**APO AND HOLO STRUCTURES OF 3α-HYDROXYSTEROID DEHYDROGENASE FROM Pseudomonas sp. B-0831: LOOP-HELIX TRANSITION INDUCED BY COENZYME BINDING**

Shota Nakamura‡, Masayuki Oda†, Sachio Kataoka†, Shigeru Ueda§, Susumu Uchiyama¶, Takuya Yoshida‡, Yuji Kobayashi‡§, and Tadayasu Ohkubo‡**

From the ‡Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan, the †Graduate School of Agriculture, Kyoto Prefectural University, 1-5 Hangi-cho, Shimogamo, Sakyo-ku, Kyoto 606-8522, Japan, the ¶Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan, the Diagnostics Department, Asahi Kasei Pharma Corporation, 632-1 Mifuku, Izunokuni, Shizuoka 410-2321, Japan, the §Graduate School of Pharmaceutical Sciences, 4-20-1 Nasahara, Takatsuki, Osaka 569-1094, Japan.

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**Address correspondence to: Tadayasu Ohkubo, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan. Tel. +81 6 6879 8223, Fax. +81 6 6879 8221; E-mail: ohkubo@protein.osaka-u.ac.jp**

Bacterial 3α-hydroxysteroid dehydrogenase, which belongs to a short-chain dehydrogenase/reductase (SDR) family and forms a dimer comprised of two 26 kDa subunits, catalyzes the oxidoreduction of hydroxysteroids in a coenzyme dependent manner. This enzyme also catalyzes the oxidoreduction of non-steroid compounds that play an important role in xenobiotic metabolisms of bacteria. We performed an X-ray analysis on the crystal of Ps3αHSD, the enzyme from Pseudomonas sp. B-0831 complexed with NADH. The resulting crystal structure at 1.8 Å resolution showed that Ps3αHSD exists as a structural heterodimer composed of apo- and holo-subunits. A distinct structural difference between them was found in the 185-207 region, where the structure in the apo-subunit is disordered while that in the holo-subunit consists of two α-helices. This fact proved that the NADH binding allows the helical structures to form the substrate binding pocket even in the absence of the substrate though the region corresponds to the so called “substrate binding loop”. The induction of α-helices in solution by the coenzyme binding was also confirmed by the CD experiment. In addition, the CD experiment revealed that the helix-inducing ability of NADH is stronger than that of NAD. We discuss the negative cooperativity for the coenzyme binding, which is caused by the effect of the structural change transferred between the subunits of the heterodimer.

Hydroxysteroid dehydrogenases are classified into three superfamilies: aldo-keto reductase (AKR); long/medium-chain dehydrogenase/reductase (MDR); and short-chain dehydrogenase/reductase (SDR). Bacterial 3α-hydroxysteroid dehydrogenase from Pseudomonas sp. B-0831 (Ps3αHSD, EC 1.1.1.50) belonging to the SDR family forms a dimer comprised of two 26 kDa subunits, and catalyzes the oxidoreduction of steroid compounds as the reversible inter-conversion between 3α-hydroxysteroid and 3-ketosteroid. In addition, Ps3αHSD can also catalyze the oxidoreduction of non-steroid compounds such as p-nitrobenzaldehyde, and thereby is also named as 3α-hydroxysteroid dehydrogenase/carbonyl reductase.
Bacteria are supposed to use this 3αHSD/CR activity for a xenobiotic metabolism which is the first step in the consecutive detoxification process in bacterial organisms (2,3).

The enzymes belonging to the SDR family form a common tertiary structure of dinucleotide binding motif, popularly known as the Rossmann fold (4). These structures have the same architecture of the Ser-Tyr-Lys triad (SYK triad) as an active site and the same coenzyme binding motif GxxxGxG at N-terminal region. Although they have quite similar coenzyme binding sites, there is a unique specificity for NAD(H) and/or NADP(H). A number of studies have been performed to explain the coenzyme specificity in SDR and illuminated that the Asp residue at the coenzyme binding site determines the preference of NAD(H). The switch of the coenzyme preference from NADP(H) to NAD(H) has been achieved by the mutation of this Asp residue (5). We have studied the coenzyme specificity for Ps3αHSD and revealed that this enzyme displayed a dual pyridine nucleotide specificity for NAD(H) and NADP(H) in spite of the existence of the Asp residue (6). Furthermore, we demonstrated that the binding mechanism of NAD(H) has been achieved by the mutation of this Asp residue (5). We have studied the coenzyme specificity for Ps3αHSD and revealed that this enzyme displayed a dual pyridine nucleotide specificity for NAD(H) and NADP(H) in spite of the existence of the Asp residue (6). Furthermore, we demonstrated that the binding mechanism of NAD(H) varies depending on its redox condition. The previous kinetic study by fluorescence stopped-flow experiment elucidated that the binding mechanism of NAD is a one-step reaction while that of NADH is a two-step reaction (7).

