The Crystal Structure and Stereospecificity of Levodione Reductase from Corynebacterium aquaticum M-13*

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The (6R)-2,2,6-trimethyl-1,4-cyclohexanedione (levodione) reductase (LVR) of the soil isolate bacterium Corynebacterium aquaticum M-13 is a NAD(H)-linked enzyme that catalyzes reversible oxidoreduction between (4R)-hydroxy-(6R)-2,2,6-trimethylcyclohexanone (actinol) and levodione. Here, the crystal structure of a ternary complex of LVR with NADH and its inhibitor 2-methyl-2,4-pentanediol has been determined by molecular replacement and refined at 1.6 Å resolution with a crystallographic R factor of 0.199. The overall structure is similar to those of other short-chain alcohol dehydrogenase/reductase enzymes. The positions of NADH and 2-methyl-2,4-pentanediol indicate the binding site of the substrate and identify residues that are likely to be important in the catalytic reaction. Modeling of the substrate binding in the active site suggests that the specificity of LVR is determined by electrostatic interactions between the negatively charged surface of Glu-103 of LVR and the positively charged surface on the re side of levodione. Mutant LVR enzymes in which Glu-103 is substituted with alanine (E103A), glutamine, (E103Q), asparagine (E103N), or aspartic acid (E103D) show a 2–6-fold increase in Km values as compared with wild-type LVR and a much lower enantiomeric excess of the reaction products (60%) than the wild-type enzyme (95%). Together, these data indicate that Glu-103 has an important role in determining the stereospecificity of LVR.

Optically active hydroxycyclohexanone derivatives, such as (4R)-hydroxy-(6R)-2,2,6-trimethylcyclohexanone (actinol), are useful chiral building blocks of naturally occurring optically active compounds, such as xanthoxin (1) and zeaxanthin (2). The (6R)-2,2,6-trimethyl-1,4-cyclohexanedione (levodione) reductase (LVR) of the soil isolate bacterium Corynebacterium aquaticum M-13 is an NAD(H)-linked enzyme that functions in the reversible oxidoreduction between actinol and levodione, which is activated by monovalent cations. Previously, we carried out a screening for microorganisms that can catalyze stereo- and regio-selective reduction of the carbonyl group at the C-4 of levodione (3). Subsequently, we reported the purification and characterization of LVR and the cloning, sequencing analysis, and expression in Escherichia coli of its gene (4). This gene contains an open reading frame of 801 nucleotides, corresponding to 267 amino acid residues, the sequence of which shows that the enzyme belongs to the family of short-chain alcohol dehydrogenase/reductase (SDR) proteins (5). These proteins contain an N-terminal Gly-X-X-Gly-X-Gly cofactor-binding motif and a Tyr-X-X-X-Lys motif, which is structurally located in the active site. This latter motif, together with a conserved serine residue (Ser-152), forms a catalytic triad in LVR.

Tropinone reductases I and II of Datura stramonium and Hyoscyamus niger belong to the SDR family and share a common substrate, tropinone. The alcohol products resulting from these two enzymes have opposite configurations at the hydroxyl group (6, 7). The reaction stereospecificity is determined by the orientation of tropinone in the substrate-binding site. In turn, the orientation is determined by electrostatic interactions between the substrate and the amino acids located in the substrate-binding site (6). To understand further the structure of LVR, a unique enzyme belonging to the same family as tropinone reductase, we have crystallized the enzyme and solved its crystal structure. As modeling of the levodione-binding mode indicated that Glu-103 might interact with the substrate, we also generated mutant enzymes in which this glutamic acid was substituted for alanine, glutamine, asparagine, glutamic acid, or aspartic acid to examine the effects of Glu-103 on LVR-substrate interactions and its role in the stereospecificity of the enzyme.

Here we report the crystal structure of C. aquaticum M-13 LVR in complex with NADH and MPD at 1.6 Å resolution and the construction and characterization of a series of mutant LVR proteins.

EXPERIMENTAL PROCEDURES

Chemicals—Levodione and actinol were kindly donated by Nippon Roche Co., Japan. All other chemicals used in this study were analytical grade and available commercially.

