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Mutational Analysis of the Three Cysteines and Active-Site Aspartic Acid 103 of Ketosteroid Isomerase from *Pseudomonas putida* Biotype B

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In order to clarify the roles of three cysteines in ketosteroid isomerase (KSI) from *Pseudomonas putida* biotype B, each of the cysteine residues has been changed to a serine residue (C69S, C81S, and C97S) by site-directed mutagenesis. All cysteine mutations caused only a slight decrease in the k_{cat} value, with no significant change of K_m for the substrate. Even modification of the sulfhydryl group with 5,5′-dithiobis(2-nitrobenzoic acid) has almost no effect on enzyme activity. These results demonstrate that none of the cysteines in the KSI from *P. putida* is critical for catalytic activity, contrary to the previous identification of a cysteine in an active-site-directed photoinactivation study of KSI. Based on the three-dimensional structures of KSIs with and without dienolate intermediate analog equilenin, as determined by X-ray crystallography at high resolution, Asp-103 was found to be located within the range of the hydrogen bond to the equilenin. To assess the role of Asp-103 in catalysis, Asp-103 has been replaced with either asparagine (D103N) or alanine (D103A) by site-directed mutagenesis. For D103A mutant KSI there was a significant decrease in the k_{cat} value: the k_{cat} of the mutant was 85-fold lower than that of the wild-type enzyme; however, for the D103N mutant, which retained some hydrogen bonding capability, there was a minor decrease in the k_{cat} value. These findings support the idea that aspartic acid 103 in the active site is an essential catalytic residue involved in catalysis by hydrogen bonding to the dienolate intermediate.

Δ^5-3-Ketosteroid isomerase (KSI) catalyzes the allylic isomerization of the 5,6 double bond of Δ^5-3-ketosteroids to the 4,5 position by stereospecific intramolecular transfer of a proton at a rate approaching the diffusion limit (23, 26). This reaction is part of the steroid hormone biosynthetic pathway from cholesterol in animals and is part of the biodegradative pathway for steroids in bacteria such as *Comamonas testosteroni*, formerly known as *Pseudomonas testosteroni* (28), and *Pseudomonas putida* biotype B, which can live on steroids as a sole source of carbon.

For the past two decades, intensive efforts have been made to study the structure and catalytic mechanism of KSI from *C. testosteroni*. By means of chemical modification and site-directed mutagenesis studies, as well as other kinetic and spectroscopic studies, it has been established that two amino acid residues, Tyr-14 and Asp-38, are critically involved in the catalytic mechanism (18, 31–33). Asp-38 is believed to be the general base responsible for shuttling the substrate 4β proton to the 6β position, while Tyr-14 is thought to function as an electrophilic catalyst, either protonating or forming a low-barrier hydrogen bond with the 3-carbonyl oxygen of the steroid to stabilize a dienolate intermediate (4, 12, 13, 34, 36) (Fig. 1).

However, the existence of an as yet unidentified functional group in the *C. testosteroni* KSI active site has been suggested. Austin et al. have proposed that a hydrogen bond from a nearby donor group to Tyr-14 exists on the basis of the results from UV resonance Raman spectroscopy (1, 2). Zhao et al. have suggested that strong hydrogen bond donation from Tyr-14 to the 3-ketosteroid ligand is compensated for by other hydrogen bond donors, such as amide NH and/or bound water molecules, during ligand binding and catalysis (36, 37). Holman and Benisek have also proposed that an unidentified functional group is present at the active site of KSI and that the electrostatic environment of the Asp-38 is hydrophobic and positively charged based on the pH dependence of the rate constants for the D38E and D38H mutants (13). Although the overall picture of the enzyme's structure and mechanism has been substantially improved by X-ray crystallographic study and nuclear magnetic resonance spectroscopy (18, 19, 29), identification of the amino acids involved in the catalytic mechanism has been elusive due to the lack, until recently, of the complete three dimensional structure at high resolution.

