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Essential Tyrosine Residues in 3-Ketosteroid- Δ^1 -Dehydrogenase from *Rhodococcus rhodochrous*

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Tetranitromethane treatment of 3-ketosteroid- Δ^1 -dehydrogenase of *Rhodococcus rhodochrous* caused loss of the catalytic activity in a time- and concentration-dependent manner. Peptides (P-81) and (PN-83) were isolated from tryptic digests of the native and tetranitromethane-treated enzyme proteins, respectively. PN-83 was the nitrated form of P-81. The amino acid sequence was GGAPLIDYLESDDDLEFMVYPWPDYFGK (positions 97-124 of the dehydrogenase sequence). PN-83 showed a low yield of PTH-Tyr of position 116, *i.e.* less than 5% of that of P-81, and instead a high yield of PTH-3-nitrotyrosine. This indicated that tetranitromethane modifies Y-116 under the experimental conditions used. Mutation of Y-104, Y-116, and Y-121 to smaller amino acid residues, Phe, Ser, or Ala, significantly changed the catalytic activity of the dehydrogenase. All of the mutants contained FAD and exhibited the same spectrophotometric properties as those of the wild type enzyme. The K_m values for 4-androstene-3,17-dione of the Y-104, Y-116, and Y-121 mutants changed to large values. The most drastic change was observed for Y116A. The K_d values for 1,4-androstadiene-3,17-dione of the Y116 mutants changed to 1.5-2.6-fold larger values than that of the recombinant enzyme. The Y-121 mutant enzymes exhibited catalytic activities like those of the recombinant enzyme, but the catalytic efficiencies of Y121F and Y121A drastically decreased to 0.014-0.054% of that of the recombinant enzyme. The present results indicate that Y-121 plays an important role in the catalytic function, and that Y-116 and Y-104 act on binding of the substrate steroid.

Key words: chemical modification, 3-ketosteroid- Δ^1 -dehydrogenase, kinetic analysis, steroid binding site, Tyr-mutants.

3-Ketosteroid- Δ^1 -dehydrogenase is a flavoenzyme catalyzing the introduction of a double bond to the C1- and C2-positions of the chemically stable, aliphatic cyclic structure of 3-ketosteroids. The reaction has been utilized in the pharmaceutical industry for the production of physiologically important steroids. The enzyme has been purified from several microorganisms, its molecular and enzymatic properties have been characterized. Recently, the enzyme genes have been cloned from four microorganisms and sequenced (1-4). The most detailed studies were carried out by us on the enzyme from *Rhodococcus rhodochrous* (4-10). The holo-dehydrogenase has a molecular mass of 54,949 Da, with 510 amino acid residues (4), and exhibits the typical spectrophotometric properties of a flavoprotein (6). We also successfully constructed the expression system of the dehydrogenase in *Escherichia coli* cells and could purify a large amount of the recombinant enzyme of high

quality (4). To clarify the reaction mechanism, we studied the chemical modification of the enzyme and reported the involvement of a histidyl residue and an arginyl one in the steroid-binding site as functional groups (9, 10). To obtain further information on the active site, we studied the modification and mutation of the tyrosyl residues of 3-ketosteroid- Δ^1 -dehydrogenase.

This paper deals with the modification of 3-ketosteroid- Δ^1 -dehydrogenase with TNM and the identification of one of the modified tyrosyl residues as tyrosine-116. We also describe the site-directed mutagenesis of three tyrosyl residues, Y-104, Y-116, and Y-121, and the clarification of the function of each residue of the dehydrogenase in the catalytic reaction.

MATERIALS AND METHODS

Materials—TNM, ampicillin and IPTG were purchased from Nacalai Tesque, and dabcyl chloride and kanamycin sulfate were from Wako Pure Chemicals. Steroids and TPCK-trypsin were obtained from Sigma. 3-Nitrotyrosine was from Research Organics. All restriction enzymes were from Boehringer Mannheim. *Taq*-DNA polymerase was from Takara Shuzo. All other chemicals used were of the highest purity available.

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Abbreviations: AD, 4-androstene-3,17-dione; ADD, 1,4-androstadiene-3,17-dione; IPTG, isopropyl- β -D-thiogalactoside; *ksd*, 3-ketosteroid- Δ^1 -dehydrogenase gene; PCR, polymerase chain reaction; PTH, phenylthiohydantoin; TNM, tetranitromethane.

