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3-Ketosteroid-Δ^1-Dehydrogenase of *Rhodococcus rhodochrous*: Sequencing of the Genomic DNA and Hyperexpression, Purification, and Characterization of the Recombinant Enzyme[1]

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The gene encoding 3-ketosteroid-Δ^1-dehydrogenase from *Rhodococcus rhodochrous* was cloned and sequenced. The gene (*ksdD*) consists of 1,536 nucleotides and encodes an enzyme protein of 511 amino acid residues. The amino terminal methionine residue was deleted in the mature protein. The amino acids involved in the flavin binding site are conserved in the dehydrogenase sequence. The deduced amino acid sequence is highly homologous to that from *Arthrobacter simplex* but less so to that from *Pseudomonas testosteroni*. Upstream of the gene was located a heat shock protein gene, *dnaJ*, and downstream, a gene of a hypothetical protein. The enzyme gene was ligated with an expression vector to construct a plasmid pDEX-3 and introduced into *Escherichia coli* cells. The transformed cells hyperexpressed the 3-ketosteroid-Δ^1-dehydrogenase as an active and soluble protein at more than 30 times the level of *R. rhodochrous* cells. Purification of the recombinant 3-ketosteroid-Δ^1-dehydrogenase from the *E. coli* cells by a simplified procedure yielded about 13 mg of enzyme protein/liter of the bacterial culture. The purified recombinant dehydrogenase exhibited identical molecular and catalytic properties to the *R. rhodochrous* enzyme.

Key words: hyperexpression, 3-ketosteroid-Δ^1-dehydrogenase, recombinant enzyme, sequence, steroid desaturation.

3-Ketosteroid-Δ^1-dehydrogenase is a flavoprotein functioning in the initial stage of steroid degradation by microorganisms. In the pharmaceutical industry, it is utilized in the production of physiologically important steroids. The enzyme is present in several bacteria, and its isolation and characterization in terms of molecular, catalytic, and spectrophotometric properties have been studied. The most extensive studies have been carried out by us with the enzyme from *Rhodococcus rhodochrous* (*1-5*). The dehydrogenase has a molecular mass of 60.5 kDa with one FAD molecule (*1*) and exhibits typical spectrophotometric properties of a flavoenzyme (*2*). It catalyzes the elimination of 1-α and 2-β hydrogen atoms from 3-ketosteroid to insert a double bond into the A-ring (*3*). One of its characteristic properties is steroid transhydrogenase activity between 3-keto-4-ene-steroid and 3-keto-1,4-diene-steroid (*3*). Chemical modification studies of the enzyme indicated the presence of a His and an Arg residue in the catalytic site for the activation of 3-ketosteroid to abstract the hydrogen atoms (*4, 5*). Structural and genomic analyses of the enzymes from *Pseudomonas testosteroni*, *Arthrobacter simplex*, and *Nocardia opaca* revealed that *Arthrobacter* enzyme comprised 515 amino acids and its sequence was 30.5 and 34.7% identical to those of the *Pseudomonas* and *Nocardia* enzymes, respectively (*6-8*). Attempts were made to express the gene (*ksdD*) of the *A. simplex* enzyme in *Streptomyces lividans* (*7, 9*) and *Pseudomonas testosteroni* (*6*) and *R. erythropolis* enzymes in *Escherichia coli* (*10*). However, these expression systems of 3-ketosteroid-Δ^1-dehydrogenase were not suitable for mutagenic study of the enzyme or for application to the production of physiologically important steroids. From *R. rhodochrous* cells, we have isolated three steroid-oxidizing enzymes, steroid-Δ^1-dehydrogenase (*1*), 3-keto-5α-steroid-Δ^4-dehydrogenase (*11*), and steroid monooxygenase (*12*), and one cytochrome P-450, P-450$_{Cor}$ (*13*). These enzyme proteins were induced with 3-ketosteroid in the cells growing in nutrient broth. The relationships among the genes of these enzyme proteins on the genomic DNA are an interesting subject on the dissimilative metabolism of steroids in the organism which can be elucidated by sequencing the DNA of these proteins.

In the present study, we have cloned and sequenced the genomic DNA encoding the 3-ketosteroid-Δ^1-dehydrogenase from *R. rhodochrous* and compared the deduced amino acid sequence with those of the enzymes from other microorganisms to determine the location of the catalytically important amino acid residues. We also designed an expression plasmid carrying the enzyme gene, which was

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Abbreviations: DCIP, 2,6-dichlorophenol indophenol; PMSF, phenylmethyl sulfonyl fluoride; ORF, open reading frame; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactopyranoside.

hyperexpressed efficiently in *E. coli* cells to yield the active enzyme. The hyperexpressed recombinant 3-ketosteroid-Δ¹-dehydrogenase was purified to homogeneity from the *E. coli* cells on a large scale and characterized in terms of its molecular and catalytic properties.

MATERIALS AND METHODS

Bacterial Strains and Plasmids—*R. rhodochrous* IFO-3338 was used as a source of DNA and protein. *E. coli* JM109 competent cells were purchased from Toyobo and used as a host strain for recombinant plasmid DNA sequencing. *E. coli* BL21(DE3) competent cells and plasmid pET28a(+) were obtained from Novagen. pUC18 and pTrc99A were from Pharmacia. pCR™II vector and *E. coli* INVαF′ competent cells were from Invitrogen.