In order to explain the molecular basis for the coenzyme binding mechanism, X-ray analysis of Ps3αHSD was carried out. Among the 3α-hydroxysteroid dehydrogenases from prokaryote, two structures, the enzyme alone and the binary complex of 3α-hydroxysteroid dehydrogenase with NAD from Comamonas teststeroni (Ct3αHSD), have been previously reported (8). On the other hand, the binary complex of bacterial 3α-hydroxysteroid dehydrogenase with NADH has not yet been reported. Here we report the crystal structure of the binary complex of Ps3αHSD obtained from co-crystallization of Ps3αHSD and NADH. The crystal structure exhibited a structural heterodimer which is formed by apo- and holo-subunits complexed with NADH. Such a dimer formation with the coenzyme binding to only one monomer has already been reported by Valencia et al. (9) as a heterodimer of cinnamyl alcohol dehydrogenase which belongs to the MDR family. The heterodimer of Ps3αHSD is the first example found in the SDR family.

Combining the heterodimer of Ps3αHSD with other structural reports of the SDR family revealed that loop-helix transition is induced by the NADH binding. Furthermore, in order to confirm whether the loop-helix transition would also occur in solution, we measured the CD spectra of the mixture of the enzyme with NADH or NAD. These results allowed us to discuss the coenzyme binding mechanism for bacterial 3α-hydroxysteroid dehydrogenases.

**EXPERIMENTAL PROCEDURES**

**Crystallization and data collection** — The crystals of Ps3αHSD were obtained by following a previously described protocol (10). Co-crystallization of Ps3αHSD (7.5 mg/mL) with the coenzyme NADH (0.6 mM) was achieved by using the hanging drop vapor diffusion method at 4 °C in the presence of 1.40 M ammonium sulphate and 0.14 M sodium chloride in 0.1M Tris buffer at pH 9.0. Rod clusters of Ps3αHSD crystal appeared within one week, which were then crushed and used for micro-seeding experiments, which led to the growth of larger single crystals. X-ray diffraction data were collected at BL38B1 in SPring-8 (Hyogo, Japan). The data were processed using DENZO and SCALEPACK from the HKL2000 package (11) and further processed using the CCP4 program suite (12). The crystals, which diffract to a resolution limit of 1.8 Å, belong to the orthorhombic space group P2_12_1, with unit cell parameters of a = 62.46, b = 82.25, c = 86.57 Å. A molecular replacement solution for Ps3αHSD was found using the program...
MOLREP (13) with the molecular model of CtαHSD (PDB code 1FK8) (8). The data statistics are shown in Table 1.

Model building and refinement — The model obtained from the molecular replacement method was subjected to the rigid body refinement in the program REFMAC (14). The modification from amino acid residues of CtαHSD to those of PsαHSD and the manual building of missing portions in the CtαHSD model were performed by using the program XtalView (15). The structure was refined with the program REFMAC, utilizing a maximum likelihood target. After the iterative refinements and the model buildings, water molecules were added to the model both by the program ARP/wARP (16) and by manual inspection of 2Fo - Fc electron density maps using the program XtalView. The structure was further refined until no improvement was observed in R and Rfree. The R-value of the final model was 0.17 (Rfree 0.21). Detailed refinement statistics are summarized in Table 1. The structure quality was evaluated using the program PROCHECK (17). Characterization of the secondary structure was carried out using the program DSSP (18). Pairwise least-squares fits of protein structure were performed by using the program PROFIT (19). The molecular figures were generated using the program PyMOL (20). A refined model of PsαHSD was deposited in PDB (PDB code: 2DKN).

CD measurements — The structural changes of PsαHSD in solution were assessed by CD spectroscopy. The CD spectra were measured in the range 195–260 nm with a 0.1 mm path length cell at 25 ºC on a JASCO J-720 spectrometer. In sample preparations, 100 μL of the protein solution at a protein concentration of 30 μM was mixed with 100 μL of the coenzyme solution containing various concentrations of NAD or NADH in the molar ratio range from 0 to 40. Both protein and coenzyme solutions were dissolved in 10 mM Tris buffer at pH 8.0.