Bacterial Strains and Plasmids—*R. rhodochrous* IFO-3338 was used as the source of the enzyme protein (5). *E. coli* JM109 competent cells were purchased from Toyobo, and *E. coli* BL21(DE3) competent cells and plasmid pET28a(+) were obtained from Novagen.

Enzyme Assay—The steroids used for the experiments were dissolved in propyleneglycol and their concentrations were determined spectrophotometrically as described (5). Dehydrogenase activity was determined by the method described (5). The assay mixture comprised 50 mM Tris-HCl buffer, pH 7.5, 200 μ M phenazine methosulfate, 200 μ M 2,6-dichlorophenol indophenol, and 200 μ M AD in a final volume of 0.5 ml. The K_m and V_{max} values were determined in 50 mM Tris-HCl buffer, pH 8.2. The dissociation constants of the TNM-modified enzyme and mutant enzymes against ADD, the reaction product, were determined by the spectrophotometric method (5).

The concentrations of the purified wild and mutant 3-ketosteroid- Δ^1 -dehydrogenases were determined spectrophotometrically as described (5). Protein concentrations were determined by the BCA method using bovine serum albumin as the standard.

Chemical Modification—The nitration of the 3-ketosteroid- Δ^1 -dehydrogenase protein was carried out in 50 mM Tris-HCl buffer, pH 8.0, at 25°C in the dark. The concentration of a freshly prepared solution of TNM in 95% ethanol was determined by adding excess tyrosine in 50 mM Tris-HCl buffer, pH 8.0, and then measuring the absorbance at 428 nm ($\epsilon_{428} = 4.2$) (11). The TNM solution was added to a final concentration of 0–400 μ M to 0.5 ml of a solution comprising 1 μ M enzyme in 50 mM Tris-HCl buffer, pH 8.0. The enzyme activity during the nitration was monitored by taking an aliquot of the sample and then assaying the residual enzyme activity. The number of nitrated tyrosyl residues was estimated from the absorbance at 428 nm after gel-filtration on a G-25 column to remove the unreacted reagent. The number of unmodified residues was determined by subtracting the number of modified residues from the 19 tyrosyl residues of the enzyme protein.

For peptide mapping, the enzyme (10 μ M) was nitrated with 180 μ M TNM in 0.2 ml of 50 mM Tris-HCl buffer, pH 8.0, for 45 min at 25°C. The nitrated protein was collected, washed with distilled water by centrifugation, and then freeze-dried. The dried protein was denatured by treatment with hot ethanol and then suspended in 400 μ l of 1% NH_4HCO_3 , pH 9.0. The protein sample was digested with TPCK-trypsin (protein/enzyme ratio=50:1) at 37°C for 15 h. The digest was freeze-dried, dissolved in 0.1% trifluoroacetic acid, and then subjected to HPLC. Chromatography on a Nucleosil-100 C_{18} column (5 μ m, 4.6 \times 250 mm) was performed with 0.1% trifluoroacetic acid (solvent A) and 0.05% trifluoroacetic acid-containing acetonitrile (solvent B), with a linear gradient of 0 to 60% of solvent B, at the flow rate of 0.4 ml/min at 38°C, and monitored at 214 nm. Peptide fractions were collected, freeze-dried, and then rechromatographed under the same conditions.

Amino Acid Analysis and Amino Acid Sequencing—The precolumn labeled method involving dabcyl chloride was used for amino acid analysis (12). A sample protein or peptide preparation was hydrolyzed by the gas-phase method for 24 h at 115°C and then evaporated to dryness. The hydrolyzate was dissolved in 20 μ l of 25 mM sodium

bicarbonate, pH 9.0, and then reacted with 300 nmol of dabcyl chloride in 40 μ l of acetonitrile at 70°C for 15 min. The dabcylated preparation was diluted with 50% acetonitrile and then injected into the HPLC. Chromatography was carried out on a C_{18} -column (5 μ m, 4.6 \times 250 mm) with 45 mM sodium acetate buffer, pH 6.3, containing 4% dimethylformamide and acetonitrile as the solvent system. 3-Nitrotyrosine was also treated with dabcyl chloride and then subjected to HPLC.