Another KSI which has been known to be analogous to the KSI from *C. testosteroni* is the isozyme from *P. putida* biotype B. The cloning and sequencing of the gene encoding KSI from *P. putida* biotype B and its subsequent overexpression have provided an alternative opportunity to study KSI (15). This dimeric enzyme consists of two identical polypeptide chains of 131 amino acids each with a calculated molecular weight of 14,536. *P. putida* KSI is highly homologous to *C. testosteroni* KSI since the analysis of a primary sequence alignment of both proteins indicates that the critical active-site residues of *C. testosteroni* KSI, Asp-38 and Tyr-14, are conserved at Asp-40 and Tyr-16, respectively, in the *P. putida* KSI polypeptide (14, 21). The catalytic mechanism of *P. putida* KSI was investigated to understand the roles that these conserved residues play by determining the activities of the mutant enzymes, and it has been proposed that Tyr-16 and Asp-40 are very similar to Tyr-14 and Asp-38 of *C. testosteroni* KSI in their roles as catalytic residues (14). These results strongly indicate that the catalytic mechanisms of the homologous enzymes are similar.

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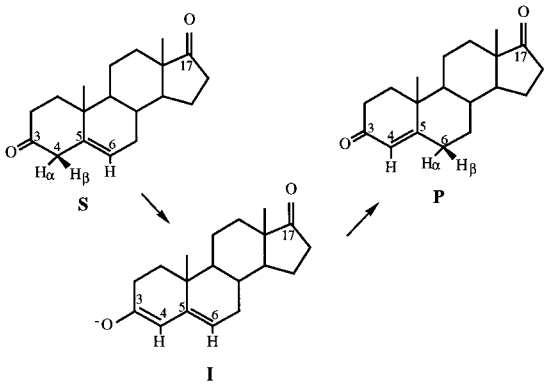

FIG. 1. Reaction catalyzed by KSI. The reaction is known to be stereospecific; the β proton at C-4 is transferred to the β side of C-6 during the isomerization reaction. The substrate (S), 5-androstene-3,17-dione, is converted to the product (P), 4-androstene-3,17-dione, via the intermediate (I). The numbering of relevant carbons is shown.

However, active-site-directed photoaffinity labeling studies of *P. putida* KSI showed that one of the cysteine residues is susceptible to modification, the extent of which correlated with the observed loss of enzyme activity (27).

The determination of the crystal structure of KSI from *P. putida* has been attempted (22), with the result that its crystal structure has been resolved to 1.9 Å and the direct observation of the unidentified amino acid with a putative functional role has been made possible (16). In the three-dimensional enzyme structure, aspartic acid 103 was found in the active-site cavity; this residue is within hydrogen bonding distance of the side chain of Tyr-16 via a bound water molecule. Furthermore, the crystal structure of KSI complexed with equilenin, an analog of the dienolate intermediate, shows clearly that the inhibitor displaces the water molecule and interacts directly with Asp-103 as well as Tyr-16. Therefore, Asp-103 located in the active site might be a new putative residue involved in catalysis by donating a hydrogen bond to the dienolate intermediate.

In order to clarify the roles of all the cysteine residues and Asp-103 in the *P. putida* KSI, these amino acids were replaced by site-directed mutagenesis and the cysteines were modified. Here we report the results of our investigation into the catalytic and structural roles of three cysteine residues and Asp-103 in *P. putida* KSI and the importance of the hydrogen bonding capability of the new active-site functional residue, Asp-103, in the catalytic mechanism of KSI.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and DNA manipulation. *Escherichia coli* XL1-Blue was grown at 37°C in Luria-Bertani medium and used for plasmid amplification. *E. coli* BL21(DE3) was used for the expression of wild-type and mutant enzymes. *E. coli* RZ1032 was used for preparing single-stranded uracil-containing DNA for site-directed mutagenesis (20). Where appropriate for plasmid maintenance, antibiotics were used at the following concentrations: ampicillin, 50 μg/ml, and tetracycline, 12.5 μg/ml. Plasmid DNA isolation, digestion, purification, ligation, electrophoresis, and bacterial transformation were carried out as described previously (24). All the enzymes for DNA manipulation were obtained from Boehringer Mannheim.

Site-directed mutagenesis. The *Eco*RI-*Hin*dIII fragments containing the *ksi* gene in the recombinant plasmid (pKK-KSI) expressing KSI (15) were ligated into the previously digested pBluescript SK(−) vector (Stratagene) to make pSK-KSI. The mutagenesis was done as described previously (14) by using uracil-containing single-stranded pSK-KSI DNA as the template and the synthetic oligonucleotide as the primer (Table 1). The entire nucleotide sequences of mutant *ksi* genes were determined by the dideoxy chain termination sequencing method (25) to verify that no other changes in the nucleotide sequences had occurred.