Fluorescence experiment — Fluorescence experiments were performed on a Spectra Max M5 spectrometer (Molecular Device Co. Ltd.). The NADH binding to Ps3αHSD was measured by the quenching of intrinsic enzyme fluorescence at 345 nm on incremental addition of NADH. Excitation was performed at 280 nm, and emission spectra were recorded in the 300 – 500 nm wavelength range at 25 ºC. The samples for measurements were prepared at the final concentration of 3 μM enzyme and 1.0 – 175 μM NADH in 50 mM Tris-HCl pH 8.5. These concentrations were determined by the molar extinction coefficients of 1.17 × 10^4 (280 nm) for enzyme and 6.22 × 10^3 (340 nm) for NADH solution. The observed data were fitted by the following three models. The first model is expressed by a simple Hill equation with dissociation constant Kd,

\[ Y = \frac{[L]}{K_d + [L]} \]  

where Y is fractional saturation of the enzyme ([occupied sites]/[total sites]), [L] is ligand concentration. The second model is expressed by a modified Hill equation in consideration of cooperativity including Hill number h, i.e.

\[ Y = \frac{[L]^h}{K_d + [L]^h} \]  

The hill number gives the degree of cooperativity: the value h = 1 indicates the absence of cooperativity, h > 1 and h < 1 mean positive and negative cooperativity, respectively (21). The third is the KNF model which is proposed to explain cooperative behavior (22),

\[ Y = \frac{(K_2 + [L])[L]}{[L]^2 + 2K_2[L] + K_1K_2} \]

where K1 and K2 are microscopic dissociation constants of the ligand-binding sites. In this model, the reaction scheme is considered to be sequential. These nonlinear fittings using three models were performed by the software Origin ver 6.0.

RESULTS

Overall structure — The crystal structure of
Ps3αHSD complexed with NADH was solved by the molecular replacement method using the structure of Ct3αHSD as the search model. The crystal asymmetric unit contained the dimer of Ps3αHSD which corresponds to the biological unit of this enzyme. The coenzyme NADH was found in only one subunit of the dimer, with well defined density as shown in Fig. 1a. In contrast, no density was observed for NADH in the other subunit. The former subunit, which is named Chain A, is therefore in the holo-form and the latter subunit, named Chain B, is in the apo-form (Fig. 1b). In these chains, we excluded from the model the following parts for which density was not observed: Met1 of Chain A; and Met1, Gly73-Ala77, and Ala185-Phe207 of Chain B. The $R$-value and $R_{free}$-value for the final model, which consists of 3406 protein atoms, 44 NADH coenzyme atoms, and 376 water molecules, were 0.17 and 0.21 at 1.8 Å resolution, respectively.

Both subunits form almost the same topology as an α/β barrel consisting of a cylindrical core of seven parallel β-strands surrounded by five long α-helices. The schematic diagrams of the topologies of apo- and holo-forms of Ps3αHSD are shown in Fig. 2, and those of 7α-hydroxysteroid dehydrogenase from Escherichia coli (Ec7αHSD) and Ct3αHSD are given for reference as well. Ec7αHSD and Ct3αHSD exist in the states of structural homotetramer and homodimer, respectively. The labeling of these secondary structural elements is according to Ghosh et al. (23). The holo-form of Ps3αHSD has six short α-helices; three α-helices (αEF1-3) between βE and αF, two α-helices (αFG1, 2) between βF and αG, and one α-helix (αCT) at C-terminal (Fig. 2a). In the apo-form, there are two distinct disordered regions; the short region 73-77 and the long region 185-207 (Fig. 2b). Note that these regions correspond to an ordered loop in the short region and the two α-helices αFG1 (189-196) and αFG2 (201-205) in the long region in the holo-form. It is often found just in this long region of SDRs that the secondary structures are different between apo- and holo-forms. Historically, this region (185-207) has been called the “substrate binding loop”.

In the structural heterodimer of Ps3αHSD, the apo- and holo-subunits interact with each other in the region (217 – 255) which include the βG strand, the helix αG, and the C-terminal helix αCT, in the manner of P-interface in tetrameric SDRs (8,24). This dimer interface is supported by the following specific interactions. The side chain-side chain interactions occur between the helices αG of the two subunits, for example, the stacking interaction between the side chains of Phe226. In the βG strands, the side chain-main chain interactions are found such that the side chain of Ser238 is hydrogen bonded to the main chain of Phe241 of the other subunit. The helix αCT is packed into the cleft formed by αG and βG of the other subunit. This interaction stabilizes the hydrophobic contacts of the dimer interface. The short loops after the helix αCT are intercrossed with each other. In this loop, there is a unique salt bridge interaction between the carboxyl group of Phe255 in the C-terminal end of one subunit and the side chain of Arg251 of the other subunit.