DNA Manipulations and Sequencing—The amino acid sequences of N-terminal and tryptic peptides of the enzyme protein were determined by Edman degradation using an Applied Biosystem Inc. gas phase protein sequencing system. The primers used for PCR were: D1-N, 5′-TGGGCNGARGARTGYGAYGT-3′, which was based on the amino terminal sequence WAEECDV; and D1-2, 5′-TCNGGRTCNCCNGCDATNGG-3′, based on the sequence of a tryptic peptide, PIAGDPE. Total DNA of *R. rhodochrous* was prepared as described previously (*14*). Using 2 μg of the DNA as template and 0.1 nmol each of primers D1-N and D1-2, PCR was carried out with *Taq*-DNA polymerase at 60°C for 1 min for annealing and in 35 cycles at 72°C for 1 min for elongation. DNA fragments of about 430 bp were obtained. The products were purified, ligated with pCR™-II-vector, and introduced into *E. coli* INVαF′ cells. Transformants were selected, and a plasmid, termed pD1430, was isolated. The nucleotide sequence of the inserted DNA was determined with a cycle sequencing kit using a model A.L.F. DNA sequencer (Pharmacia). The 431-bp DNA fragment containing the desired base sequence for the N-terminal amino acid sequence of the dehydrogenase was labeled with ^{32}P using a DNA labeling system kit and [α-^{32}P]dCTP (3,000 Ci/mmol). The total DNA from *R. rhodochrous* was digested with six endonucleases, and analyzed by Southern blot hybridization with ^{32}P-labeled 431-bp DNA fragment. *Bam*HI DNA fragments of about 4 kbp that gave positive signal were purified, ligated with pUC-18 plasmid digested previously with *Bam*HI, and introduced into *E. coli* JM109. Plasmid screening was carried out by colony hybridization with the ^{32}P-labeled probe, and a plasmid, named pD1, containing the desired DNA fragment was isolated. A deletion series of the plasmid was constructed and sequenced following the manufacturer's instructions.

Computer analyses of the obtained sequence data were carried out with DNASIS-MAC (Hitachi Software).

Construction of Expression Plasmid—Primers DE-1 and DE-2 were designed from the nucleotide sequence shown in Fig. 1 and synthesized: DE-1, 5′-AAGGCAACAGCCATGGCGGA-3′, based on the base sequence from position 442 to 461, in which thymine at position 452 was replaced with cytosine to make a restriction site for *Nco*I; and DE-2, 5′-ACCATGAATTCGAGGTCGTC-3′, based on the complementary sequence from position 778 to 797. PCR with the primers and pD1 was carried out and the product was digested with *Nco*I and *Eco*RI. The obtained fragment was ligated with pTrc99A vector digested previously with *Nco*I and *Eco*RI to construct pDEX-1. The plasmid was digested with *Eco*RI and *Bam*HI, then ligated with a DNA fragment prepared from pD1 by digestion with *Eco*RI and *Bam*HI to produce a plasmid pDEX-2. The plasmid was digested with *Nco*I and *Bam*HI, the fragment containing the *ksdD* gene was inserted into the pET28a(+) digested previously with *Nco*I and *Bam*HI, and the expression plasmid pDEX-3 was obtained.

Hyperexpression—*E. coli* BL21(DE3) was transformed with pDEX-3 and cultured in 4 ml of LB medium containing 0.003% kanamycin for 15 h at 37°C. The grown cells were transferred to 50 ml of fresh LB medium and cultured with shaking. After 2 h of culture, IPTG was added to a final concentration of 100 μM to induce the expression of the 3-ketosteroid-Δ¹-dehydrogenase. The culture was continued for 8 h and the cells were harvested. The gene expression was confirmed by analyzing the dehydrogenase activity (*1*) and with SDS-PAGE and native PAGE.

For purification of the enzyme, the recombinant bacterial cells from the stock culture were directly inoculated into 50 ml of LB medium in a 200-ml flask. After incubation overnight at 37°C, the cells were transferred to 1 liter of fresh LB medium containing 30 mg/liter kanamycin and 10 mg/liter riboflavin. After 2 h of culture, IPTG was added in a final concentration of 100 μM to induce expression of the enzyme, and culture was continued for 7 h at 37°C. Cells were then harvested, the yield being about 23 g/5 liters, and stored at −80°C.

