The Crystal Structure of 3α-Hydroxysteroid Dehydrogenase/Carbonyl Reductase from Comamonas testosteroni Shows a Novel Oligomerization Pattern within the Short Chain Dehydrogenase/Reductase Family*

Received for publication, August 18, 2000, and in revised form, September 25, 2000
Published, JBC Papers in Press, September 27, 2000, DOI 10.1074/jbc.M007559200

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The crystal structure of 3α-hydroxysteroid dehydrogenase/carbonyl reductase from Comamonas testosteroni (3α-HSDH) as well as the structure of its binary complex with NAD+ have been solved at 1.68-Å and 1.95-Å resolution, respectively. The enzyme is a member of the short chain dehydrogenase/reductase (SDR) family. Accordingly, the active center and the conformation of the bound nucleotide cofactor closely resemble those of other SDRs. The crystal structure reveals one homodimer per asymmetric unit representing the physiologically active unit. Dimerization takes place via an interface essentially built-up by helix α6 and strand βG of each subunit. So far this type of intermolecular contact has exclusively been observed in homotetrameric SDRs but never in the structure of a homodimeric SDR. The formation of a tetramer is blocked in 3α-HSDH by the presence of a predominantly α-helical subdomain which is missing in all other SDRs of known structure.

Gram-negative bacteria of the genus Comamonas have been isolated from soil, mud, and water. These strictly aerobic, non-fermentative, chemoorganotrophic bacteria rarely attack sugars, but grow well on organic acids and amino acids (1). Comamonas testosteroni is able to use steroids as a sole carbon source, but grow well on organic acids and amino acids (1). Isolated from soil, mud, and water. These strictly aerobic, non-enzymes, 3 genes encoding steroid catabolizing enzymes (3). One of these testosterone induces in this organism the expression of a set of these stable compounds from the environment. The presence of a source (2) and may be an attractive means for the removal of radiation of these relatively inert substrates. In addition, 3α-HSDH accepts a wide spectrum of xenobiotic molecules such as metyrapone or the significantly smaller p-nitrobenzaldehyde as substrates (4, 5). As a consequence, in the presence of a steroid inducer not only the resistance of C. testosteroni to the steroid antibiotic fusidic acid but also the metabolic capacity of insecticide degradation are significantly enhanced (6).

3α-HSDH from C. testosteroni is a member of the short chain dehydrogenase/reductase (SDR) family. Despite the fact that the amino acid identity among the members of this family is only in the 15 to 30% range, they reveal a striking similarity in their tertiary structure. Their overall structure is based upon a typical dinucleotide binding motif or Rossmann fold composed of βββ units, which build up a parallel β-sheet sandwiched between two arrays of parallel α-helices (7). Most SDR enzymes are either homodimers or -tetramers and all require NAD(H) or NAPD(H) as a cofactor (8). The 3α-HSDH primary structure shows two sequence motifs which are common to the members of the SDR family. These are the amino-terminal GlyX-Gly12-X-Gly14 cofactor binding motif and the Tyr155-X-Y-Lys159 motif (5) which is structurally located in the active site and forms together with the conserved Ser141 a catalytic triad (numbering according to the 3α-HSDH primary structure). Probably all SDRs share a common reaction mechanism, which follows a compulsory ordered pathway with the cofactor binding first. Upon binding of the hydroxyl or carbonyl substrate a so-called substrate binding loop, which is highly flexible in most apo-SDRs, becomes well ordered and covers the substrate as well as the catalytic center from aqueous environment. The transfer of a hydride ion is accompanied by a proton transfer via the Tyr residue of the catalytic triad. The catalytic Ser orients the substrate and stabilizes the transient reaction intermediate. Alternatively, it has been proposed that the deprotonated Ser may act as a catalytic base with Tyr playing a subsidiary role during catalysis (9). The dual role of the Lys in the catalytic triad is the proper orientation of the cofactor by forming hydrogen bonds to the nicotinamide-ribose moiety and to lower the pKₐ of the catalytic Tyr via electrostatic interaction (10–12).

Here we report the crystal structure of C. testosteroni 3α-HSDH in its apo, as well as, in its NAD+ bound form. While the catalytic triad of 3α-HSDH and the conformation of the bound dinucleotide cofactor are well superimposable with those found in other SDRs of known structure, the enzyme reveals an oligomerization mode which is so far unique within the SDR family.