In the structural heterodimer, both structures of the apo- and holo-subunits share the typical features of SDRs such as an active site and a coenzyme binding site. The active site of Ps3αHSD includes the well characterized SYK triad (25-27), which consists of Ser114 in the loop connecting βE and αEF1, Tyr153 and Lys157 in the helix αF. In the holo-subunit, NADH is located at the C-terminal ends of the parallel β-strands of the Rossmann fold, one of which carries a coenzyme binding motif GxxxGxG (Gly8 – Ser9 – Ala10 – Ser11 – Gly12 – Ile13 – Gly14) (8,25,26). This coenzyme binding site remains in the apo-subunit with almost the same conformation.

The structural differences between apo- and holo-subunits — The Ca atoms of apo- and holo-subunits, excluding the two disordered regions, almost overlapped with an average RMSD of 0.45Å. Large local differences arose around the two disordered regions, which are
clearly shown by the plot of RMSD against residue numbers in Fig. 3a. Other local collective differences over the average RMSD are found in the following regions: αEF1-3 region (123-150) and the C-terminal region (243-255). RMSD values mapped on the structural heterodimer of Ps3αHSD are shown in Fig. 3b. The structural changes of the substrate binding loop (185-207), the short disordered region βD-αE loop (73-77), and αEF1-3 are concentrated in the vicinity of the coenzyme NADH. Some conformational changes are also observed in the C-terminal region though there is no direct interaction with NADH. These differences between apo- and holo-subunits of Ps3αHSD indicate that the NADH binding causes the following three structural changes: (i) the drastic change from loop to helix in the region of the substrate binding loop (185-207); (ii) the βD-αE loop formation; and (iii) the local conformational changes in αEF1-3 and the C-terminal region.

The interactions between Ps3αHSD and NADH — The interactions between Ps3αHSD and NADH were analyzed by the program LIGPLOT (28) as shown in Fig. 4. There are many interactions between Ps3αHSD and NADH found as follows. The residues of Asp41 and Leu42 interact with the adenine moiety of NADH. The residues of Asp32 and Arg33 participate in hydrogen bonding with the adenosine ribose moiety. The residues Ser11, Ile13, and Thr188, interact with the diphosphate group. The residues Cys69, Tyr153, and Lys157, are hydrogen bonded to the nicotinamide ribose moiety. These interactions and other hydrophobic contacts, which are mainly formed in the Rossmann fold, play a role in anchoring NADH in almost the same manner as other SDRs. The nicotinamide moiety of NADH interacts with the main chain (NH, CO) of Val186 and the side chain (OH) of Thr188 to stabilize the substrate binding loop (185-207). As Shi and Lin have pointed out, the anchoring of NADH in which the main chain atoms get involved appears to be the common feature of the SDR family (29).

The formation of the helices, αFG1 and αFG2 — The orientation of the helix αFG1 (189-196), which is formed following Thr188, is dictated by Pro189 at the turning point from loop to helix. The side chain hydroxyl group of Thr188 forms a bifurcated hydrogen bond with the amide group of nicotinamide and the main chain NH group of Leu191 (Fig. 5a). In addition, the distance between the nearest oxygen atom of the phosphate group of NADH and the NH group of Leu191 is 3.2 Å. The hydrogen bond network formed among Thr188, Leu191, and NADH would act as the stabilizing factors analogous to an N-cap motif for the positive dipole of N-terminal of the helix to help the helix αFG1 formation.

Succeeding αFG1, a turn Asp197 - Pro198 - Arg199 - Tyr200 is formed. Generally, Asx - Pro - Xxx - Yyy sequences occur frequently in protein structures and take a quite stable turn structure (30). The helix αFG2 (201-205) comes after this turn (Fig. 5b). The relative orientation of αFG1 and αFG2 is determined by this turn. Consequently, αFG1 and αFG2 form an L-shaped structure. This L-shaped structure provides a large pocket for substrate (substrate binding pocket) surrounded by NADH, the βD-αE loop, and SYK triad. In this pocket, 15 water molecules are found. These water molecules in the pocket form a hydrogen bond network, which stabilizes the helix αFG2 and the βD-αE loop. This formation of the substrate binding pocket was caused by the interactions of the water bridging and the helices αFG1 and αFG2 which were induced by the NADH binding. These results from the structural analysis of Ps3αHSD clearly showed that the substrate binding pocket was formed in spite of no substrate binding.