Overexpression and Purification of LVR Proteins—Overexpression of the gene encoding C. aquaticum M-13 LVR in E. coli and purification of the recombinant protein were performed as described previously (4). For crystallization, a final stage of purification was carried out by chromatography using RESOURCE ISO (Amersham Biosciences) after the second chromatographic step using a MonoQ HR10/10 column (Amersham Biosciences). Chromatography was performed in buffer A (10 mM potassium phosphate buffer containing 2 mM (NH₄)₂SO₄ and 0.1 mM

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dithiothreitol, pH 7.0), with a 0–100% gradient of buffer B (10 mM potassium phosphate buffer containing 0.1 mM dithiothreitol, pH 7.0) at a flow rate of 1 ml/min. The purified protein was dialyzed against 10 mM Hepes buffer (pH 7.0) containing 50 mM KCl.

Preparation of 4(S)-[4-2H]NADH and 4(R)-[4-2H]NADH—4(S)-[4-2H]NADH and 4(R)-[4-2H]NADH were synthesized by enzymes as described previously (9–11). The synthesized nucleotides were not de-salted because LVR is activated by monovalent cations. Purified LVR was incubated in a reaction mixture (3.33 ml) containing 830 μmol of potassium phosphate buffer (pH 7.0), 3.1 μmol of (4R)-[4-2H]NADH or (4S)-[4-2H]NADH, and 2.0 μmol of levodione for 1 h at 20 °C. The reaction products were extracted with 1 ml of ethyl acetate. After the ethyl acetate layer was diluted with 9 ml of ethyl acetate, the extracts were analyzed by gas chromatography-mass spectrometry (GC-MS) on an HP 5973MSD system (Hewlett-Packard) with an HR-20M capillary column (0.25 mm × 30 m; Shinwa Chemical Industries, Japan). The GC conditions have been described previously (3). The GC-MS was operated with a mass range from m/z 20 to 200, and the ionization voltage was 70 eV.

Crystalization—The enzyme was concentrated to 15 mg/ml and incubated with a 5-fold molar excess of NADH for a few hours. Crystals were grown by the hanging-drop vapor diffusion method at 15 °C with the reservoir solution containing 18–20% (w/v) PEG MME 2000, 0.2 M magnesium chloride, 5% (v/v) MPD, and 5% (v/v) glycerol in 0.1 M MES buffer at pH 6.0. The crystals belong to the primitive triclinic crystal system, with space group P1 and cell parameters of a = 66.7 Å, b = 79.2 Å, c = 112.7 Å, α = 103.9°, β = 91.4°, γ = 105.5°. The asymmetric unit contains two tetramers of 112 kDa, giving a Vm value (12) of 2.5 Å³/Da.

Data Collection and Processing—The crystals used for cryo-crystallographic experiments were directly picked up from the droplet and flash-cooled using liquid N2. Diffraction data were collected on both a Quantum-4R CCD detector (Area Detector System Corporation) at beamline BL38B1, and an R-AXIS-V imaging plate detector (Rigaku Corp.) at beamline BL24XU of Spring-8 in Hyogo, Japan. All data were collected from one crystal at low temperature (100 K). Two data sets were processed and merged with the programs DPS/MOSFLM (13, 14) and SCALA of the CCP4 program suite (15). A total of 1,031,771 reflections were collected to 1.6 Å resolution, with 261,097 unique reflections having an Rmerge of 0.081 and a completeness of 92.2%. Data collection and processing statistics are given in Table I.

Molecular Replacement—The structure was solved by molecular replacement with the program CNX.2 Coordinates of 3α,20β-hydroxy-2 CNX, DelPhi, and Insight II; Accelrys, Inc., 9685 Scranton Rd., San Diego, CA 92121-3752.

FIG. 1. Conversion of (6R)-2,2,6-trimethyl-1,4-cyclohexanedi-one to (4R)-hydroxy-(6R)-2,2,6-trimethylcyclohexanone by levodione reductase.

FIG. 2. Comparison of eight monomers in the asymmetric unit. A, root mean square deviations by residue. Average, largest, and smallest differences between monomers are shown in thick, dashed, and thin lines, respectively. B, average B-factors by residue. C, superposition of residues 194–235 with NADH and MPD for four monomers. Ca atom models of four monomers (chain C, E, G, and H) are colored in yellow, red, green, and blue, respectively. NADH, MPD, and side-chain atoms that have intermolecular interactions are also depicted in the same colors. Chain names are labeled, and intermolecular hydrogen bonds are denoted by broken lines.
Steroid dehydrogenase (Protein Data Bank accession code 2HSD (17)), which has 39% sequence identity with LVR, was used as a search model. A complete tetramer was modeled as a search model on the basis of the amino acid sequence of LVR by the program XSAE. Cross-rotation functions and subsequent translation functions were calculated with data between 10 and 4 Å resolution, and the crystal packing was analyzed for each solution. Two definite solutions with higher correlation coefficients were identified which gave an R factor of 0.47 at 3.0 Å resolution after rigid-body refinement.