Overexpression and purification. The expression and purification of the wild-type and mutant KSIs were carried out as described previously (14) with the following modification: each *Eco*RI-*Hin*dIII fragment containing the mutated *ksi* gene was ligated to previously digested plasmid pKK223-3 (Pharmacia), and the recombinant plasmid was introduced into *E. coli* BL21(DE3). The cultures were grown in 200 ml of Luria-Bertani medium containing 100 mg of ampicillin per liter for 16 to 20 h at 37°C, and the expression of mutant KSIs was induced by the addition of 0.75 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at the start of growth. The purification of the wild-type and mutant KSIs was carried out with affinity chromatography as the principal step according to procedures described previously (14). The KSI from *C. testosteroni* (5) was overexpressed and purified by a method similar to that described previously (12).

UV spectroscopy and determination of protein concentration. UV absorption spectra of wild-type and mutant KSIs were obtained with a computer-interfaced UV-visible spectrophotometer (Varian Cary 3E). The difference spectrum between the spectra produced at high and low pH was obtained as follows. A pair of identical quartz cuvettes with a 1-cm light path were filled with 1.0 ml of 50 mM Tris buffer, pH 7.0, and used to produce a baseline absorption spectrum from 350 to 250 nm. After the sample cuvette was removed, cleaned, and dried, it was filled with 1.0 ml of a ca. 10 μM solution of KSI in the same buffer, and then the absorption spectrum of the sample was recorded and stored digitally. Fifty microliters of 10 N NaOH was then added to the sample cuvette. This solution was mixed well, and its absorption spectrum was recorded as described above. The difference spectrum of the sample was then generated by digitally subtracting the spectrum obtained without NaOH from the one obtained with NaOH after the latter was corrected for the dilution of the added NaOH. Protein concentration was determined by use of the difference extinction coefficient between tyrosinate and tyrosine at 295 nm.

DTNB titration. The sulfhydryl contents of the wild-type and Cys-to-Ser mutant KSIs were determined by Ellman's method (8). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB; 500 μM) was added to a solution containing 6 μM KSI, 50 mM Tris, and 1 mM EDTA at pH 7.0, either without denaturant for the determination of accessible sulfhydryl groups in the native conformation or with 8 M urea for the determination of total sulfhydryl content. The increase in absorbance at 412 nm due to the release of 5-thio-nitrobenzoate was monitored for 40 min, during which time the reaction of the accessible cysteine was completed. The same solution without the enzyme was used to measure the spontaneous breakdown of the reagent for subsequent correction of the value determined in the titration of each enzyme. An extinction coefficient of 14,000 M^{-1} cm^{-1} was used to calculate the number of sulfhydryl groups per molecule of protein (6).

Kinetic analysis. The KSI activity was determined spectrophotometrically at 248 nm in a system consisting of a 3-ml final solution containing 34 mM potassium phosphate (pH 7.0), 2.5 mM EDTA, 58.2 μM 5-androstene-3,17-dione in methanol (1.7% final concentration) and enzyme as described previously (14). The reactions were initiated by the addition of enzyme, and all rates were corrected for the blank rates which were measured prior to the addition of enzyme. Enzymes were diluted appropriately in a solution containing 1% bovine

TABLE 1. Oligonucleotides used for site-directed mutagenesis

Mutation[a]	Mutagenic oligonucleotide[b]	Eliminated restriction site[c]
C69S	5′-GGC GGG GGC AAG GTG CGC GCC AGC CTG ACC GGG C-3′	*Ava*II
C81S	5′-GG GCC AGC CAT AAC GGG AGC GGG GCG ATG C-3′	*Bgl*I
C97S	5′-TGG AAC GGC CAG CCC AGC GCA CTG GAT GTC-3′	*Apa*LI
D103N	5′-CTG GAT GTC ATC AAT GTG ATG CGC-3′	*Cla*I
D103A	5′-CTG GAT GTC ATC GCT GTG ATG CGC-3′	*Cla*I

[a] Mutations are identified by amino acid and position number in wild-type KSI followed by the substituted amino acid. Amino acids are denoted by the single-letter code.

[b] Base changes are underlined.