Purification—Purification was carried out at 4°C by the method used for the purification of *R. rhodochrous* 3-ketosteroid-Δ¹-dehydrogenase (*1*). The Tris-HCl buffers contained 30 mM Tris-HCl, 50 μM dithiothreitol, 100 μM EDTA, and 1 μM FAD, at pH 7.4. The cells (23 g) were suspended in 100 ml of Tris-HCl buffer and disrupted by sonication for 15 min in an ice bath. The extraction procedure was carried out twice and the crude cell extract was obtained by centrifugation at 15,000×*g* for 15 min. PMSF (10 mg/liter) and DNase (0.2 mg/liter) were added to the combined supernatants. The solution was diluted with the same volume of cold distilled water and applied onto a DEAE-cellulose column (3.5×30 cm) equilibrated with Tris-HCl buffer. The column was developed with a 2-liter linear gradient of 0-0.9 M KCl in the Tris-HCl buffer with a flow rate of 0.73 ml/min. The active fraction was collected, solid ammonium sulfate was added to the concentration of 1.7 M, and 1 M tris[hydroxymethyl]aminomethane solution was added to keep the pH at 7.4. The enzyme preparation was loaded onto a phenyl Sepharose column (2.0×30 cm) equilibrated with 1.7 M ammonium sulfate (pH 7.4), and eluted with a 600-ml reversed linear gradient of 1.7-0 M ammonium sulfate in the Tris-HCl buffer, then with 100 ml of the Tris-HCl buffer at a flow rate of 0.45 ml/min. The active fractions were combined, concentrated, and applied on a column (1.5×110 cm) of Ultrogel AcA44 equilibrated with 0.1 M KCl in the Tris-HCl buffer. The gel filtration was carried out at a flow rate of 0.1 ml/min, and the fractions containing 3-ketosteroid-Δ¹-dehydrogenase were collected, concentrated, and stored at −80°C.

N-Terminal Sequence Determination—The amino terminus sequence of recombinant 3-ketosteroid-Δ¹-dehydrogenase was determined in an Applied Biosystems model 476A protein sequencer, and phenylthiohydantoin deriva-

tives were detected *via* an on-line system.

Western Blotting—Polyclonal antibody against *R. rhodochrous* 3-ketosteroid-Δ^1-dehydrogenase was generated in BALB/c mice. Western blotting was carried out using horseradish peroxidase-conjugated rabbit antimouse IgG serum.

Spectrophotometric Experiments—Spectral studies of recombinant 3-ketosteroid-Δ^1-dehydrogenase were performed on a Hitachi model U-3400 spectrophotometer at 25°C. Fluorescent spectra were observed on a Hitachi model F-7000 fluorospectrometer.

Gel Electrophoresis, Enzyme Assay, and Protein Determination—SDS-PAGE was performed with 9% polyacrylamide gel containing 1% SDS (*15*). The proteins were visualized with Coomassie Brilliant Blue R-250 staining. Gel electrophoresis without SDS was performed as described previously (*16*). The active enzyme protein was visualized with nitroblue tetrazolium staining in the presence of phenazine methosulfate and androstenedione (*1*). The enzyme activity of cultured cells was assayed by incubation of the cells with androstenedione for 20 min at 37°C and quantitative analysis of the product steroid with HPLC (*1*). The enzymatic activity was usually measured spectrophotometrically using 2,6-dichlorophenol indophenol as an electron acceptor as described (*1*). The reaction product of 3-ketosteroid-Δ^1-dehydrogenase was identified by HPLC and TLC.

Concentration of the purified 3-ketosteroid-Δ^1-dehydrogenase was determined spectrophotometrically as described (*1*). Protein concentrations were determined by the method of Lowry *et al.* (*17*) using bovine serum albumin as the standard.

Materials—All restriction enzymes were obtained from Boehringer Mannheim. *Taq*-DNA polymerase and Deletion kit for Kilo-sequencing were purchased from Takara Shuzo. Autocycle™ Sequencing kit was from Pharmacia, and [α-^{32}P]dCTP and *redi*prime™ DNA labeling system were from Amersham. Ampicillin-Na was from Wako, and isopropyl β-D-thiogalactopyranoside, 5-bromo-4-chloro-3-indolyl-β-D-galactoside, riboflavin, dithiothreitol, and kanamycin were from Nacalai Tesque. Horseradish peroxidase-conjugated rabbit antimouse IgG serum was obtained from Zymed Lab. PMSF, DNase, nitroblue tetrazolium, androstenedione, and androstadienedione were from Sigma. All other chemicals used were of the highest purity available.

RESULTS

Cloning and Sequencing of the 3-Ketosteroid-Δ^1-dehydrogenase Gene—A 4.2-kbp *Bam*HI DNA fragment on pD1 was sequenced and the middle part of the determined nucleotide sequence, a 2,268-bp region encompassing the *ksdD* gene of 3-ketosteroid-Δ^1-dehydrogenase, is shown in Fig. 1. The ORF of 1,536 bp encoding the 511 amino acid residues of the dehydrogenase, located from position 454 to 1989, was identified and designated *ksdD*. Except for the initial amino acid, the deduced amino acid sequence of the N-terminus of 3-ketosteroid-Δ^1-dehydrogenase is in perfect agreement with that determined by Edman degradation. The predicted methionine residue is not present in the mature enzyme, in which N-terminus initiates with Ala. The calculated molecular mass is 54,295 Da, and thus the