Structure Refinement—Crystallographic refinement of the two tetramers was performed with CNX at 1.6 Å resolution. A few cycles of simulated annealing refinement using the slow-cooling protocol, followed by model building with the program Moloc (18), were carried out, applying bulk solvent correction. At this stage, the bond molecules NADH and MPD were fitted into the electron density in the catalytic site of each monomer, and a total of 954 well defined water molecules were incorporated in the model. The first nine residues of the polypeptide chain for each monomer could not be located because of poor electron density and are not included in the final model. The model has been refined to an R factor of 0.199 and a free R of 0.218 for all of the reflections between 40 and 1.6 Å resolution, with good stereochemistry. The statistics for refinement and model geometry are listed in Table I. Theatomic coordinates have been deposited in the Protein Data Bank (accession code IITY). The figures were generated with the programs MOLSCRIPT (19), CONSCRIPT (20), and Raster3D (21).

Docking Study—The ab initio Hartree-Fock calculation with a 6-31G* basis set was carried out by the program Gaussian98 to optimize the conformation of levodione. We used the program FlexX to dock the geometrically optimized structure of levodione into the active site of the crystal structure. The orientation of the nicotinamide ring of NADH was modified without altering the protein structure to allow transfer of the pro-S hydride to the substrate. One of the docked structures with the lowest energy score was optimized in the active site by adjusting the NADH structure with the program Moloc (18). All molecular modeling was performed on a Silicon Graphics Maximum Impact (R10000).

Calculation of Electrostatic Potential—The atomic partial charges calculated by Gaussian98 were used for levodione, and a set of standard protein charges was assigned to the LVR atoms. The estimated electrostatic potential surface and shift in pK of Tyr-165 for the wild-type LVR and each mutant were calculated by DelPhi of the Insight II program package. The mutant structures were modeled by Insight II.

Site-directed Mutagenesis—Mutation was introduced into the LVR-coding regions of the expression vector pKKLVR, which expresses the wild-type LVR enzyme of C. aquaticum M-13 under control of the tac promoter. The substitution of Glu-103 by Ala, Gin, Asn, and Asp was performed by PCR with the following oligonucleotide primers: E103A/H11032 (5'-TACGCGGCGGA-3' /H11032)-GGGCTGCTGCAGTCAG-3') and E103N/H11032 (5'-TCTGCTTGCCTTGGATGCCGGCTTGTGTT-3' /H11032). The fragments were ligated to the new plasmid pKK223-3 digested with Pst I. The unique amplified band was digested with EcoRI and PstI. The fragments were ligated to the new plasmid pKK223-3 digested with EcoRI and PstI. The mutations were confirmed by DNA sequencing.

Enzyme Assay and Kinetic Analysis—The activity of LVR was determined spectrophotometrically by measuring the levodione-dependent decrease in the NADH content (3). For kinetics analysis, the photometric cell was used because of the accuracy in measuring the steady-state velocity of the reaction. K_m and V_max values were determined from 1/v versus 1/[S] plots using at least five different [S] values, where [S] is the concentration of the substrate and v is the activity at each substrate concentration.

Circular Dichroism Spectroscopy—CD spectroscopy was carried out using a Jasco model J-710 circular dichroism spectropolarimeter (Jasco, Tokyo, Japan). Preparations of purified recombinant enzyme were analyzed in 10 mm potassium phosphate buffer (pH 7.0), 1 mM KCl, and 0.1 mM dithiothreitol at a concentration of 0.5 mg/ml. For each protein, the ellipticity was measured as a function of wavelength at 0.5-nm increments between 240 and 200 nm at 18 °C.