Structural changes in solution — In order to confirm that the structural changes caused by coenzyme binding also occur in solution, the CD spectra of Ps3αHSD were measured at various concentrations of NAD or NADH. As shown in Fig. 6a, the presence of an isodichroic point near 203 nm in the spectra is an indicative of a two-state structural transition. The changes in molar residue ellipticity at 222 nm, which are plotted against the concentration
of coenzymes in Fig. 6b, are negatively increased. This indicates that the binding of the coenzymes enhanced the helicity of Ps3αHSD. The saturated molar residue ellipticities for NAD and NADH are -1.85 x 10^-4 and -1.98 x 10^-4 deg·cm^2·dmol^-1, respectively. It showed that both coenzymes have the ability to induce the loop-helix transition in solution, and that NADH has higher inducing ability than NAD.

Negative cooperativity in the NADH binding — The binding profile of NADH to Ps3αHSD was examined by the fluorescence experiment. In Fig. 7, the fraction of bound NADH against the NADH concentration is plotted. The observed data were analyzed by the three models, simple Hill equation, and two models with consideration of cooperativity; modified Hill equation, and KNF equation. The simple Hill model poorly fit while the other two models fit pretty well indicating the cooperative profile of the binding (Fig. 7). The parameters obtained from eq(2) of the cooperative Hill equation are 8.80 μM for \( K_d \) and 0.779 for \( h \). This \( h \) value of less than 1.0 demonstrates that there is a negative cooperativity. Furthermore eq(3) of the KNF model gave clearly the microscopic dissociation constants, 9.35 and 28.50 for \( K_1 \) and \( K_2 \), respectively. Thus these results confirmed the negative cooperativity in the NADH binding to Ps3αHSD.

**DISCUSSION**

In this study, we revealed that the crystal structure of Ps3αHSD, complexed with NADH at 1.8 Å resolution, exhibits a structural heterodimer which is composed of apo- and holo-subunits.

Ps3αHSD uses both NAD(H) and NADP(H) as a coenzyme. Such a character is called “dual pyridine nucleotide specificity” (31-34). On the other hand, the structures of Ct3αHSD which are incapable of accepting NADP(H) as a coenzyme were determined in different crystals, with and without NAD, by Grimm et al. (8). Ps3αHSD and Ct3αHSD with high sequence homology showed high structural similarities in both forms taking the Rossmann fold as pyridine nucleotide binding motif.

The pyridine nucleotide specificities of SDRs have been attributed to Asp32, which is highly conserved among SDRs preferring NAD(H) (5). The side chain of this Asp32 was hydrogen bonded to 2'- and 3'-hydroxyl groups of the adenosine ribose in the same manner in both proteins. In the case of NADP(H), the 2'-phosphate group of the adenosine moiety would be electrostatically repelled by Asp32. These interactions are considered to cause the NAD(H) preference of Ps3αHSD and Ct3αHSD.

Grimm et al. mentioned that, in Ct3αHSD, the NADP(H) binding would be impeded further by Ile33 with its steric exclusion (8). However, the counterpart of Ile33 in Ps3αHSD is Arg33, which formed hydrogen bonds to the 2'-hydroxyl group of the ribose as described above. Due to the flexible side chain and the positive charge of Arg33, Ps3αHSD would interact with the 2'-phosphate group of NADP(H) avoiding the steric exclusion. Arg33 in Ps3αHSD contributes to reduce the unfavorable electrostatic interaction with Asp32 and provides the dual pyridine nucleotide specificity. Therefore, Ps3αHSD can catalyze the redox reaction not only by using NAD(H) but also by using NADP(H) as a coenzyme with lower affinities for NADP (\( K_d \) 192 μM) and NADPH (\( K_d \) 120 μM) compared to NAD (\( K_d \) 173 μM) and NADH (\( K_d \) 7.6 μM), respectively (7). The binding affinity of NADH is significantly stronger compared with NAD. To prove how this difference of affinity is brought about, we compared the structures among NADs in Ct3αHSD and NADH in Ps3αHSD. Even though quantum mechanics calculations of NAD and NADH alone showed that both coenzymes take in-plane conformations between the carboxyamide group and the aromatic ring as a lowest energy conformation (data not shown), a distinct difference was found in the carboxyamide group conformation as shown in Fig. 8. The dihedral angles of the carboxyamide groups (NO7-NC7-NC3-NC4)
are 175° for NADH in Chain A of Ps3αHSD, -35° for NAD in Chain A, and -69° for NAD in Chain B of Ct3αHSD. The carboxyamide group of NADH, which is found to take an in-plane conformation against the aromatic ring, is hydrogen bonded with carbonyl and amide groups of Val186. This hydrogen bonding formation provides NADH with the strong affinity. On the other hand, the carboxyamide group of NADs is not in-plane so that it cannot form the hydrogen bonds as found in NADH. It is noteworthy that the concentration of NADH is lower than that of NAD in the bacterial intracellular environments (35). The difference of this affinity between NADH and NAD might compensate for such a concentration difference to maintain the equilibrium of the enzymatic reaction in the intracellular environments.