* This work was supported in part by Deutsche Forschungsgemeinschaft Grants SFB 286/A11 (to R. F.) and SFB 395 (to E. Mas) and European Community Program Biotech 2 contract BIO4-97-2123 (to E. Maser). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† The abbreviations used are: HSDH, hydroxysteroid dehydrogenase/carbonyl reductase; SDR, short chain dehydrogenase/reductase; PEG, polyethylene glycol; SA, simulated annealing; NCS, noncrystallographic symmetry.
**EXPERIMENTAL PROCEDURES**

**Protein Purification**—Overexpression of the gene encoding *C. testosteroni* 3α-HSDH in *Escherichia coli* and purification of the recombinant protein were performed as described previously (5).

**Crystalization**—All crystallization experiments were carried out in Linbro cell culture plates using the hanging drop vapor-diffusion technique. Initial crystallization conditions were obtained using the Crystal Screen kit from Hampton-Research. For this initial screen 1.5 μl of protein solution (5 g/liter 3α-HSDH protein in 10 mM Tris/HCl, pH 7.5, and 0.1 mM dithiothreitol) was mixed with 1.5 μl of reservoir solution which was then sealed against the reservoir. The screen was carried out in parallel at 277 and 293 K. After a period of 1 week, small single crystals were obtained at 277 K with screening solution number 22 (30% PEG 4000, 0.1 M cacodylate (30% PEG 4000, 0.1 M Tris/HCl, pH 8.5, 0.2 M sodium acetate). With refined crystallization conditions (25–30% PEG 4000, 0.1 M cacodylate buffer, pH 6.5, and 0.2 M ammonium acetate) crystals grew to a size of 0.3 × 0.3 × 0.4 mm within a period of 3 weeks. The size of the crystals was further improved by macroseeding and lowering the precipitant concentration to 20%, which resulted in crystals with dimensions of approximately 0.5 × 0.5 × 0.8 mm.

**Data Collection and Processing**—Data sets were collected on a Rigaku rotating-anode generator (Rigaku/MSC) operating at 50 kV and 100 mA and equipped with focusing mirrors (MSC/Yale) and a RAXIS IV image plate detector. Crystals were flash-cooled in nylon-fiber loops in a 100 K nitrogen gas stream provided by an X-stream cryo system. As a cryo buffer reservoir solution with 20% (v/v) glycerol was used. Raw data indexing and processing was done within DENZO and XDISP (13). The pre-processed data were then scaled and merged using SCALEPACK (13).

**Structure Determination and Refinement**—Initial phasing of the native dataset was carried out by the molecular replacement method (14) by means of the program AMoRe (15) from the CCP4 suite (16). Patterson rotation and translation search were carried out with the coordinates of the poly-alanine trace of one monomer of tropinone reductase II (Protein DataBank code Zae1) (17) after removal of helix c and the substrate binding loop. The best solution from the rotation function search (Table I) was kept fixed for the translation search of the second molecule. The best solution emerging from the translation function search (Table II) could be clearly distinguished from all other (wrong) solutions by means of correlation coefficients and R-factor. With two monomers oriented according to the two solutions a sensible dimer could be formed. The model was then subjected to a rigid body and a 3000 K simulated annealing (SA) refinement with the program CNS (18) using data from 8 to 3.5 Å and 8 to 2 Å, respectively. Refinement was carried out without non-crystallographic symmetry (NCS) restraints. After a first visual inspection of this initial model in conjunction with an electron density map calculated with the model phases, 110 residues creating some difficulties with respect to the dimerization contacts and crystal packing were removed. Appropriate side chains corresponding to the 3α-HSDH sequence were then introduced. The side chain conformation was chosen similar to the conformation of homologous residues in other SDR structures, when possible. After another 300 K SA refinement and an individual B-factor refinement in CNS (data in the resolution range from 8 to 1.68 Å) a 2Fcalc − Fobs map and an Fcalc − Fobs map were calculated. Due to insufficient quality of these maps for model building and manual model refinement, density modification techniques (solvent flattening, histogram matching, 2-fold non-crystallographic averaging with phase extension) were applied using the programs DM, the CCP4 and the CCP4 suite (18) using data from 8 to 3.5 Å and 8 to 2 Å, respectively. Refinement was carried out with non-crystallographic symmetry (NCS) restraints. With DM, the resulting map and the model were again inspected with graphic averaging with phase extension. A bulk solvent correction was then introduced and a 1000 K SA refinement and an individual B-factor refinement in the CNS using data from 8 to 1.68 Å were carried out. Phases and figure of merit estimates were calculated using the improved phase and figure of merit estimates. For all density modifications and subsequent map calculations the full resolution range (99 to 1.68 Å) was used. The resulting map enabled us to build an additional helix-loop-helix domain in the residue range from 120 to 149 and to accomplish some minor corrections to the model. Some 40 residues which did not fit the density were removed. After these manual corrections a 3000 K SA refinement and an individual B-factor refinement in the CNS using data from 8 to 1.68 Å were carried out. Phases and figure of merit estimates were calculated from the improved model and new averaging and solvent-flattening masks were generated. After running 35 cycles of density modification with DM, the resulting map and the model were again inspected manually. Within a total of four of these refinement cycles the whole model could be built, lacking only residues 188 to 208 forming a putative loop region where no appropriate density could be found. In this stage, water molecules were fit into the model using the appropriate CNS script. A bulk solvent correction was then introduced and a 1000 K SA refinement including an individual B-factor refinement were performed in CNS using all available data within the 99 to 1.68 Å range. NCS restraints were released at this stage. After inclusion of a total of 405 water molecules, some additional manual fine tuning and automatic refinement, including an overall anisotropic B-factor refinement, a final Rmerge of 20.0/22.9% has been achieved.