The amino acid sequences of the peptides and protein were determined with an Applied Biosystems protein sequencer model 476A. The retention time of PTH-3-nitrotyrosine was determined by using free 3-nitrotyrosine and a tyrosine-containing peptide nitrated before the sequence analysis.

Site-Specific Mutagenesis—Site-specific mutagenesis of the gene encoding 3-ketosteroid- Δ^1 -dehydrogenase from *R. rhodochrous* was performed using plasmid pDEX-2 described in the previous paper (4). The oligonucleotide, 5'-ACCTCGAATTCATGGTGXXXCCGTG-3' [where XXX denotes the replacement of TAC for TTC (Y116F), TCC (Y116S), and GCC (Y116A), respectively], was used as a primer for the construction of Y-116 mutants. The oligonucleotide, 5'-ACCTCGAATTCATGGTGTACCCGTGGCCCGACXXXTTCCGG-3' [where XXX denotes the replacement of TAC for TTC (Y121F), TCC (Y121S), and GCC (Y121A), respectively], was used as a primer for the construction of Y-121 mutants. An oligonucleotide, 5'-GC-CGAGCGGGCCGCGGATCG-3', was used as a primer with one of the above primers for PCR. The reaction product was digested with *EcoRI* and *KspI* to yield a 142-bp DNA fragment, which was then purified. The plasmid, pDEX-2, was also digested with the same restriction enzymes and ligated with the 142-bp fragment to obtain pDEX-2M. The plasmid was digested with *NcoI* and *BamHI*, and the DNA fragment containing the mutated *ksdD* gene was ligated to pET28a(+) digested previously with *NcoI* and *BamHI* to obtain the expression plasmid for the mutated enzyme.

For preparation of the Y104A mutant, oligonucleotides 5'-AAGGCAACAGCCATGGCGGA-3' and 5'-CCATGAA-TTCGAGGTCGTCTCGGACTCGAGGGCGTTCGAT-3' were used as the primers for PCR with plasmid pD1 DNA as a template (4). The product was digested with *NcoI* and *EcoRI*, and a 330-bp DNA fragment was obtained and ligated with plasmid pDEX-2 digested previously with the same restriction enzymes. The plasmid containing the Y104A-mutated *ksdD* gene was digested with *NcoI* and *BamHI*, a mutant plasmid, pDEX3-Y104A, being obtained as described above. All mutants were confirmed by nucleotide sequencing. Each mutant plasmid was introduced into *E. coli* BL21(DE3) and then the mutant enzyme was hyperexpressed by the reported method (4).

Enzyme Purification—The mutant enzymes were purified by a slight modification of the purification protocol developed for the recombinant enzyme (4). The cell extract was chromatographed on a DEAE-cellulose column, and then the enzyme fractions were combined, concentrated, and applied on a column of Ultrogel AcA44. The eluted enzyme fractions were concentrated and then subjected to preparative gel electrophoresis on acrylamide gels (8.5 \times 5 \times 0.2 cm). The purified mutant enzymes were concentrated and stored at -80°C.

RESULTS

Modification of 3-Ketosteroid- Δ^1 -Dehydrogenase with TNM—TNM markedly inactivated the dehydrogenase in time and concentration dependent manners (Fig. 1A). With a low concentration of TNM, the plot of $\log v_i/v_0$ against the incubation time gave a curved line, suggesting that the inactivation proceeds through modification of more than one tyrosyl residue with different reactivities. The inactivation rate constant was estimated to be $500 \text{ min}^{-1} \cdot \text{M}^{-1}$ by plotting the apparent rate constants of the fast reaction against the TNM concentrations (insert in Fig. 1A). The number of modified tyrosyl residues of the enzyme increased with a longer incubation time with TNM (Fig. 1B), and reached 3 mol Tyr/mol enzyme. When the first of the tyrosyl residues was modified, the catalytic activity decreased to about 30% of the original level. This suggested that this tyrosyl residue is essential for the catalytic function of the enzyme. The apparent dissociation constant for ADD of the TNM-modified enzyme preparation was examined and found to be elevated to about 4 times greater than that of the native enzyme preparation ($K_d = 36.2 \mu\text{M}$).