Among the SDR family, various secondary structures of the region corresponding to the substrate binding loop have been reported as disordered loop, ordered loop, α-helix, 3_10 helix, β-strand and so on. Ps3αHSD exhibits two α-helices in the holo-form and the disordered loop in the apo-form. The formation of two α-helices in the holo-form like Ps3αHSD was reported for Ec7αHSD which is tetrameric SDR complexed with NAD (27). However, it has been quite recently reported that tetrameric D-3-hydroxybutyrate dehydrogenase from Pseudomonas fragi shows the disordered loop in the holo-form complexed with NAD and two α-helices in the apo-form (36). This is just a reverse situation to that found in Ps3αHSD. This variety of conformations in the region of the substrate binding loop might be caused by experimental conditions where structural analyses have been carried out. These include crystals with or without substrate or substrate analogue and/or coenzymes, states in monomer, dimer or tetramer, and more combinations. Thus, the factors to determine the structure in this region have been controversial so far.

However, we would like to emphasize that, in this study, we confirmed by CD experiments that coenzyme binding to Ps3αHSD induces the loop-helix transition in solution. At least, this fact showed that this transition could occur even in the absence of substrate. It has been considered so far that the coenzyme binding preceding the substrate binding does not induce a significant conformational change and that the ordered structure in the substrate binding loop is induced by the substrate binding. Thus, the scenario elucidated here is different from widely accepted figures of the reaction process of SDR.

RMSD plot between the apo- and the holo-forms of Ps3αHSD showed large differences of structure not only around the region of the substrate binding loop but also in the C-terminal region which contributes to the dimer formation. These facts suggest that the structural change of one subunit affects the conformation of the other subunit through C-terminal interface, and provides negative cooperativity, where binding of the first coenzyme disfavors the binding of the subsequent coenzyme. This expectation is supported by the fluorescence experiment which clearly indicates negative cooperativity in the NADH binding to Ps3αHSD. The possibility of such cooperativity caused by the structural changes of the substrate binding loop was prefigured for Ct3αHSD by the comparison of the structures of SDRs by Grimm et al (8). In spite of their expectation, they did not obtain the experimental evidence because the Ct3αHSD crystal they investigated showed little conformational difference between apo- and holo-forms. Even though the substrate binding loops of the structural homodimer seemed to be disordered regardless of the NAD binding, they predicted that the effect of the conformational change is transferred between the subunits. Here, we proved this hypothesis using the structure of Ps3αHSD complexed with NADH. Furthermore, we obtained additional evidence which would explain the difference in the effect of the coenzyme binding between the cases of Ct3αHSD and Ps3αHSD. Comparing NADH and NAD, our CD experiments revealed that the ability of helix induction of NADH is stronger than that of NAD. We could
delineate the following scheme of the coenzyme binding mechanism. NADH as a strong helix maker induces the first α-helices in the region of the substrate binding loop in one subunit of the dimer, and then this information is transferred to the other subunit through the mutual interaction of the C-terminal regions. Consequently, a conformational change occurs in the region of the substrate binding loop in the second subunit to reduce its binding ability. However, NAD which has less capability of helix formation induces little conformational change on Ps3αHSD. This is likely in the case of Ct3αHSD. The NADH binding to Ct3αHSD would also lead to a similar information transduction as Grimm et al. speculated.