Stereo-specificities of Mutant Enzymes—The stereospecificity of the mutant enzymes was determined by analyzing the reaction products by gas-liquid chromatography (GC). The enzymatic reduction of levodione was carried out as follows. A 3.5 ml reaction mixture containing 830 mmol of potassium phosphate buffer (pH 7.0), 1.0 unit of the enzyme, 65.5 μmol of NADH, and 50 μmol of levodione was incubated at 20 °C. After 10 min of incubation, the reaction mixture was extracted with 1 ml of ethyl acetate, and the extract was analyzed by GC using a Shimadzu model GC-14B GC equipped with a flame ionization detector and a type CB-G, 0.25 μm, 30 mm, SGE Analytical, Agilent, Switzerland. The column temperature program was 107 °C for 30 min, 5–129 °C for 15 min, 5–180 °C for 5 min. Helium was used as the carrier gas at a flow rate of 1 ml/min. Under these conditions, levodione, actinol, (4R,6S)-, (4S,6R)-, and (4S,6S)-hydroxy-2,2,6-trimethylcyclohexanone eluted at 18.3, 37.5, 39.0, 39.4, and 40.2 min, respectively.

RESULTS

Sterospecificity of Hydrogen Transfer in LVR—The reaction products formed in parallel incubations of either (4R)-[4-2H]NADH or (4S)-[4-2H]NADH with levodione and LVR were analyzed by GC-MS. Levodione produced by LVR in the presence of (4S)-[4-2H]NADH yielded a molecular ion of m/z 157, whereas the corresponding alcohol, produced in the presence of (4R)-[4-2H]NADH, yielded an ion of m/z 156. This shows that LVR transfers the pro-S hydrogen from the C-4 of NADH to levodione, as do tropinone reductase I and II from H. niger (24). Accordingly, LVR is classified as a B-specific oxireductase.

Quality of the Model—The crystal structure of the LVR-NADH-MPD ternary complex was determined by molecular replacement and refined at 1.6 Å resolution. The refined model includes 267 of the 276 amino acid residues and one molecule each of NADH and MPD per subunit. A total of 88.1% of non-glycine residues are in the most favored region of the Ramachandran plot (25). The molecule has well defined electron density except for the N terminus. The root mean square

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**Table I**

| Data collection statistics |  |
|---------------------------|--|
| Resolution (Å)            | 50–1.6 |
| No. observations          | 1,051,771 |
| Completeness (%)          | 92.1 (82.7) |
| Redundancy                | 0.081 (0.263) |
| R_mol                     | 7.0 |

| Refinement statistics     |  |
|---------------------------|--|
| Resolution range (Å)      | 40–1.6 |
| No. reflections           | 16,602 |
| Completeness (%)          | 92.0 (83.2) |
| No. non-hydrogen atoms    | 261,092 |
| R factor                  | 0.199 (0.254) |
| R_mol                     | 0.218 (0.281) |
| r.m.s. deviations         | 0.006 |
| Bond lengths (Å)          | 1.534 |
| Ramachandran plane        | 10.9 |
| Most favored region (%)   | 88.1 |
| Allowed region (%)        | 14.2 |
| Average B-factors (Å)     | 21.3 |

*Values in parentheses are for the highest resolution shell (1.7–1.6 Å).

Calculated with 5% of the reflections set aside randomly.
differences (r.m.s.d.) and temperature factors by residue of the main-chain atoms of the eight subunits (labeled A–H) in the asymmetric unit is shown in Fig. 2, A and B, with an average of 0.11 and 15.1 Å², respectively. The region encompassing residues 210–225, corresponding to the helix-loop-helix region discussed in the text, are shown in yellow. The N and C termini are labeled. Possible hydrogen bonds are denoted by broken yellow lines.

FIG. 4. Stereoview of the residual electron density map superimposed on models of the NADH and MPD molecules. The $F_o - F_c$ simulated annealing omit electron density map was calculated with data at 1.6 Å resolution and contoured at the 3.5σ level. NADH and MPD were excluded for computation of the calculated structure factors and phases. Atoms are shown in standard colors as detailed in Fig. 2.

FIG. 5. The catalytic site and its interactions with the NADH and MPD molecules. A, stereoview. Atoms are shown in standard colors, except for carbon atoms of the NADH and MPD, which are colored green. Broken yellow lines indicate hydrogen bonds. B, schematic drawing. The ligand and residues that have a hydrogen interaction with the ligand are depicted in ball and stick models. The figure was generated using the program LIGPLOT (23).