The apo-3α-HSDH model was used as an initial model for refinement against all datasets measured from crystals grown in the presence of enzyme substrates. A 1500 K SA and individual B-factor refinement against each dataset were carried out using data sampled over the whole resolution range in conjunction with a bulk solvent correction. 2Fcalc − Fobs and Fcalc − Fobs maps were then inspected for density corresponding to bound substrate.

The quality of the models was assessed using the programs PROCHECK (19), MOLSCRIPT (20) and RASTER3D (21).

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**Table I**

| No. | α   | β   | γ   | corr |
|-----|-----|-----|-----|------|
| 1   | 315.65 | 92.13 | 266.30 | 8.4  |
| 2   | 109.97 | 107.36 | 229.52 | 7.8  |
| 3   | 43.50  | 29.00  | 269.00 | 7.3  |
| 4   | 270.78 | 76.97  | 79.10  | 7.3  |
| 5   | 222.34 | 108.95 | 63.57  | 7.3  |
| 6   | 58.94  | 150.29 | 149.29 | 7.3  |
| 7   | 233.89 | 121.26 | 43.14  | 7.2  |
| 8   | 303.32 | 92.59  | 260.45 | 7.2  |
| 9   | 216.00 | 149.01 | 211.67 | 7.0  |
| 10  | 119.78 | 127.54 | 61.25  | 6.8  |

**Table II**

| a   | β   | γ   | tx  | ty  | tz  | corr | R % |
|-----|-----|-----|-----|-----|-----|------|-----|
| 222.34 | 108.95 | 63.57 | 0.4000 | 0.0541 | 0.4708 | 17.6 | 52.7 |
| 95.18  | 77.79  | 52.45  | 0.5694 | 0.8683 | 0.6529 | 11.4 | 54.9 |
| 58.94  | 150.29 | 149.29 | 0.6131 | 0.2077 | 0.6778 | 11.3 | 54.8 |
| 279.35 | 138.26 | 257.63 | 0.8500 | 0.9000 | 0.7410 | 11.2 | 54.5 |
| 303.32 | 92.59  | 260.45 | 1.0545 | 0.8608 | 0.1718 | 11.0 | 54.6 |
| 350.25 | 139.20 | 338.23 | 0.0250 | 0.8860 | 0.2738 | 10.9 | 54.1 |
| 238.35 | 51.79  | 297.20 | 0.7913 | 0.0008 | 0.7179 | 10.7 | 54.6 |
| 233.78 | 106.53 | 117.32 | 0.1845 | 0.0538 | 0.9078 | 10.6 | 54.1 |
| 109.97 | 107.36 | 229.52 | 0.8683 | 0.0065 | 0.1130 | 10.5 | 54.6 |
| 233.89 | 121.26 | 43.14  | 0.5348 | 0.0427 | 0.1931 | 10.5 | 54.7 |
CHECK (20) (Ramachandran diagrams) and O (21) (rotamer analysis and peptide orientation analysis). The contact area of the two NCS related monomers as well as the area of the three major crystal contacts of the complete dimer was calculated using the program GA_FIT (22, 23). The coordinates have been deposited with the Protein Data Bank (codes: 1fjh for apo-3α-HSDH and 1fk8 for the 3α-HSDH NAD$^+$ complex).