In the structure of the holo-subunit of Ps3αHSD, the existence of the substrate binding pocket was evident even in the absence of the bound substrate. NADH and αFG2 compose the two side walls of the pocket, respectively, with αFG1 lying on the bottom. This prebuilt pocket, which has enough room to accommodate hydroxysteroid moiety of the substrate, was filled with ordered water molecules. Almost 15 molecules form a cluster by hydrogen bonding with each other. This configuration of the binding pocket could determine the broad substrate specificity of Ps3αHSD. For large substrates like hydroxysteroid, most of the water molecules are replaced by the substrate which is fixed by the direct interaction with the two α-helices, αFG1 and αFG2, to face the active site consisting of SYK triad and NADH. On the other hand, in the case of small substrates like non-steroid compounds, the ordered water molecules support the substrate to take suitable spatial arrangements. For much larger hydroxysteroid molecules like glycine-conjugated hydroxysteroid, the hydroxysteroid moiety is trapped in the pocket and the remaining part of the molecules protrude into solvent through the mouth of the pocket (27).

We discussed the reaction process of bacterial 3α-hydroxysteroid dehydrogenase in relation to its structure. It remains uncertain if the scheme proposed here could be applied in much more complex systems in other SDRs. If we could assume the state of the structural heterodimer in solution as an intermediate state, it might be possible to interpret the two step reaction of the NADH binding on Ps3αHSD that has been discussed in our previous work (7).

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**FOOTNOTES**

Abbreviations: AKR, aldo-keto reductase; MDR, long/medium-chain dehydrogenase/reductase; SDR, short-chain dehydrogenase/reductase; Ps3αHSD, 3α-hydroxysteroid dehydrogenase from *Pseudomonas* sp. B-0831; Ct3αHSD, 3α-hydroxysteroid dehydrogenase from *Comamonas* testosterone; Ec7αHSD; 7α-hydroxysteroid dehydrogenase from *Escherichia coli*.

PDB: The atomic coordinates and structure factors (PDB code: 2DKN for crystal structure of Ps3αHSD) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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**FIGURE LEGENDS**

**Figure 1.** (a) The electron density of the coenzyme NADH in Chain A. This electron density map is 2Fo - Fc map contoured at the 1-sigma level. (b) Cartoon-type representation of Ps3αHSD. In the holo-subunit, α-helices are shown in red, and β-sheet in orange. In the apo-subunit, α-helices are shown in blue and β-sheet in cyan. The disordered loop (185-207) in the apo-subunit is shown as a broken line. NADH is represented by the yellow surface model.

**Figure 2.** Comparison of the folding topologies found in the crystal structures of (a) the holo-form of Ps3αHSD, (b) the apo-form of Ps3αHSD, (c) the holo-form of Ec7αHSD, and (d) the holo-form of Ct3αHSD. α-helices are represented as circles, short α-helices as cylinders, β-strands as triangles, and disordered loops as dotted lines. Greek numbers indicate multimeric state.

**Figure 3.** (a) Plot of RMSD of the backbone structures of apo- and holo-forms for Ps3αHSD versus residue numbers. The values of RMSD are based on the overlaying of two monomers excluding disordered residues. The secondary structure elements of Ps3αHSD are shown with different colored backgrounds (red, α-helices; cyan, β-strands; grey, loops). (b) RMSD values mapped on the structural heterodimer of Ps3αHSD. The regions in holo-subunit corresponding to
disordered regions in \textit{apo}-subunit are colored in red.

\textbf{Figure 4.} Schematic presentation of coenzyme-protein interactions produced by the program LIGPLOT. Val186 and Thr188 are emphasized by red background. SYK triad is emphasized by cyan background.

\textbf{Figure 5.} (a) The hydrogen bond network among $\alpha$FG1, $\alpha$FG2, and NADH. (b) Stereo view of the box in Fig. 5a. The residues around $\alpha$FG1 and $\alpha$FG2 are shown in stick model.

\textbf{Figure 6.} (a) CD spectra of Ps3$\alpha$HSD with coenzymes. The spectra are colored by concentration of NADH from pink (lowest) to red (highest) and of NAD from cyan (lowest) to dark blue (highest). Black line indicates the CD spectrum of Ps3$\alpha$HSD only. (b) The plot of molar ellipticities at 222 nm against coenzyme concentrations. These error bars represent the standard deviations estimated for three experiments carried out independently.

\textbf{Figure 7.} The binding curves obtained from the fluorescence analysis. Observed data are plotted by closed squares. Fitting curves are colored by the following color code: Red, simple Hill model; Green, Hill model including Hill number (Hill(h)); and Cyan, KNF model.