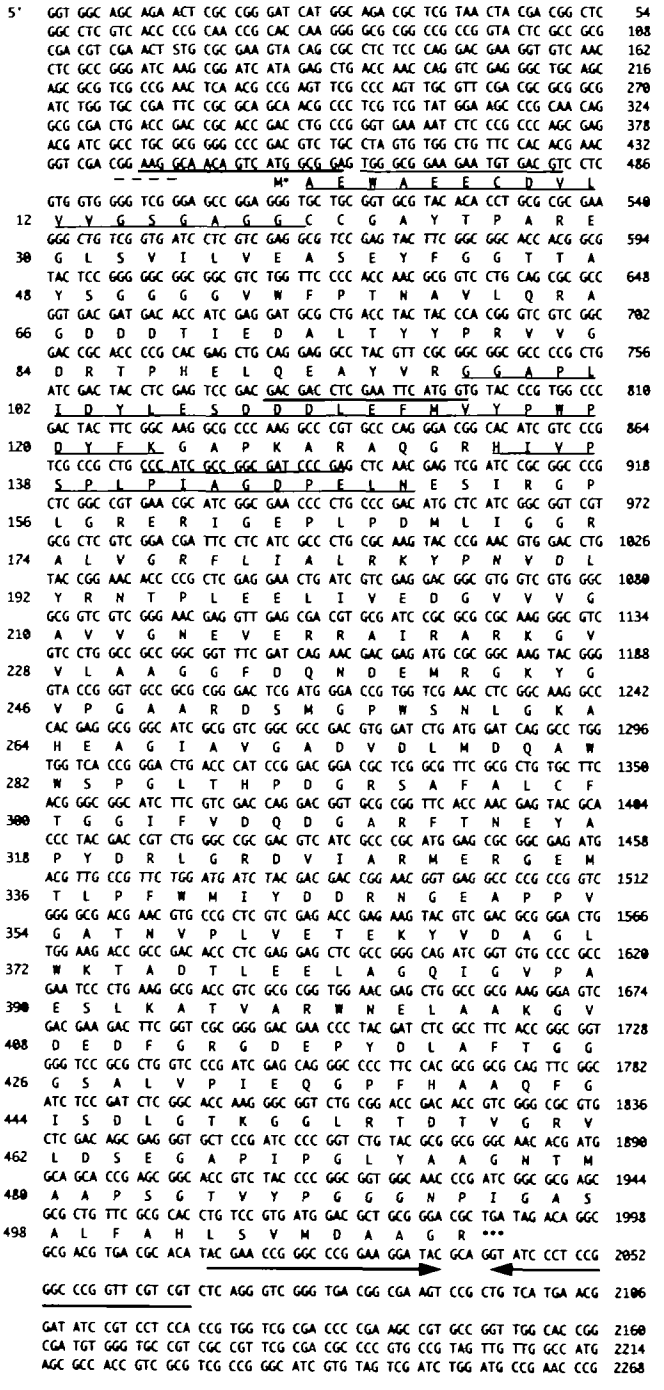

Fig. 1. **Nucleotide sequence of 2,268 nucleotides of *Bam*HI insert in plasmid pD1 and deduced amino acid sequence of the *Rhodococcus rhodochrous* 3-ketosteroid-Δ^1-dehydrogenase.** The numbering at the left refers to the amino acid position and that at the right to the nucleotide position. The underlined amino acid sequences indicate the sequences determined by Edman degradation of the enzyme protein or peptides. The putative ribosome-binding site is shown by dotted-underline (----) upstream from the initiation codon. An inverted repeat (possible terminator) sequence is indicated by inverted arrows. The underlined nucleotide sequences indicate the sequences of primers D1-N and D1-2. The bold lines under the nucleotide sequence indicate the sequences of primers DE-1 and DE-2 used for construction of the expression vector.

size of the apoenzyme is 54,164 Da with 510 residues and the holoenzyme bound with an FAD molecule is 54,949 Da. Comparison of the predicted amino acid sequence of *R. rhodochrous* 3-ketosteroid-Δ¹-dehydrogenase with sequences of the enzymes from other organisms revealed 67.8% identity with the dehydrogenase from *A. simplex* (*7*) and 72.25% identity in the nucleotide sequence. The sequence shows lower similarities (35 and 25% identity) with the enzymes from *N. opaca* and *P. testosteroni*, respectively. Sequence comparison showing the conserved amino acids of these enzymes (Fig. 2) reveals a consensus sequence [G-S-G-A(G)-G-G(A)-X_2-G(A)-] of a nucleotide-binding site at position 14. This sequence corresponds to a putative region to bind the ADP moiety of FAD (*18*). The *ksdD* gene is preceded by a typical ribosome-binding site, GGAAGG, at position 440 (Fig. 1), and a palindrome sequence is located just downstream (position 2015) from the termination codon. In the extended sequence upstream of the *ksdD* gene (A in Fig. 3), in the same direction as the dehydrogenase gene, was found the carboxy terminal region of the ORF of a protein whose amino acid sequence is highly similar to the heat-shock protein of *dnaJ* of *Mycobacterium tuberculosis* (*19*). Downstream, the carboxy-terminal region of the ORF of another protein was found in the region indicated as B in the figure. Its deduced amino acid sequence is similar to that of a hypothetical protein (YHBW) found in *E. coli* (*20*).