[c] Restriction sites were eliminated by either mutations (D103N and D103A) or silent mutations (C69S, C81S, and C97S) to facilitate the identification of clones carrying the desired mutations.

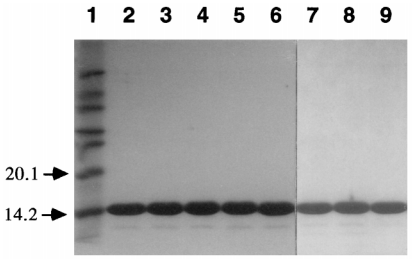

FIG. 2. SDS-PAGE analysis of wild-type and mutant KSIs of *P. putida* and *C. testosteroni* KSI. The 15% polyacrylamide gel containing 0.1% SDS was stained with Coomassie blue R 250. All the samples loaded were boiled in 0.1% SDS prior to electrophoresis. Molecular weights (in thousands) are shown at the left. Lanes: 1, molecular weight markers; 2, wild-type *P. putida* KSI; 3, D103N KSI; 4, D103A KSI; 5, C69S KSI; 6, C81S KSI; 7, wild-type *P. putida* KSI; 8, C97S KSI; 9, *C. testosteroni* KSI; The weak bands migrating faster than the KSIs are from the dye in the sample.

serum albumin and 10 mM EDTA, pH 7.0, just prior to use. All assays were performed at 30°C.

Kinetic data were analyzed with Lineweaver-Burk reciprocal plots under the assay condition in which the methanol concentration was raised to 3.3% by volume while the substrate was added at concentrations of 11.6, 34.9, 58.2, 81.5, and 116.4 μM. The V_{max} and K_m values were determined by regression analysis, and the k_{cat} values (first-order rate constants for the reaction of enzyme-substrate complex to yield product) were derived from V_{max} intersects of the double reciprocal plots. Mean values (±2 standard deviations) from three to five separate determinations were obtained for the comparison of kinetic parameters.

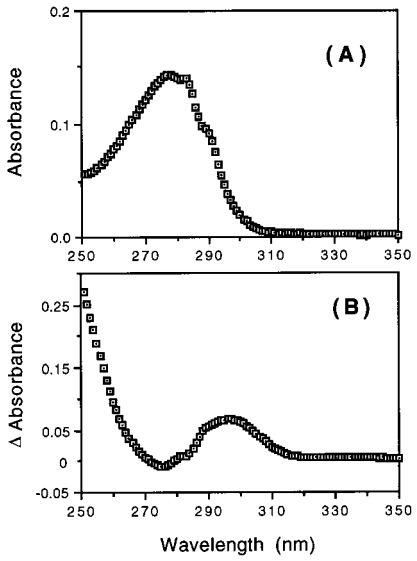

FIG. 3. (A) Ultraviolet absorption spectrum of purified wild-type KSI from *P. putida* (ca. 10 μM) in 50 mM Tris buffer, pH 7.0. (B) The difference spectrum of KSI, obtained by subtracting the spectrum at pH 7.0 from that at alkaline pH. The spectrum exhibits the absorption maximum at 295 nm originating from the difference extinction coefficient between tyrosinate and tyrosine.

RESULTS

Overexpression and purification. The overexpression of the mutant KSIs was as great as that of the wild-type KSI since the predominant 14-kDa band corresponding to the size of KSI was evident in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the extract from *E. coli* transformed with the expression vector containing the mutant *ksi* gene. The mutant KSIs and the wild-type enzyme were both found to bind specifically to the deoxycholate affinity resin during affinity chromatography. After purification, the preparations of wild-type and mutant KSI proteins were nearly homogeneous as judged by SDS-PAGE analysis (Fig. 2). The wild-type and each mutant KSI exhibited a protein band of identical size on SDS-PAGE gel, corresponding to a molecular mass of 14-kDa for the monomer. Although *P. putida* KSI has six more amino acids per subunit (calculated molecular weight = 14,536) than *C. testosteroni* KSI (calculated molecular weight = 13,399), purified *C. testosteroni* KSI had mobility similar to those of *P. putida* KSI and mutant KSIs on SDS–15% PAGE gel. The yield of the enzyme purified from a 200-ml culture of bacterial cells was variable, but it was always greater than 10 mg.