The largest r.m.s.d. (about 1.2 Å) among the subunit structures occurs in the region between residues 212 and 216, which is in the vicinity of the substrate-binding site (Fig. 2A). The largest difference described above is due to the intermolecular interaction between Glu-213 of chain E and Arg-27 of chain A, which is observed in only one pair of these monomers. This region also has intermolecular interactions by the crystal packing, mainly a charged interaction between Arg-216 and Glu-233, which is observed in only half of eight monomers, such as chains C, E, and G. In the other half of the monomers, such as chain H, no intermolecular interactions were observed to facilitate its polypeptide chain conformation. After all, superposition of all monomers in the asymmetric unit showed that polypeptide chains are essentially identical among them and that the NADH and MPD molecules are bound to the enzyme in the same configuration. The average r.m.s.d. values for the NADH and MPD molecules of eight monomers are comparable with that of the polypeptide chain, with 0.09 ± 0.02 and 0.13 ± 0.03 Å, respectively. Thus, the structure of subunit G is considered hereafter to be representative of all eight subunits and is used...
in the modeling experiments described below.

Overall Structure—A ribbon diagram of the subunit of LVR with bound MPD and NADH is shown in Fig. 3. The MPD molecule, which was added to aid crystallization, binds unambiguously at the active site of the enzyme in close proximity to the NADH molecule. LVR assembles into a homotetramer in solution (3) and has an overall dimension of about 700 x 800 x 600 Å with 222 molecular symmetry in its P, Q, and R axes. Each subunit of LVR forms a single domain structure comprising a seven-stranded parallel \( \beta \)-sheet flanked by three \( \alpha \)-helices on each side, which constitutes a dinucleotide-binding motif called the “Rossman” fold, which is also found in other SDRs. Here we refer to the secondary structure of the subunit in terms of the elements that are commonly used for the SDR family. Two other short helices (\( \alpha FG1 \) and \( \alpha FG2 \)) form the substrate-binding site (residues 201–225).

The Cofactor-binding Site—NADH binds in an extended conformation at the C-terminal side of the parallel \( \beta \)-sheet. A \( \sigma \)-weighted \( F_o - F_c \) simulated annealing omit map for the NADH molecule is shown in Fig. 4. A close-up view of its binding site is shown in Fig. 5. The adenosine portion of the NADH molecule part is positioned in an \( \alpha \)-helix conformation and is embedded in a pocket made up of three \( \beta \)-strands, and the ribose ring adopts a 3-endo conformation (Fig. 3). The adenine moiety forms hydrogen bonds with the side chain of Asp-72. Two hydroxyl groups of the ribose group form hydrogen bonds with the side chain of Asp-44. The pyrophosphate moiety interacts with the enzyme through hydrogen bonds with the side chains of Ser-23 and Thr-200, the main-chain nitrogen of Leu-25, and two water molecules. These water molecules are located in equivalent positions in each subunit in the asymmetric unit, which suggests that they have definite structural roles.

The nicotinamide mononucleoside portion is located between two \( \beta \)-strands with a puckered C-2-endo conformation. The ribose moiety forms hydrogen bonds with the side chains of Tyr-165 and Lys-169 and with the main-chain oxygen of Asn-99. The nicotinamide ring is buried in the protein interior close to the loop between Trp-199 and Val-203, which is consistent with the well ordered electron density. The hydroxyl groups of Ser-152 and Tyr-165 form hydrogen bonds with the main-chain oxygen of the nicotinamide ring. The hydroxyl of Tyr-165 also forms hydrogen bonds with the 2-hydroxyl of NADH, which in turn forms hydrogen bonds with the side-chain nitrogen of Lys-169. The nicotinamide ring is bound to the enzyme in an \( \alpha \)-helix conformation, in contrast to the \( \beta \) conformation that is needed for SDRs to transfer the B-face substrate. The average \( B \)-factors of the nicotinamide ring of eight monomers are in the range of 15–17 Å², indicating that this moiety is well ordered for all monomers as well as the polypeptide chain. Our interpretation of this observed orientation of the nicotinamide ring is described below.

The Substrate-binding Site—The unbiased simulated annealing omit electron density map was intended to identify
unequivocally the bound MPD molecule at the catalytic site of the enzyme (Fig. 4), in close proximity to the catalytically important residues. It seems reasonable to attribute this density to MPD, which was included in the crystallization solution. Therefore, the electron density is interpreted to be an MPD molecule through the structure of the surrounding residues and the hydrogen-bond pattern of the hydroxyl groups of MPD, although two hydroxyl groups at positions 2 and 4 are indistinguishable from methyl groups at the present resolution. We also confirmed that MPD binds LVR with a $K_i$ value of 23.8 $\pm$ 4.5 mM at pH 7.0.