\textbf{Figure 8.} The comparison between structures around NADH in Ps3$\alpha$HSD (red). Two NADs in Ct3$\alpha$HSD (green) were superimposed to NADH in Ps3$\alpha$HSD. The water molecules are represented as spheres. The hydrogen bonds between the carboxyamide group of NADH and the carbonyl and amide groups of Val186 are shown by cyan dotted lines.
Table 1. Data collection and refinement statistics

<table>
<thead>
<tr>
<th>Data collection</th>
<th>SPring-8 BL38B1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (K)</td>
<td>100</td>
</tr>
<tr>
<td>Space group</td>
<td>P2₁2₁2₁</td>
</tr>
<tr>
<td>Cell dimensions a, b, c (Å)</td>
<td>62.46, 82.25, 86.57</td>
</tr>
<tr>
<td>Resolution limits (Å)</td>
<td>50.00 – 1.80 (1.90-1.80)</td>
</tr>
<tr>
<td>No. unique/observed reflections</td>
<td>41,605 / 308,293</td>
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<tr>
<td>Average redundancy</td>
<td>7.41 (7.10)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.5 (98.7)</td>
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<tr>
<td>$R_{\text{merge}}$</td>
<td>0.17 (0.25)</td>
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</table>

<table>
<thead>
<tr>
<th>Refinement statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Refinements resolution range (Å)</td>
</tr>
<tr>
<td>$R / R_{\text{free}}$ c</td>
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<tr>
<td>RMSD from ideal Bonds (Å)</td>
</tr>
<tr>
<td>angles (°)</td>
</tr>
<tr>
<td>$&lt;B&gt;$ for atomic model d (Å²)</td>
</tr>
<tr>
<td>for water molecules</td>
</tr>
<tr>
<td>for NADH molecule</td>
</tr>
<tr>
<td>Ramachandran plot</td>
</tr>
<tr>
<td>Most favored regions (%)</td>
</tr>
<tr>
<td>Additional allowed regions (%)</td>
</tr>
<tr>
<td>Generously allowed regions (%)</td>
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<tr>
<td>Disallowed regions (%)</td>
</tr>
</tbody>
</table>

aValues in parentheses indicate the data in the highest resolution shell. b$R_{\text{merge}} = \Sigma |I_h - <I_h>| / \Sigma I_h$, where $<I_h>$ is the average intensity of reflection h and symmetry-related reflections. c$R = R_{\text{free}} = \Sigma ||F_o||-|F_c||/\Sigma |F_o|$ calculated for reflections of the working set and test (5%) set, respectively. dB$<B>$ is the average temperature factor for all protein atoms.
Table II. Obtained best fit parameters.

<table>
<thead>
<tr>
<th>Model</th>
<th>Hill</th>
<th>Hill (h)</th>
<th>KNF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameters</td>
<td>$K_d/\mu M$ 17.97</td>
<td>$K_d/\mu M$ 8.80</td>
<td>$K_1/\mu M$ 9.35</td>
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<tr>
<td>$h$</td>
<td>0.779</td>
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<tr>
<td>$\chi^2$a</td>
<td>0.00214</td>
<td>0.00036</td>
<td>0.00041</td>
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<tr>
<td>$R$ a</td>
<td>0.966</td>
<td>0.9948</td>
<td>0.9941</td>
</tr>
</tbody>
</table>

*a Fitting statistics, $\chi^2$: accumulated deviation between observed data and calculated curve. $R$: correlation coefficient.
Figure 2

(a) Ps3αHSD(II) *holo*-form

(b) Ps3αHSD(II) *apo*-form

(c) Ec7αHSD (IV) *holo*-form

(d) Ct3αHSD(II) *holo*-form
Figure 3

(a) RMSD (Å) vs Residue Numbers

(b) Structural diagram with labeled regions:
- Substrate binding loop
- αEF2 (126-133)
- αEF3 (136-146)
- βD-αE loop (69-78)
- αCT (246-250)
- βG (239-242)
- αFG2 (201-205)
- αFG1 (189-196)
Figure 4
Figure 5
Figure 6

(a)

(b)
Figure 7

Fraction

Coenzyme concentration / µM

Hill
Hill (h)
KNF
Apo and holo structures of 3α-hydroxysteroid dehydrogenase from Pseudomonas sp. B-0831: loop-helix transition induced by coenzyme binding
Shota Nakamura, Masayuki Oda, Sachio Kataoka, Shigeru Ueda, Susumu Uchiyama, Takuya Yoshida, Yuji Kobayashi and Tadayasu Ohkubo

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