UV spectroscopy and determination of protein concentrations. A UV absorption spectrum obtained for the purified wild-type KSI at pH 7.0 is shown in Fig. 3A. The spectrum exhibits the 278-nm absorption maximum typical for a protein containing both tyrosine and tryptophan. All the mutants showed UV spectra with shapes similar to that of wild-type KSI.

In order to accurately determine the protein concentrations for wild-type and mutant KSIs, we have made use of the tyrosinate-tyrosine difference spectrum method (7). The difference spectrum obtained by subtracting the spectrum obtained at neutral pH from that at alkaline pH displays a shallow trough at 275 nm with a maximum peak at ca. 295 nm, as shown for the case of wild-type KSI in Fig. 3B. By utilizing the difference extinction coefficient (2,330 M^{-1} cm^{-1} per tyrosine residue) and the numbers of tyrosine residues in the amino acid sequences of wild-type and mutant KSIs, the concentration of the enzyme was calculated from the absorbance value for the 295-nm band in the difference spectrum. The determinations of protein concentration by this method were in good agreement with estimates for the same solutions based on the colorimetric assay of Bradford (3), but the former method was more reproducible than the latter.

Thermostability of enzymes. Relative thermal stabilities of wild-type and mutant KSIs were examined by measuring the changes of enzyme activity upon heat treatment. We have estimated the thermostability of each enzyme, as shown in Table 2. When kept at 60°C for 30 min, the wild-type enzyme retained about 74% of the initial activity; the corresponding values for mutant KSIs at the same conditions were 63% (C69S), 50% (C81S), 43% (C97S), 79% (D103N), and 91% (D103A). While the mutations of the three cysteines somewhat destabilized the enzyme, the D103A mutation were found to stabilize the protein significantly. The cysteine-free *C. testos-*

TABLE 2. Thermostability of KSI and mutants

Enzyme	Remaining activity[a] (%) at:		
	55°C	60°C	70°C
Wild type	90 ± 3.6	73 ± 1.1	23 ± 2.2
C69S mutant	84 ± 1.4	63 ± 1.3	19 ± 3.0
C81S mutant	78 ± 1.7	50 ± 3.5	19 ± 1.9
C97S mutant	90 ± 2.0	43 ± 4.0	33 ± 3.9
D103N mutant	92 ± 1.8	79 ± 0.4	20 ± 0.4
D103A mutant	95 ± 0.6	91 ± 0.6	70 ± 0.8
TI[b]	5 ± 0.6	ND[c]	ND

[a] Remaining activities after heating for 30 min at the indicated temperatures were determined under the standard assay condition with 58.2 μM 5-androstene-3,17-dione. Values are means ± 2 standard deviations for three to five separate determinations.

[b] TI, *C. testosteroni* KSI.

[c] ND, not detected.

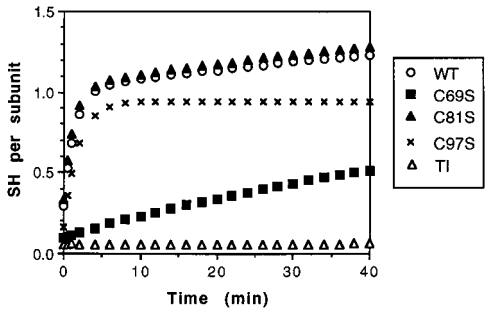

FIG. 4. Time course of the reactions of wild-type and Cys-to-Ser mutant KSIs with DTNB. The cysteine-free KSI from *C. testosteroni* was also reacted with DTNB as a control. The reactions were carried out in a solution containing 50 mM Tris (pH 7.0), 1 mM EDTA, 500 μM DTNB, and 6 μM KSI. Each reaction was monitored by the change in absorbance at 412 nm and 25°C. The *y* axis represents the number of modified sulfhydryl (SH) groups, corresponding to the change of absorbance at 412 nm. WT, wild-type KSI from *P. putida*; TI, cysteine-free KSI from *C. testosteroni*.

teroni KSI seems to be less stable than the wild-type and mutant KSIs of *P. putida* since *C. testosteroni* KSI had no detectable activity when kept at 60°C for 30 min.