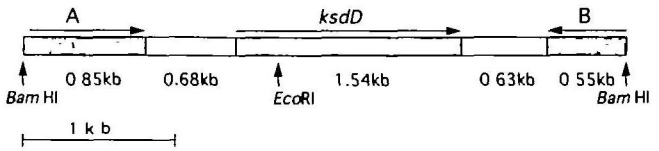

Fig. 3. **Schematic presentation of the gene structure of the *Bam*HI-DNA fragment of *R. rhodochrous*.** *ksdD*, 3-ketosteroid-Δ¹-dehydrogenase. A and B are similar regions to the heat shock protein gene *dnaJ* of *Mycobacterium tuberculosis* and the gene of a hypothetical protein, YHBW, of *Escherichia coli*, respectively. Arrows indicate the direction of transcription of each gene.

Expression of ksdD Gene of R. rhodochrous in E. coli—

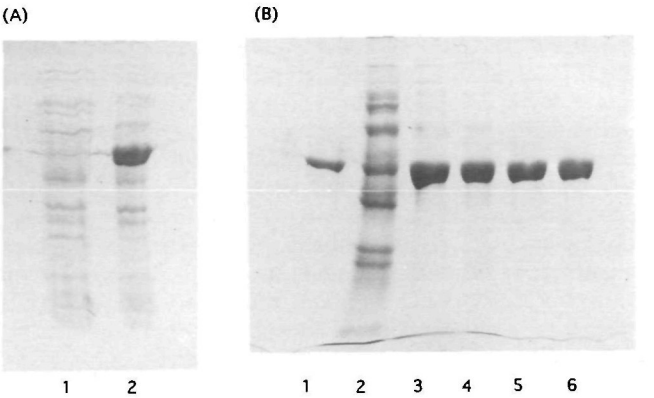

Fig. 4. **SDS-PAGE of SDS-dissolved cells of *E. coli* containing pDEX-3 and the recombinant 3-ketosteroid-Δ¹-dehydrogenase at various purification steps.** (A) Lane 1, cell lysate (6 μg protein/lane) of *E. coli* containing pET28a(+); lane 2, cell lysate of *E. coli* containing pDEX-3 after an 8-h incubation at 37°C with 100 μM IPTG. (B) Lane 1, purified *R. rhodococcus* enzyme; lane 2, crude extract; lane 3, after DEAE-cellulose chromatography; lane 4, after phenyl-Sepharose chromatography; lane 5, after concentration by centriflow; and lane 6, after Ultrogel AcA44 gel filtration (10 μg protein/lane).

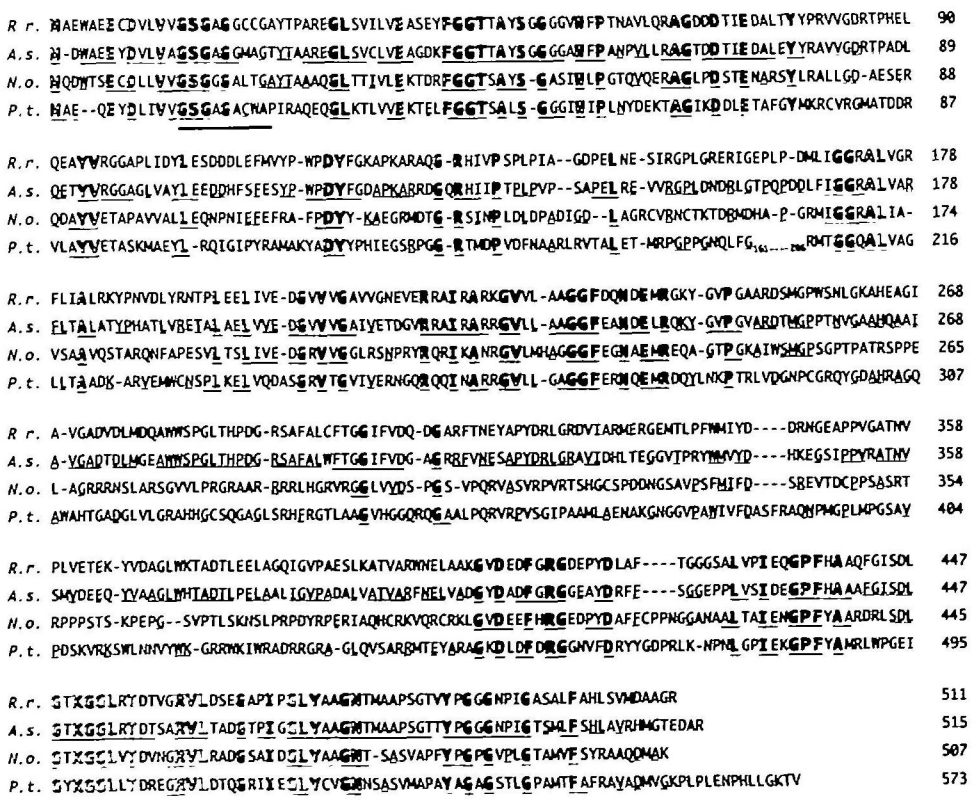

Fig. 2. **Alignment of the *Rhodococcus rhodochrous* 3-ketosteroid-Δ¹-dehydrogenase protein sequence with those from *Arthrobacter simplex* (*7*), *Nocardia opaca* (*8*), and *Pseudomonas testosteroni* (*6*).** The *P. testosteroni* enzyme sequence was aligned by introducing a gap from residue 162 to 205 to maximize identities. R.r., *Rhodococcus rhodochrous*; A.s., *Arthrobacter simplex*; N.o., *Nocardia opaca*; and P.t., *Pseudomonas testosteroni*. Identical residues to those of *R. rhodochrous* enzyme protein are indicated by underlines. The conserved amino acid residues among the enzyme proteins of four bacterial sources are indicated by outlined letters. The bold underline from residue 14 to 20 indicates the flavin-binding region.