The MPD molecule is located in the catalytic cleft formed by residues Glu-103, Ser-152, Val-153, Ile-159, Gln-162, Tyr-165, Met-202, Val-203, Ser-206, Met-207, and Phe-222 (Fig. 5). The 2- and 4-hydroxyl groups form hydrogen bonds with the carboxamide group of the nicotinamide and the side chain of Gln-162, respectively. The MPD molecule is positioned between the side chain of Glu-103 on one side and the nicotinamide ring of NADH on the other side. Some of these residues maybe involved in substrate recognition and stereoselective reduction. Nearly 95% of the 285-Å$^2$ solvent-accessible surface area of the MPD molecule is buried in the catalytic cleft through contacts with both the polypeptide chain and the NADH molecule, which indicates that its binding might be controlled by some conformational changes of the enzyme.

**Comparison with Other SDRs**—A structural comparison of LVR with other SDRs demonstrated that LVR exhibits a high degree of overall structural homology, with some conformational differences on the molecular surface and the terminal region of the polypeptide chain, as found previously in a structural study in the SDR family, such as 3α,20β-hydroxysteroid dehydrogenase (17, 27), glucose dehydrogenase (GluDH) (28), and meso-2,3-butanediol dehydrogenase (meso-BDH) (29). The subunit interactions found in LVR are also essentially similar to those in other tetrameric SDRs of known structure (data not shown). Due to low sequence homology, the substantial differences among them are found close to the substrate-binding site, which may be related to substrate specificity. Superpositions of LVR with other SDRs in the substrate-binding site is shown in Fig. 6. In LVR, the conserved catalytic residues, Ser-152, Tyr-165, and Lys-169, are located in the active-site cleft with a spatial arrangement that is common to those seen in other SDRs. These observations indicate that LVR probably shares the same mechanistic features and uses this triad for catalysis. In our structure, however, the binding conformation of the nicotinamide moiety of NADH is different from those in other SDRs, although the binding conformation of the adenine dinucleotide moiety is identical to those in other SDRs. Furthermore, the MPD molecule does not directly form hydrogen bonds with residues of the catalytic triad. The presence of the carboxamide group of the nicotinamide precludes a direct contact between MPD and the catalytic residues.

In comparison with meso-BDH, α-helix (αFG1) is located in the same orientation, and the side chain of Thr-200 makes a hydrogen bond with the di-phosphate moiety of NADH as well as meso-BDH. But the side chain of Val-203 of LVR is located...
close to the position of the carboxamide group of meso-BDH, so that there is a little space to bind in the syn conformation for the nicotinamide nucleotide. In the case of meso-BDH, the valine residue is replaced by tryptophan (Trp-190), which has no repulsive interaction with the carboxamide group. In the case of GluDH and 3α,20β-hydroxysteroid dehydrogenase, although the valine residue is substituted with aspartate (Asn-196) and threonine (Thr-190), respectively, αFG1 is shifted to the external region to compensate for the steric hindrance by the side-chain atoms. The hydrogen bond interaction between Thr-200 and the di-phosphate moiety is conserved in the GluDH structure. In LVR, Ser-206 in the helix has a hydrogen bond with the side chain of Glu-103. This hydrogen bond interaction was not observed in other SDRs and was specific to LVR due to amino acid differences.

Predicted Model of Levodione Binding—The crystal structure of LVR bound to levodione has not been solved. Thus, the position of the MPD molecule at the active site is insufficient to understand the binding of levodione, although the MPD molecule generally occupies a similar position within the active site. The observed three-dimensional architecture of the active site can shed light on the residues implicated in levodione binding, however, because the spatial organization of the surrounding residues is completely defined by this structure. Therefore, in order to understand the substrate stereospecificity, the binding mode of levodione can be modeled into this structure, based on the catalytic mechanism of the SDR enzymes, which was proposed previously using crystal structures and modeled structures (30, 31). It may be assumed that the 4-keto oxygen of levodione occupies the similar position of the oxygen atom of the carboxamide group of NADH, which interacts with Ser-152 and Tyr-165, the highly conserved residues of the catalytic triad.