DTNB titration. *P. putida* KSI is a dimer which contains six cysteine residues. In denatured conformation, the wild-type KSI was found to have 2.9 ± 0.08 free sulfhydryl groups per subunit that could be titrated by DTNB, while C69S, C81S, and C97S mutant KSIs were found to contain 1.9 ± 0.06, 1.8 ± 0.05, and 1.8 ± 0.04 free sulfhydryl groups per subunit, respectively. The wild-type enzyme in the denatured state gave the expected titer of three sulfhydryl groups per subunit when titrated with DTNB, suggesting that three sulfhydryl groups of KSI exist as free thiols. The denatured C69S, C81S, and C97S mutant enzymes also gave the expected titer of two sulfhydryl groups per subunit, which is consistent with the expected mutagenesis.

The Cys-to-Ser mutants allow the identification of each sulfhydryl group in the enzyme with the extent of its accessibility by DTNB in the native conformation. When wild-type, C69S, C81S, and C97S KSIs were reacted with excess DTNB, distinct DTNB titration curves for these four enzymes could be obtained at 25°C (Fig. 4). For wild-type and C81S KSIs, about one sulfhydryl group per subunit was rapidly titrated after exposure to 500 μM DTNB and another sulfhydryl group was slowly titrated after 5 min, while for C97S mutant KSI only one sulfhydryl group reacted rapidly with DTNB, reaching a saturation level after 5 min. The C69S KSI has a sulfhydryl group that reacts slowly with DTNB, as judged by the titration curve in which half of the sulfhydryl group was modified in 40 min. The cysteine-free KSI from *C. testosteroni* was also reacted with excess DTNB as a control, but the absorbance change at 412 nm could not be detected since it has no cysteine residue.

The slow titration of C69S KSI reveals that Cys-69 is the most reactive of the Cys residues in the enzyme, suggesting that it is positioned on the exterior of the protein. The C97S KSI reached a saturation level 5 min after the start of the DTNB reaction showing that no further titration of free sulfhydryl groups occurred. The titration curve of C97S KSI suggests that Cys-81 is the cysteine residue least reactive to DTNB in KSI, and the titration patterns of wild-type and C81S KSIs indicate that Cys-97 has an intermediate reactivity to DTNB among the three cysteines in KSI. Therefore, the distribution of the three sulfhydryl groups in KSI might be as follows: Cys-69 has an exposed sulfhydryl group, readily accessible to DTNB, Cys-97 has a semiburied sulfhydryl group, and Cys-81 has the most-buried sulfhydryl group.

We have also investigated the effect of the modified sulfhydryl group on the activities of wild-type and mutant enzymes. Modification of the exposed sulfhydryl groups with DTNB had little or no effect on the enzyme activities (data not shown).

Kinetic analysis. Specific activity, k_{cat}, and K_m values for each purified mutant were compared with those of the wild-type enzyme and are presented in Table 3. All Cys-to-Ser mutations resulted in very slight changes in the kinetic parameters of the enzyme: the k_{cat} values for Cys mutant enzymes were 89 to 97% of that for the wild-type enzyme. The effect of the cysteine-to-serine mutations on the K_m for 5-androstene-3,17-dione was either minor decreases of 1.04-fold (C81S) and 1.1-fold (C69S) or a slight increase of 1.2-fold (C97S). The largest change among the Cys mutants was observed for C97S KSI, whose k_{cat}/K_m value was 1.4-fold less than that of wild-type KSI. Since mutations of the three cysteine residues did not show any significant effect on the catalytic efficiency of the isomerase, the possibility that any of the cysteine residues is involved in the catalytic reaction is remote.

However, k_{cat} values are significantly reduced for D103A KSI (ca. 85-fold) relative to that of wild-type KSI, while D103N KSI has a slightly decreased k_{cat} value (3.8-fold) relative to the wild-type enzyme. Both mutations of aspartic acid 103 give decreased K_m values for substrate 5-androstene-3,17-dione, implying that the hydrophobic interaction at the active site is important in binding the hydrophobic steroid molecule.