We designed and constructed an efficient heterologous hyperexpression system, plasmid pDEX-3, from pD1. It was introduced into *E. coli* BL21(DE2) cells to express the *ksdD* gene. The gene product exhibiting the expected molecular mass (55 kDa) on SDS-PAGE was hyperproduced upon induction of the expression system with IPTG (Fig. 4A, lane 2). Western blotting analysis with an antibody raised against the dehydrogenase protein of *R. rhodochrous* also gave a clear band. The protein was produced at a lower level without the treatment by IPTG, because the T7-promoter of the plasmid is almost silent in the absence of IPTG (Fig. 4A, lane 1). On analysis by native PAGE, the lysate from cells containing pDEX-3 was confirmed to have the active enzyme by the migration distance and enzyme-activity staining (the data, not shown). The enzyme activity was also confirmed by detection of androstadienedione on HPLC-analysis of the reaction product after the incubation with androstenedione of the cell suspension of *E. coli* transformed with pDEX-3. The time course kinetics of IPTG-induction of the enzyme by the cells carrying pDEX-3 showed that the enzyme activity reached 0.4 nmol androstadienedione produced/min/10^8 cells after incubation for 8 h with the inducer.

Comparison of SDS-PAGE of cell-lysate and soluble supernatant of the hyperproducing bacteria showed that a part of the 3-ketosteroid-Δ^1-dehydrogenase is located in the membrane fractions. The enzyme activity located in the soluble extract of hyperproducing cells is more than 30 times that in the soluble extract from *R. rhodochrous*.

Purification and Characterization of the Recombinant 3-Ketosteroid-Δ^1-dehydrogenase—The recombinant 3-ketosteroid-Δ^1-dehydrogenase was purified from *E. coli* cells harboring pDEX-3 by a three-step procedure simplified from the methods reported (*1*). The degree of purity of fractions obtained at each step was analyzed by SDS-PAGE (Fig. 4B) and the activity of these samples was measured by DCIP reduction assay (Table I). The purification protocol involved a DEAE-cellulose ion-exchange chromatography with a linear gradient elution, followed by a reversed linear chromatography on a phenyl-Sepharose column, and finally gel filtration on an Ultrogel column. 3-Ketosteroid-Δ^1-dehydrogenase was efficiently obtained in a high degree of purity with a yield of 2.8 mg protein/g wet cells. The purified enzyme had a high specific activity of 25 units/mg protein. The 3-ketosteroid-Δ^1-dehydrogenase activity of the crude extract seemed to be decreased by inhibitor present in the bacterial lysate, as the total activity increased to 143% of the crude extract after the chromatography on the DEAE-cellulose column.

The size of the recombinant enzyme protein was confirmed to be 55 kDa by SDS-PAGE, on which the enzyme migrated with the same distance of that of the *R. rhodo-*

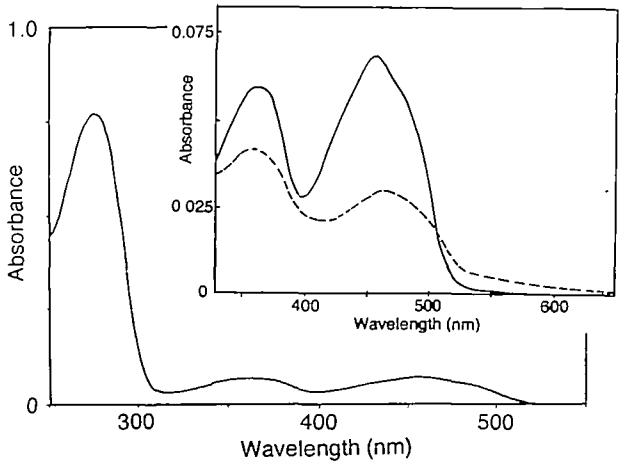

Fig. 5. **Spectrophotometric analysis of the recombinant 3-ketosteroid-Δ^1-dehydrogenase.** Ultraviolet/visible absorption spectrum of oxidized recombinant enzyme was monitored at 25°C in the 250–650 nm range. The sample consisted of 6.42 μM dehydrogenase in 30 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA in a 2-ml volume. The insert shows the enlarged absorption spectra of oxidized and androstenedione-reduced enzyme in the visible region. The reduced form was prepared by addition of 10 nmol of androstenedione under anaerobic conditions in a Thunberg cuvette.