Isolation of Nitrated Tryptic Peptides—The enzyme treated with $180 \mu\text{M}$ TNM, which lost about 85% of the original catalytic activity, was denatured and digested with TPCK-trypsin. The digest was subjected to HPLC, with development with a solvent system of trifluoroacetic acid-acetonitrile. Figure 2 shows the elution patterns of peptides of the native and modified enzymes; a peptide (P-81) was eluted for the native enzyme protein digest at 81 min (Fig. 2A). In contrast, in the elution pattern of the nitrated protein digest, the amount of the peptide decreased considerably to less than 20% in the case of that of the native enzyme and a new peptide (PN-83) was eluted at 83 min (Fig. 2B). Peptides P-81 and PN-83 were isolated, purified, and analyzed as to their amino acid compositions and sequences. Both peptides have the same amino acid composition with the exception of the number of tyrosyl residues. Peptide P-81 contains 3 mol of Tyr, and peptide PN-83 2 mol of Tyr and 1 mol of 3-nitrotyrosine, which was identified by chromatographic analysis. The amino acid sequence of peptide P-81 was determined to be GGAPLIDY-LESDDLEFMVYPWPYFGK. This sequence corresponds

well to that of positions 97-124 of the deduced amino acid sequence of the dehydrogenase (4). The sequence of peptide PN-83 was determined to be GGAPLIDY-LESDDLEFMVY*PWPYFGK, where Y* is a 3-nitrotyrosine residue. PN-83 showed a low yield of PTH-Tyr at position 116, i.e. less than 5% of that of P-81, and instead a high

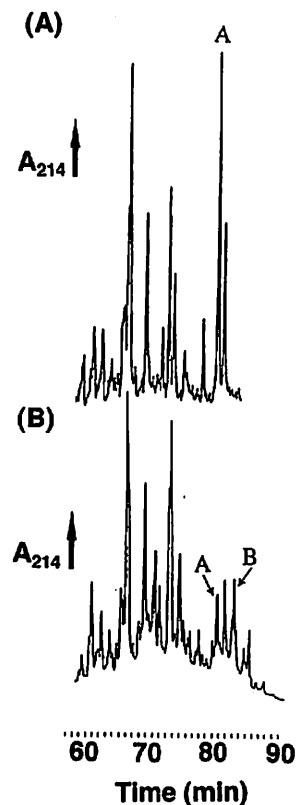
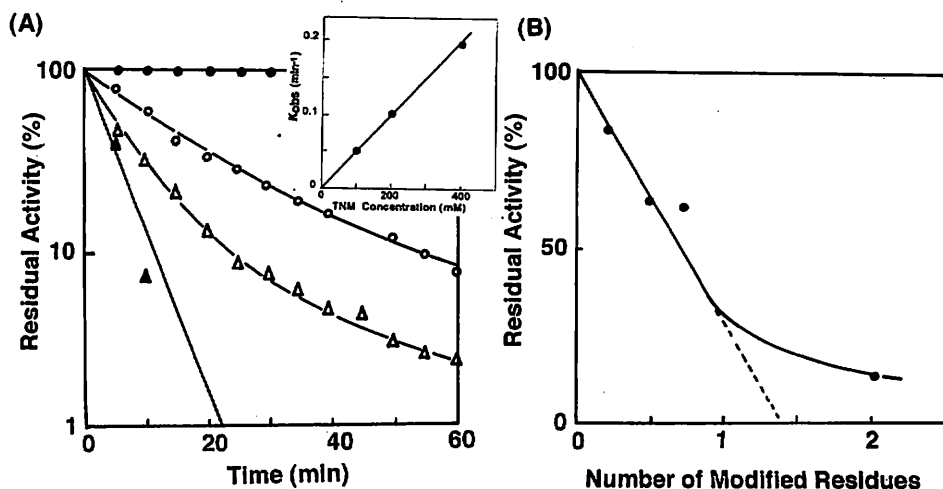


Fig. 2. Chromatographic separation of the tryptic peptides of TNM-modified 3-ketosteroid- Δ^1 -dehydrogenase. The native enzyme ($10 \mu\text{M}$) was treated with $180 \mu\text{M}$ TNM in 50 mM Tris-HCl buffer, pH 8.0, for 45 min at 25°C. After denaturation, the protein sample was digested with TPCK-trypsin and then subjected to HPLC. The elution pattern was monitored at 214 nm. The figures show part of the elution patterns from 60 min to 90 min. (A) Peptides of the native enzyme and (B) peptides of the TNM-modified enzyme. Peaks A and B represent peptides P-81 and PN-83, respectively.