DISCUSSION

In the work presented here, the roles of all cysteine residues and Asp-103 in the catalytic mechanism of *P. putida* biotype B KSI were investigated by site-directed mutagenesis. The catalytic contribution of cysteine residues in *P. putida* KSI has been suspected even though active-site-directed photoaffinity labeling studies of *P. putida* KSI showed that one of the cysteine residues is susceptible to a modification that correlates to the

TABLE 3. Kinetic parameters of KSI and its mutants[a]

Enzyme	Sp act (μmol min^{-1} mg^{-1})[b]	k_{cat} (s^{-1})[b]	K_m (μM)[b]	k_{cat}/K_m (M^{-1} s^{-1})	Relative k_{cat}	Relative K_m
Wild type	48,048 ± 1,054	21,496 ± 724	50.8 ± 2.3	4.23×10^8	1.00	1.00
C69S mutant	48,337 ± 1,655	20,888 ± 934	45.3 ± 2.3	4.61×10^8	0.97	0.89
C81S mutant	47,009 ± 2,095	20,928 ± 1,298	48.2 ± 3.4	4.34×10^8	0.97	0.95
C97S mutant	38,053 ± 1,388	19,068 ± 1,069	60.2 ± 6.4	3.17×10^8	0.89	1.19
D103N mutant	14,592 ± 801	5,709 ± 271	36.8 ± 0.7	1.55×10^8	0.27	0.72
D103A mutant	700 ± 30	254 ± 15	31.8 ± 2.0	7.99×10^6	$10^{-1.9}$	0.63

[a] The assays were performed in 34 mM potassium phosphate buffer containing 2.5 mM EDTA, pH 7.0.
[b] Values are means ± 2 standard deviations from three to five separate determinations.

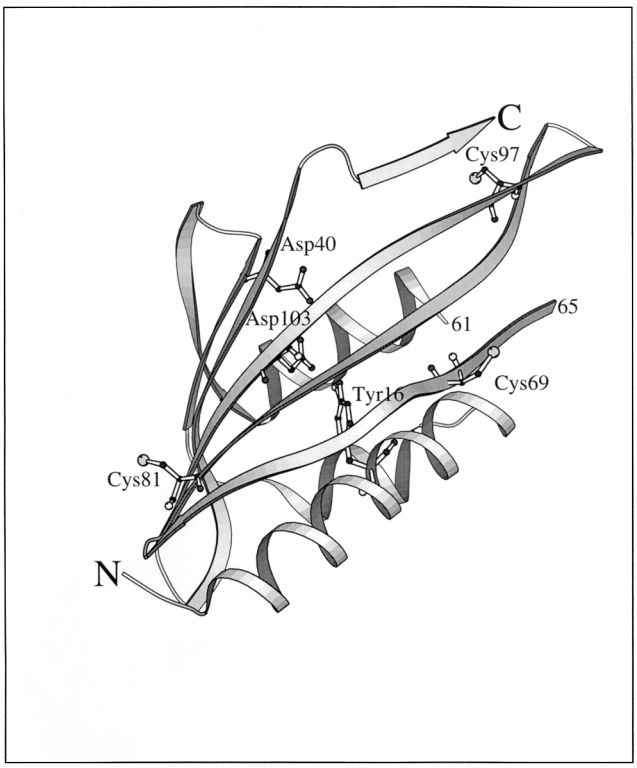

FIG. 5. Ribbon diagram of a monomer of *P. putida* KSI. Three cysteine residues and the active-site residues in the enzyme are shown in a ball-and-stick model. The catalytic residues (Tyr-16, Asp-40, and Asp-103) are included to show the active-site cavity. The ribbon diagram of the KSI structure does not contain residues 1 and 62 to 64 and C-terminal residues 128 to 131 since no or very weak electron densities were observed for these residues. The residues at positions 62 to 64 are glycines. Those residues are supposed to be very flexible. This figure was prepared by using MOLSCRIPT (17).

loss of enzyme activity (27). The position of the photolabeled cysteine in the primary structure also remains unknown. This earlier investigation left open the question of whether one or more cysteines are really involved in catalysis. Moreover, the cysteine residues of *P. putida* KSI are not conserved in the homologous *C. testosteroni* KSI, which does not have any cysteine residue at all, and a previous report (14) suggested that the catalytic mechanism of *P. putida* KSI seems to be similar to that of the homologous *C. testosteroni* KSI, on the basis of site-directed mutagenesis of conserved active-site residues.

The *P. putida* KSI has three sulfhydryl groups per subunit. But neither any replacement of each cysteine with serine by site-directed mutation nor modification of accessible sulfhydryl groups with DTNB resulted in any sizable loss of enzyme activity. Furthermore, the three-dimensional crystal structure of *P. putida* KSI clearly shows that none of the cysteines can be found in the active-site cleft of the KSI (Fig. 5).

