTD2 (strain RG7) results in significant temporary accumulation of 9OHAD during AD bioconversion, and in low rates of 9OHAD degradation. In *vivo*, KSTD1 thus is considerably less efficient in Δ\(^{1}\)-dehydrogenation of 9OHAD than KSTD2. Incubation of strain RG8 with AD resulted in high accumulation levels of 9OHAD (75% of AD converted into 9OHAD) within 24 h after addition of AD. The 9OHAD formed was not degraded during further incubation. Thus, inactivation of both kstD and kstD2 is necessary to inactivate KSTD activity in *R. erythropolis* SQ1 and to block 9OHAD degradation.

**KSTD2 mutants Ser325Phe and Thr503Ile**

In addition to strain RG1-UV29, three other mutants (strains RG1-UV45, RG1-UV50 and RG1-UV51) were identified during screening for mutants blocked in Δ\(^{1}\)-dehydrogenation. Conceivably, these four mutant strains possess mutant kstD2 genes, containing point mutations as a result of UV irradiation, resulting in inactive KSTD2 enzymes. Nucleotide sequencing revealed that kstD2 from RG1-UV29 contained an opal mutation at amino acid residue Trp122. Mutant kstD2 gene from strain RG1-UV45 contained the Thr503Ile mutation. Thr503 is a fully conserved residue among all KSTD enzymes known to date (Fig. 3). Replacement of the Thr503 residue by isoleucine, a hydrophobic residue of comparable size, renders KSTD2 non-functional, suggesting that the hydroxyl group of threonine is important in either substrate binding or in catalysis. The kstD2 gene of strain RG1-UV51 contained the Ser325Phe mutation. Ser325 is positioned in a poorly conserved region of KSTD enzymes, although the low similarity is mostly due to the aberrant amino acid sequence of KSTD from *Comamonas testosteroni* and *Pseudomonas aeruginosa* in this region. Amino acid
sequences of actinomycetal KSTD enzymes, however, are rather conserved over a stretch of 8 aa (322-MDQSWWFQ-329). Amino acid residue position 325 in KSTD2 thus is important for activity in actinomycetal KSTD enzymes. Ser325 of KSTD2 is occupied by an alanine or threonine in other actinomycetal KSTD enzymes, indicating that a small residue at this position is favoured. The presence of a phenyl moiety in KSTD2 could inactivate KSTD activity, for instance by steric hindrance of substrate binding. No mutation was found in kstD2 of strain RG1-UV50. Inactivation of KSTD2 in this strain could be the result of a mutation in sequences needed for transcription (e.g. promoter sequences), which was not further investigated.

Heterologous expression of mutant KSTD2 protein encoded by kstD2 (Thr503Ile) or kstD2 (Ser325Phe) in E. coli BL21(DE3) resulted in full-length protein on SDS-PAGE having no detectable KSTD enzyme activity.

Conclusions

R. erythropolis SQ1 employs two KSTD isoenzymes that in vivo may have different functional roles in AD degradation. AD degradation may occur via either ADD or 9OHAD (Fig. 1). Considering the observed mild toxicity of ADD to R. erythropolis SQ1 [growth on agar medium is considerably slower in the presence of kstD2 encoded by kstD2 of strain RG1-UV50. Inactivation of KSTD2 in this strain could be the result of a mutation in sequences needed for transcription (e.g. promoter sequences), which was not further investigated.

Heterologous expression of mutant KSTD2 protein encoded by kstD2 (Thr503Ile) or kstD2 (Ser325Phe) in E. coli BL21(DE3) resulted in full-length protein on SDS-PAGE having no detectable KSTD enzyme activity.

Acknowledgements

This work was partly funded by Diosynth (Oss, The Netherlands) and BTS grant BIO94049 (Bedrijjsgerichte Technologie Stimulerin). We thank Rick van Gerwen, Jos Gielis and Peter van der Meijden (Diosynth) for their contributions to this work.

References


Received 21 March 2002; revised 14 June 2002; accepted 28 June 2002.