Fig. 1. Inactivation of 3-ketosteroid- Δ^1 -dehydrogenase with TNM. (A) Effects of the concentration of TNM and the incubation time on the inactivation. The enzyme ($0.97 \mu\text{M}$) was incubated at pH 8.0 at 25°C with TNM; 0 (\bullet), 100 (\circ), 200 (Δ), and 400 (\blacktriangle) μM . The inset in the figure shows secondary plots of k_{obs} against the concentration of TNM. (B) Plots of the residual activity against the number of modified tyrosyl residues. The enzyme ($10 \mu\text{M}$) was incubated in 50 mM Tris-HCl buffer, pH 8.0, at 25°C with 0-400 μM TNM for 20 min, and then the residual enzyme activity and the absorbance at 428 nm, to estimate the number of modified tyrosine residues, were determined.



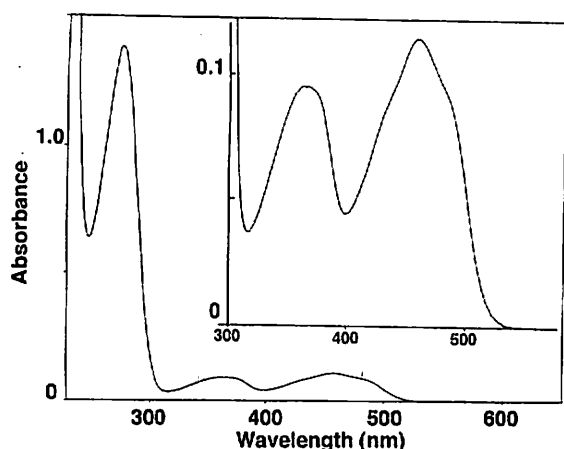


Fig. 3. Absorption spectrum of the Y116F mutant. The purified mutant enzyme (10 μ M) in 30 mM Tris-HCl buffer, pH 7.4, was used to obtain the spectrum.

yield of PTH-3-nitrotyrosine. It is noteworthy that three tyrosyl residues located in the isolated peptides are Y-104, Y-116, and Y-121, and Y-116 was modified to 3-nitrotyrosine with TNM. Other small differences in the elution patterns can be observed in the figures. These might be due to small differences in the digestion conditions. One or two modified peptide(s) might be present in them but they could not be found in this analysis.

Preparation and Characterization of Tyr-Mutated Enzymes—Based on the results described above and comparison of the amino acid sequences of the 3-ketosteroid- Δ^1 -dehydrogenases from 4 species (4), 3 tyrosyl residues were selected to prepare mutant enzymes. All of the Tyr mutant enzymes, *i.e.* Y104A, Y116A, Y116S, Y116F, Y121A, Y121S, and Y121F, were expressed at high levels in *E. coli* BL21(D3) harboring the mutated pDEX plasmid, and were purified by means of simplified procedures in high yields (about 1–2.5 mg/g wet cells). The Y-104, Y-116, and Y-121 replacements did not cause any changes in the optical properties of the flavin prosthetic group: the flavin absorption spectra of all mutants were nearly the same as those of the recombinant and also the wild type enzyme, showing absorption peaks at 458 and 362 nm in the visible region (Fig. 3) (4, 6). Moreover, the flavin perturbation difference spectra of the mutants in the presence of ADD were also similar to that of the wild type enzyme, indicating no significant change in the flavin conformation in the enzyme molecule. The steady state kinetic parameters of the Tyr mutants were studied at pH 8.3, the optimum pH for the turnover of the wild type enzyme (5). The Y-116 mutants showed elevated apparent K_m values for AD of one or two orders of magnitude greater than that of the recombinant enzyme (Table I), and the dissociation constants for ADD of the Y-116 mutants became larger values of 46 to 99 μ M, like the dissociation constant of the TNM-modified enzyme described above. In particular, the K_m value of Y116A changed to become the largest obtained. These results indicate that the type of amino acid residue engineered at position 116 drastically affects the substrate binding.