Without altering the structure of the enzyme except for the position of NADH, we are able to model a levodione molecule in the substrate-binding cleft (Fig. 6A). The electrostatic potential surface of the active site with a stick model of levodione and NADH is shown in Fig. 7A to illustrate the complementarity of the binding cavity for the levodione molecule. The lowest energy conformation of levodione in the active site has a chair shape, with the C-6 methyl group in an equatorial orientation. This conformation is essentially identical to that of the crystal structure (32) from Cambridge Structure Database, with an r.m.s.d. of 0.04 Å. In this model, the aliphatic rings of levodione can be mapped roughly onto the MPD molecule, and the 4-keto oxygen of levodione can accept a hydrogen from the side chains of Ser-152 and Tyr-165, thus allowing the 4-keto oxygen to be mapped onto the carboxamide oxygen of nicotinamide. Hydrogen bonds between the catalytic Ser and/or Tyr and the substrate have been reported previously, such as human 7β-hydroxysteroid dehydrogenase (30), tropinone reductase II (33), and E. coli 7α-hydroxysteroid dehydrogenase (34). Nicotinamide can liberate the pro-S hydride at C-4 for nucleophilic attack on the 4-keto carbon of levodione, which is positioned at a distance of about 4 Å.

We tried to determine the stereoselectivity of hydrogen transfer. The neighboring residues Glu-103, Ile-159, Val-203, Ser-206, Met-207, and Phe-222 might be responsible for recognition of levodione through van der Waals and electrostatic interaction. In particular, Glu-103 might constitute the negatively charged surface seen at the bottom of the substrate-binding site, which is more clearly suggested after comparison with a model of the E103Q mutant protein (Fig. 7A). By contrast, positively charged surface is distributed in the middle of the levodione molecule because of two polarized carbonyl groups. Fig. 7B suggests that there is slightly more positive charge on the re side than on the si side of levodione. Therefore, Glu-103 may have a role in preserving the stereoselectivity of the enzyme. To test this hypothesis, four LVR enzymes containing different substitutions at Glu-103 were prepared, and their stereoselectivities were measured (see below).

CD Spectra of Wild-type and Mutant LVR Enzymes—To verify that the mutant enzymes were folded correctly, we obtained the CD spectra of the wild-type and the four mutant LVR proteins (E103A, E103Q, E103N, and E103D). All five enzymes had virtually superimposable CD spectra (data not shown), indicating that any observed decrease in enzyme activity or enantiomeric excess of the reaction products for these four mutants was not due to a major structural change in the protein.

Characterization of Mutant LVR Enzymes—From the crystal structure of LVR complexed with MPD, it was predicted that Glu-103 of LVR would possibly come in contact with the levodione molecule bound to the enzyme. Mutant LVR enzyme was purified under essentially identical conditions to those used for the corresponding wild-type enzyme (4). The two chromatographic steps result in a nearly homogenous preparation of LVR proteins (Fig. 8). The purified mutant enzymes were analyzed for their affinity for levodione and NADH (Table II). All four mutant enzymes had a K_m value for levodione that was several orders of magnitude higher than that of the wild-type enzyme; however, their affinity for NADH was not greatly affected, with K_m values of the same order of magnitude as those of the wild-type enzyme. This observation is in keeping with the fact that mutations were made only to the putative substrate-binding site.

Stereospecificities of the Mutant Enzymes—The substitution of Glu-103 with glutamine, aspartate, asparagine, or alanine resulted in a 2–6-fold increase in K_m. In addition, the enantiomeric excess of the reaction products was about 60% enantiomeric excess, a value much lower than that of wild-type (95% enantiomeric excess; Table II).
In the LVR crystal structure, the orientation of the nicotinamide ring of the NADH molecule differs substantially from those of other enzymes of the SDR family for which structures are available. The side chain of Val-203 is located in close proximity to the binding site of the nicotinamide ring of the NADH molecule and certainly has little space for the cofactor to approach. The side chain of Val-203 is located in close proximity to the binding site of the nicotinamide ring of the NADH molecule differs substantially from those of other enzymes of the SDR family for which structures are available. The side chain of Val-203 is located in close proximity to the binding site of the nicotinamide ring of the NADH molecule and certainly has little space for the cofactor to approach. The side chain of Val-203 is located in close proximity to the binding site of the nicotinamide ring of the NADH molecule and certainly has little space for the cofactor to approach.