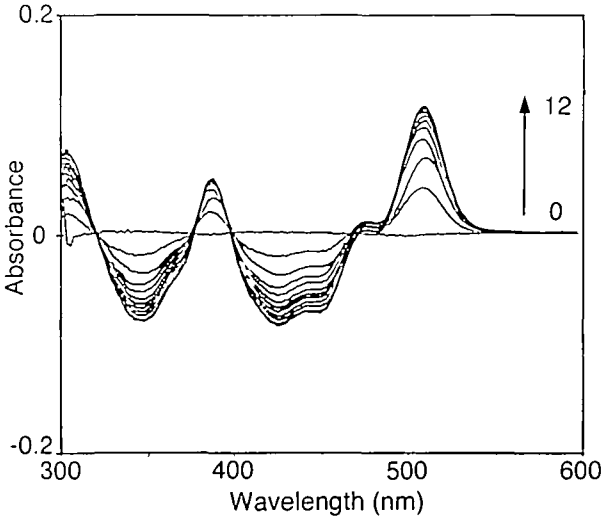

Fig. 6. **Perturbation spectra of the purified recombinant 3-ketosteroid-Δ^1-dehydrogenase by binding of androstadienedione.** 3-Ketosteroid-Δ^1-dehydrogenase (30 μM) in 50 mM potassium phosphate buffer (pH 7.2) was titrated with androstadienedione dissolved in propyleneglycol. The spectra of samples were recorded against the spectrum of the enzyme at the same concentration at 25°C. The spectra 1 to 12 were recorded with concentrations of androstadienedione of 0, 15.7, 31.1, 46.3, 61.1, 75.7, 90.0, 104, 117, 131, 144, 157, and 170 μM, respectively.

TABLE I. **Summary of the purification of recombinant 3-ketosteroid-Δ^1-dehydrogenase.**

Step	Total activity (U[a])	Total volume (ml)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification factor
Crude extract	2,190	245	3,283	0.67	100	1
DEAE-cellulose	3,151	299	523	6.0	143	9
Phenyl-Sepharose	2,664	136	263	10.2	122	15.2
Ultrogel AcA44	1,625	3.7	65	25.0	74	37.5

[a]1 U = 1 μmol androstadienedione produced/min. The purification was carried out with 23.4 g wet cells.

TABLE II. **Kinetic properties of recombinant 3-ketosteroid-Δ¹-dehydrogenase.**

Enzyme	Turnover number (mol/min/mol FAD)	K_m' (μM)	V_{max}' (mol/min/mol FAD)	K_d for ADD (μM)
Wild type	1.6×10^3	45.1	2.81×10^3	36.2
Recombinant	1.5×10^3	45.6	2.85×10^3	38.3

The enzyme assay was carried out in 50 mM potassium phosphate buffer (pH 7.2) by using DCIP as the hydrogen acceptor at 25°C.

chrous enzyme. The protein had immunologically the same reactivity against mouse polyclonal antibody raised against the *R. rhodochrous* enzyme (data not shown). The native recombinant dehydrogenase migrated with a high migration coefficient near the position of a marker dye, bromophenol blue, indicating that it has the feature of an acidic protein, like the native *R. rhodochrous* enzyme (*1*).

The spectroscopic ultraviolet/visible absorption characteristics of the recombinant 3-ketosteroid-Δ¹-dehydrogenase were recorded in different redox states (Fig. 5). Absorption maxima were observed at 276, 362, and 458 nm, being typical of a flavoprotein and similar to those of the *R. rhodochrous* enzyme. The absorption ratio A_{280}/A_{458} of 10.64 was measured with the purified recombinant enzyme. Upon reduction with androstenedione under anaerobic conditions, a dramatic decrease occurred in the absorbance in the 320–550 nm range due to the reduction of the prosthetic group, as observed with the wild-type enzyme (Fig. 5, insert) (*2*). The substrate-reduced enzyme was reoxidized by bubbling air through the solution. The spectrum of 3-ketosteroid-Δ¹-dehydrogenase was changed by binding of 3-ketosteroids (*2*). The recombinant enzyme exhibited similar perturbation spectra on binding of androstadienedione, with increasing absorption intensities at 507, 472, 392, and 308 nm and decreasing intensities at 450, 431, and 358 nm (Fig. 6). The fluorescence spectrum of the recombinant enzyme was identical to that of the *Rhodococcus* enzyme. These spectrophotometric properties indicate that the FAD molecule in the recombinant enzyme orients in the same manner to that of the *Rhodococcus* flavoprotein.

The purified recombinant 3-ketosteroid-Δ¹-dehydrogenase catalyzed the conversion of androstenedione to androstadienedione, identified by HPLC and TLC, with the apparent K_m and V_{max} values for androstenedione of 45.6 μM and 2.85×10^3 mol/min/mol enzyme FAD (pH 7.2), respectively. These are near the values of the wild-type dehydrogenase under the same assay conditions (Table II). The K_d value of 38.3 μM for androstadienedione was obtained by analysis of the perturbation spectra shown in Fig. 6. The value is almost same as that of the *R. rhodochrous* 3-ketosteroid-Δ¹-dehydrogenase (Table II).