The Y104A mutant exhibited high catalytic activity corresponding to that of the recombinant enzyme, and some change in the K_m value (Table I), suggesting that Y-104

TABLE I. Summary of the kinetic parameters of the tyrosine-mutants of 3-ketosteroid- Δ^1 -dehydrogenase.

| Enzyme | K_m^a | V_{max}^b | V_{max}/K_m | Ratio ^c | K_d for ADD ^d |
|-------------|---------|--------------------|-----------------|----------------------|----------------------------|
| Recombinant | 21 | 4.6×10^3 | 219 | 100 | 38.3 |
| Y104A | 117 | 5.0×10^3 | 43 | 19.6 | nd. |
| Y116F | 166 | 6.5×10^3 | 39 | 18 | 46.1 |
| Y116S | 600 | 13.3×10^3 | 22 | 10 | 71.6 |
| Y116A | >3,000 | — ^e | ~4 ^f | ~1.8 | 99.2 |
| Y121F | 420 | 12 | 0.03 | 1.4×10^{-2} | nd. |
| Y121S | 270 | 4.4×10^3 | 16 | 7.3 | nd. |
| Y121A | 695 | 80 | 0.12 | 5.4×10^{-2} | nd. |

^a $K_m = \mu$ M AD. ^b $V_{max} = \text{mol/min/mol enzyme}$. ^c V_{max}/K_m of the mutant against that of the recombinant enzyme. ^d $K_d = \mu$ M ADD, nd. = not determined. ^eThe value could not be determined by the large K_m value. ^fThe value was estimated from the slope of a double reciprocal plot of v/s .

does not directly function in the catalytic center of the enzyme. Y-121 is one of the conserved residues in 3-ketosteroid- Δ^1 -dehydrogenase (4). The Y-121 mutants exhibited more drastic changes in the catalytic activity and also the V_{max} value (Table I), indicating that this tyrosyl residue functions differently from Y-116 and Y-104, and plays a crucial role in the catalytic reaction. Mutagenic effects were also demonstrated on the catalytic efficiency of the enzyme; the mutation at Y121 largely caused the lowering of the efficiency. Among them, Y121S exhibited 7.3% of the catalytic efficiency of the recombinant enzyme but had a high V_{max} value, which is almost the same as that of the recombinant enzyme. This suggests that the hydroxyl group of the seryl residue functions as that of Y-121.

DISCUSSION

In the previous study, histidyl and arginyl residues were suggested to be involved in the active site of 3-ketosteroid- Δ^1 -dehydrogenase (9, 10), but their locations are not clear in the enzyme molecule. We proposed that the arginyl residue plays a role in the interaction with the C3-carbonyl oxygen of a substrate steroid as an electrophile to activate 2β -hydrogen, and the histidyl residue, as a nucleophile, acts to abstract 2β -hydrogen from the substrate in the active site to facilitate the transfer of 1α -hydrogen to the alloxazine ring of the flavin prosthetic group.

Regarding tyrosyl residues of the enzyme, we attempted modification of the enzyme with TNM to obtain useful data for mutagenic experiments.

The results obtained on chemical modification did not agree well with those of mutagenic experiments. The enzyme preparation with Y-116 modified with TNM greatly lost the catalytic activity and did not show rapid reduction of FAD of the enzyme with AD under anaerobic conditions (data not shown). The Y-116 mutants, however, did not change the catalytic activities greatly. This may be due to that properties of the mutated amino acid residues are different from those of the nitrated tyrosyl residue in structure, size and polarity. Nitration of the enzyme changed the K_d value for ADD, the reaction product steroid, to a fourfold larger value; indicating that the binding of ADD to the enzyme molecule is hindered by nitration of tyrosyl residue(s) of the active site. This evidence suggests that the large and polar nitro group considerably affects the interaction between the substrate steroid and amino acid

residues in the active site. In contrast, the mutation of Y-116 caused lower increases, 1.2-2.6-fold, in the K_m values.