The structures of other SDR enzymes have shown that the nicotinamide-ribose end of the cofactor has several possible conformations or is dynamically disordered (17, 35) and that the helix-loop-helix region, called flexible lid, is indicative of enhanced mobility in the apoenzyme and the binary complex of protein with cofactor (16, 22, 27). Thus, it has been assumed that this flexible region of the catalytic cleft, which is the most variable region among SDRs, acts as a lid that controls access of the substrate to the active site. In our structure, residues Thr-200 to Asn-226 is very well defined in the electron density maps, although the loop region between residues 211 and 216 has relatively higher B-factor values than the rest of the protein. The polypeptide chain folding is consistent in the substrate-binding pocket, regardless of the intermolecular interactions by the crystal packing, such as a charged interaction between Arg-216 and Glu-233. Thus, the conformation of this region in LVR is independent to protein assembly and crystal packing.

The nicotinamide binding in an anti conformation has not been observed in other SDR enzymes. Our structure may provide an obvious means of visualizing the substrate within the active site. The complex offers the closest analogy to the keto group of substrates, in which the carboxamide oxygen of the nicotinamide ring forms hydrogen bonds with both Ser-152 and Tyr-165. The model can account for hydride delivery from NADH to the B-face of the substrate, forming an R-configuration product with high stereoselectivity. The model places the 4-keto carbon at a distance of about 4 Å from the nicotinamide, which means that some conformational changes must occur in the enzyme to accommodate levodione. The necessary movements are presumably brought about by structural rearrangements in the flexible region described above.

In comparison with wild-type LVR, the E103A, E103Q, and E103N mutant enzymes exhibited only a low level of stereospecificity, in keeping with our proposed binding mode of levodione. In addition, the low stereospecificity of the mutants indicates that Glu-103 has an important role in this process; the negatively charged surface of Glu-103 interacts with the positively charged surface on the re side of levodione in the determination of stereospecificity. The marked change in sidechain volume and electrostatic potential caused by substitution of Glu-103 with alanine suggests that this mutation may cause minor structural modifications in the region of these residues, thus preventing the binding of the substrate. The substitutions of aspartic acid and glutamine were also not sufficient for maintaining the stereospecificity of LVR. The side chain of aspartic acid is about 1 Å shorter than that of glutamic acid, which may alter the optimum position for accepting the levodione molecule in the E103D mutant enzyme. It is interesting to note the shift in pK for the Tyr-165 hydroxyl group in the mutant LVR proteins, which is estimated and given in Table III. Substitution of Glu-103 with Ala, Asn, and Gln can cause changes of 0.4–1.6 pK units at different ionic strengths. Substitution with Asp also affects the pK but alters it negatively. These results are consistent with the decrease in the kinetic parameter kcat of the mutants (Table II). Although we now have a detailed structural description of the active site, it still remains unclear which factors underlie its stereospecific oxidation. Further structural analysis of the crystallized mutants will help to clarify the subtle structural differences at the active site with the expectation of deepening our understanding of the enzyme stereospecificity.

CONCLUSIONS

The crystal structure of LVR confirms that this enzyme belongs to the SDR family of proteins, which share a common fold, termed the Rossman fold, and the invariant sequence Tyr-X-X-Lys, which is known to be essential for catalysis by the enzyme. Fortuitously, LVR was crystallized with MPD bound in the active-site pocket facing the nicotinamide ring of the NADH cofactor. This MPD-bound structure sheds light on the location of the cofactor- and substrate-binding sites. The so-called “Ser-Tyr-Lys catalytic triad” (Ser-152, Tyr-165, and Lys-169 in LVR) is a highly conserved geometrical feature of the active site that is proposed to be central to the catalytic mechanism of SDRs. In LVR, we found that the hydroxyl groups of both Ser-152 and Tyr-165 form hydrogen bonds with the carboxamide oxygen of the nicotinamide. In addition, the side chain of Lys-169 forms a hydrogen bond with the side chain of Tyr-165 through the hydroxyl group of the nicotinamide ribose. These observations provide insights into the structural basis that underlies substrate binding. The composition of the active site most probably reflects the specific interactions that LVR makes with the substrate and also the stereospecificity of the catalytic reaction. This has allowed us to propose a
model for levodione binding in the active site and for participation of the surrounding residues in substrate recognition. The residue Glu-103 seems to be involved in the high stereospecificity of the enzyme; however, a clear picture of how levodione binds to LVR is not yet available. Structure determination of the complex bound to its substrate will be critical to understand fully the mechanisms underlying substrate recognition and stereospecific oxidoreduction in this enzyme.

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The Crystal Structure and Stereospecificity of Levodione Reductase from *Corynebacterium aquaticum* M-13

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