The three-dimensional structure of *P. putida* KSI shows that the three cysteines in the native conformation of the enzyme exist as free sulfhydryl groups. Cys-69 is located on the surface of the molecule, Cys-81 is located near the dimer interface, and Cys-97 is located near the surface of the active-site cleft. The locations of three cysteine residues in the crystal structure are consistent with the results of the DTNB titration experiments. Calculations based on the three-dimensional X-ray structure of *P. putida* KSI confirmed that all three cysteines per subunit are located either between 13.6 and 18.0 Å away from one of the active-site residues, Tyr-16, or between 15.1 and 17.0 Å away from the other active-site residue, Asp-40. The crystal structure of the KSI docked with a competitive inhibitor, equilenin, also supports the hypothesis that all the cysteine residues are not located at the binding site of the inhibitor, which is positioned very close to such catalytic residues as Tyr-16 and Asp-40 and to the putative catalytic residue, Asp-103 (16). Thus, the results presented here demonstrate that none of the cysteines in KSI from *P. putida* is essential for catalytic activity.

The KSI reaction is initiated by abstraction of the 4β proton adjacent to a carbonyl group by a general base catalyst. This type of reaction, which involves proton abstraction from a carbon atom adjacent to a carbonyl or carboxylic acid group (α proton of a carbon acid), is very common in many enzyme systems. Structurally characterized examples include mandelate racemase, triose-phosphate isomerase, and citrate synthase (9, 10). Since the α protons of carbon acids are not very acidic, the precise mechanism by which the α proton is labilized during catalysis is of considerable interest. It is proposed that an electrophilic catalyst positioned adjacent to the carbonyl oxygen of the substrate is primarily responsible for stabilizing the intermediates (10). The available three-dimensional structure of KSI complexed with equilenin confirms that, invariably, electrophilic catalysts are positioned proximal to the carbonyl group of the substrate at the active site as suggested previously (11).

Tyr-16 in *P. putida* KSI is the only amino acid that has been identified as an electrophilic catalyst for the stabilization of the dienolate intermediate so far. However, analysis of the recently determined high-resolution X-ray structure of KSI from *P. putida* biotype B allowed the determination of the precise locations for all the residues at the active site, and Asp-103 was found to be located close to the oxygen of the dienolate intermediate analog. Thus, it was suggested that Asp-103 was another electrophilic catalyst for the stabilization of the dienolate intermediate by hydrogen bonding. Therefore, in order to test the importance of the hydrogen bonding capability of Asp-103, we have prepared the D103N and D103A mutant isomerases.

The D103A mutation in the isomerase resulted in a significant decrease in the k_{cat} value (85-fold), while the D103N mutation resulted in a rather slight decrease in the k_{cat} value (3.8-fold), implying that the hydrogen bonding capability of Asp-103 is very important. The effect of mutations is not likely to come from any change of the overall structure of each mutant protein since the following results confirmed that no significant conformational change in the mutant enzymes occurred: (i) both mutants bound specifically to a deoxycholate affinity resin and (ii) the UV spectra of both mutants showed the same shape as that of the wild-type KSI.

It was also found that the replacement of Asp-103 by alanine had a significant effect on the thermostability of the enzyme. When kept at 60°C for 30 min, D103A KSI was more stable than wild-type KSI. The substitution of aspartic acid with the nonpolar amino acid at the active site seems to stabilize the protein significantly since the hydrophobic interaction can induce favorable packing of amino acids at the active site. Moreover, the high-resolution crystal structure also showed that the active-site environment of KSI is extremely apolar (16).

Newly identified catalytic residue Asp-103 of *P. putida* KSI is conserved at Asp-99 in *C. testosteroni* KSI (14). Recently, the secondary structure and three-dimensional structure of *C. testosteroni* KSI were determined by nuclear magnetic resonance spectroscopy (30, 35). The overall three-dimensional structure of *C. testosteroni* is remarkably identical to that of *P. putida* KSI even though the sequence identity between the two isozymes is only 34%. From the results of a mutational study, Wu et al.

suggest that Asp-99 plays an important role in stabilizing the intermediate and transition states (30). On the basis of these results, it is reasonable to propose that the catalytic reactions of both *P. putida* and *C. testosteroni* KSIs have nearly identical mechanisms and that the structural information about *P. putida* KSI seems to be directly applicable to the study of KSI from *C. testosteroni*.

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