Analysis of tryptic peptides of the native and TNM-modified enzyme proteins revealed that a peptide (P-81) comprising positions 97-124 contains three tyrosyl residues, of which Y-121 is well conserved in the 3-ketosteroid- Δ^1 -dehydrogenases from four microorganisms (4), and another peptide (PN-83) contained one 3-nitrotyrosine residue at position 116. This peptide (PN-83) could be detected with the enzyme protein digest modified for a short period and retained more than 50% of the original enzyme activity (data not shown). These results indicate that the Y-116 residue was preferentially nitrated in the fast phase of TNM modification. We could not obtain the 2nd modified peptide; perhaps the peptide was produced in the slow phase reaction, and thus its amount was too low to be detected with the HPLC system.

To understand the role of these tyrosyl residues in the catalytic reaction, we constructed expression plasmids of Tyr-mutant enzymes, and could hyperexpress the mutant enzymes in *E. coli* and obtain the purified enzymes. The molecular and spectrophotometric properties of the Y-104, Y-116, and Y-121 mutant enzymes showed no significant changes on engineering of the residues to other amino acid species. However, alteration of the catalytic properties was clearly observed (Table I).

The results suggest that Y-104 is located inside the protein molecule as a buried tyrosyl residue, which does not react with TNM, and is not involved directly in the catalytic function, as evidenced by that the mutation of the residue to Ala did not affect the V_{max} value, but did the K_m one. It is assumed to be involved in the binding of a substrate steroid. Y-116 reacts easily with TNM. The results suggest that the residue is located near the surface of the protein molecule and is in direct contact with the solvent.

The K_m' value of the Y-116 mutant without the hydroxyl group (Y116F) or without an aromatic ring (Y116S) became a one order of magnitude larger value than that of the recombinant enzyme. The influence was further observed with the Y116A mutant, whose K_m' was more than 150-fold greater than that of the recombinant enzyme. These facts indicate that the phenolic group of Y-116 is indispensable for binding of the substrate in the catalytic site.

Y-121, which is a conserved residue in 3-ketosteroid- Δ^1 -dehydrogenase, is more essential for the catalytic function. The replacement of this residue with Phe or Ala cause a great loss of the catalytic activity, but with Ser almost the same V_{max} value was observed. This suggests that the hydroxyl group of the Ser residue at position 121 functions in the catalytic cycle as the hydroxyl group of the Tyr residue in the wild type enzyme. Since the phenyl group of Phe can not be replaced as to the function of the enzyme, the hydroxyl group is important and indispensable for the enzyme. As reported in the case of steroid isomerase (13), it seems to form a hydrogen bond system with other functional amino acid residues to abstract α,β -hydrogens from the substrate. To elucidate more clearly the function of each tyrosyl residue, it is necessary to carry out a crystallographic study on this enzyme.

Acyl CoA dehydrogenase catalyzes the same type of *trans*-axial dehydrogenation and has a Glu residue that

functions in the active site to interact with the α -hydrogen of a substrate (14). Steroid isomerase also has an Asp residue in the active site to bind a substrate steroid (13). We examined these acidic amino acid residues with carbodiimide and glycine ethylester (15), but could not obtain information concerning their involvement in the catalytic site of 3-ketosteroid- Δ^1 -dehydrogenase. Recently, yeast old yellow enzyme was reported to catalyze the *trans*-axial dehydrogenation of quinones (16). The ligand binding site of this enzyme contains Y-375 and H-191 (17). The abstraction of a hydrogen atom as a proton might be carried out in different manners in these enzymes. *p*-Hydroxybenzoate hydroxylase of *Pseudomonas fluorescens* contains tyrosyl residues in the active site, which form a hydrogen-bond network to activate *p*-hydroxybenzoic acid (18).

In the present study, the functions of three tyrosyl residues of 3-ketosteroid- Δ^1 -dehydrogenase from *R. rhodochrous* were clarified; they act differently in the catalytic reaction. Although this study was carried out on the tyrosyl residues on a TNM-modified tryptic peptide, the enzyme contains four more conserved tyrosyl residues. When the functions of these residues are examined, more information on the interaction with 3-ketosteroids will be obtained, and which will facilitate understanding of the reaction mechanisms.

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