DISCUSSION

The present study has examined the nucleotide sequence of the *ksdD* gene of 1,536 nucleotide length and the deduced amino acid sequence of 3-ketosteroid-Δ¹-dehydrogenase of *R. rhodochrous*. The dehydrogenase gene showed a high content of C+G of 68% in the coding region, and a high preferential usage (87.25%) of C- and G-ending codons; these values for *ksdD* of *A. simplex* were also high, 73.94 and 96.7%, respectively (*7*). The amino acid sequence of *R. rhodochrous* enzyme is highly homologous to that of the *A. simplex* enzyme but less so to those of the enzymes of *P. testosteroni* and *N. opaca* (*6, 8*).

The amino terminal residue of 3-ketosteroid-Δ¹-dehydrogenase of *R. rhodochrous* is Ala, which is different from the deduced amino acid sequences of other three bacterial enzymes (*6–8*). This indicates that the *R. rhodochrous* enzyme is processed to remove the methionine residue from the translated product during maturation. A consensus sequence, GSGA(G)GG(A), at position 14–19 is found in all of four known sequences of the enzymes and is confirmed to be the binding motif for adenine of FAD in flavoproteins. It is preceded and followed by many hydrophobic amino acids, residues 8–13 and residues 30–36, to make a βαβ structure (*21*). Eight well-conserved regions are seen in the four enzymes: residues 42–59, 171–175, 218–241, 406–419, 429–439, 448–462, 466–477, and 487–500 in *R. rhodochrous*. The functions of these regions are not yet known. The polypeptide chain of *R. rhodochrous* enzyme is shorter by 4 and 62 residues in total length than those of the *A. simplex* and *P. testosteroni* enzymes, respectively, and longer by 4 residues than that of the *N. opaca* enzyme (Fig. 2). The enzyme protein of *P. testosteroni* has an especially long extra domain of 44 residues from position 162 to 205. Comparison of the four amino acid sequences in Fig. 2 resulted in better alignment of the extra domain of *P. testosteroni* than that previously reported (*7*), and it could be placed between positions 162 and 166 of the *R. rhodochrous* protein.

No genes involved in the metabolism of steroids were found upstream and downstream of the 3-ketosteroid-Δ¹-dehydrogenase gene on the *Bam*HI-DNA fragment of *R. rhodochrous* encompassing it (Fig. 3). This suggests that the organism has no genomic orientation of the operon of genes for steroid-metabolizing enzyme proteins. In contrast, it is interesting that the 3-ketosteroid-Δ¹-dehydrogenase gene (*Δ¹dh*) and 3-ketosteroid-Δ⁴(5α)-dehydrogenase gene [*Δ⁴(5α)dh*] are located close to each other on the genomic DNA of *Comamonas testosteroni* ATCC17410 (*22*), and that the 3-ketosteroid isomerase gene is transcriptionally coupled with the 3-ketosteroid-Δ¹-dehydrogenase gene (*ksdD*) through a four-base overlap located at the end of the dehydrogenase gene on the *A. simplex* chromosome (*7*).

A desire to study the functional amino acid residues in the active site of the dehydrogenase by chemical modification prompted us to develop an expression system of 3-ketosteroid-Δ¹-dehydrogenase in *E. coli* cells. Expression systems of 3-ketosteroid-Δ¹-dehydrogenase were reported for *P. testosteroni* and *R. erythropolis* enzymes in *E. coli* (*6, 10*) and *A. simplex* enzyme in *S. lividans* (*7, 9*). These three expression systems, however, are not suitable for study of site-directed mutation of the enzyme or crystallographic analysis of the protein structure by distribution and yield of the overproduced enzyme. The *ksdD* gene of *A. simplex* was expressed at a low level in *S. lividans* as extracellular and intracellular products (*7*), and *ksdD* of *N. erythropolis* was expressed in *E. coli* as an insoluble and non-catalytic form reacting with the antibody against the dehydrogenase protein (*10*). In the present study, the active enzyme protein was produced efficiently with an expression system of the *ksdD* gene. The product was extractable from the *E. coli* cells by sonication or lysis

with lysozyme as a soluble protein, in the same way as the *Rhodococcus* enzyme, and purified by simple chromatographic procedures. The gene product of 3-ketosteroid-$\Delta^4(5\alpha)$-dehydrogenase of *C. testosteroni* was also distributed in the cellular membrane component (*22*). In *R. rhodochrous*, nitrile hydratase was expressed in the presence of cobalt ions, but in *E. coli* host cells, the enzyme gene was not expressed (*23*). In contrast, hyperproduction of *R. rhodochrous* nitrilase in *E. coli* was reported under the control of the *lac* promoter (*24*). When we constructed a 3-ketosteroid-Δ^1-dehydrogenase expression system, pDEX-2, under the control of the *lac* promoter gene, production of the enzyme was not observed in *E. coli* cells. By changing the control of the expression vector to the T7 promoter, we achieved the hyperexpression of the 3-ketosteroid-Δ^1-dehydrogenase gene. The purified recombinant enzyme has the same molecular mass, and electrophoretic and catalytic properties as the *R. rhodochrous* enzyme. Thus, the DNA fragment cloned from total DNA of *R. rhodochrous* is confirmed to contain the dehydrogenase gene, *ksdD*, whose nucleotide sequence has been determined. The deduced amino acid sequence and the hyperexpression system will be useful for studying the structure of the active site of the enzyme and also for the crystallographic study.

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