RESULTS AND DISCUSSION

Overall Structure and Topology—The structures of 3α-HSDH in its apo and in its NAD$^+$ bound form were solved by molecular replacement using tropinone reductase II (17) as a search model. The structures were refined at a resolution of 1.68 and 1.95 Å, respectively, and show good stereochemistry with 91% of all model residues in the most favored and no residues in disallowed regions of the Ramachandran plot. The data collection and refinement statistics are summarized in Table III. Comparison of both structures clearly shows that binding of NAD$^+$ has no influence on the conformation of 3α-HSDH and therefore both structures are virtually identical apart from the presence or absence of the cofactor. The crystal structure reveals one homodimer per asymmetric unit representing the physiologically active entity (24). Although the monomeric subunit of 3α-HSDH shows a typical SDR architecture, its topology differs in some details from that of other members within this protein family (Figs. 1 and 2). The canonical structural motif of SDR proteins is a dinucleotide binding or Rossmann fold consisting of $\alpha\beta\beta$ units forming a central six-stranded parallel $\beta$-sheet sandwiched between two arrays of $\alpha$-helices (7). In all SDRs this fold is extended at its COOH

FIG. 2. Comparison of the folding topologies found in the crystal structures of S. hydrogenans 3α,20β-HSDH (10) (A), Drosophila lebanonensis alcohol dehydrogenase (34) (B), and C. testosteroni 3α-HSDH (C). $\alpha$-Helices are represented as circles and $\beta$-strands as triangles. Cylinders indicate short $\alpha$-helices and the dotted lines disordered regions. The labeling convention used with respect to the secondary structural elements is according to Ghosh et al. (35).

TABLE III

| Crystallographic data and refinement statistics |
|-----------------------------------------------|
| **Apo-protein** | **Binary complex** |
| Max. resolution (Å) | 1.65 | 1.95 |
| Wavelength (Å) | 1.5418 | 1.5418 |
| Temperature (K) | 100 | 100 |
| Space group | P1 | P1 |
| Unit cell parameters Å | | |
| $a$ | 43.3 | 42.8 |
| $b$ | 46.2 | 46.2 |
| $c$ | 65.3 | 65.0 |
| $\beta$ | 106.5 | 107.5 |
| $\gamma$ | 98.8 | 97.6 |
| No. of observed reflections | 374,072 | 233,945 |
| No. of unique reflections | 50,140 | 30,053 |
| Completeness (%) | | |
| Overall | 97.7 | 93.3 |
| Last shell | 74.0 | 90.5 |
| $R_{cryst^a}$ (%) | | |
| Overall | 4.3 | 4.2 |
| Last shell | 27.1 | 27.5 |
| Refinement statistics | | |
| $R$-factor$^b$ (%) | 20.0 | 18.5 |
| $R$-free (%) | 22.9 | 22.0 |
| Deviations from ideal geometry | | |
| Bond lengths (Å) | 0.005 | 0.006 |
| Bond angles (°) | 1.2 | 1.3 |

$^a$ $R_{cryst} = \Sigma |I - \langle I\rangle|/\Sigma I$, where $I$ is the observed intensity and $\langle I\rangle$ is the average intensity for multiple measurements.

$^b$ $R$-factor = $\Sigma |F_{obs} - F_{calc}|/\Sigma F_{obs}$, where $F_{obs}$ and $F_{calc}$ are the observed and calculated structure factors, respectively. $R$-free is the cross-validation $R$-factor calculated for 15% of the reflections omitted in the refinement process.

FIG. 1. Three-dimensional structure of the 3α-HSDH monomer with bound NAD$^+$ cosubstrate. The disordered substrate binding loop is indicated by a dotted line. The ribbon representation was produced using the program MOLMOL (33). of the complete dimer was calculated using the program GA_FIT (22, 23). The coordinates have been deposited with the Protein Data Bank (codes: 1fjh for apo-3α-HSDH and 1fk8 for the 3α-HSDH NAD$^+$ complex).

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Crystal Structure of 3α-HSDH

Fig. 3. Three-dimensional structure based sequence alignment of C. testosteroni 3α-HSDH and two further representatives of the SDR enzyme family. The 3α-HSDH secondary structure shown on top of the alignment was assigned according to PROCHECK (20). The section representing the missing part of the substrate binding loop allowing no structural alignment is indicated by italic letters. 3α,20β-HSDH, 3α,20β-HSDH from S. hydrogenans; ADH, alcohol dehydrogenase from D. lebanonensis.

The quaternary structure of 3α-HSDH was clearly shown to be a homodimer by gel filtration of the purified protein (24). To demonstrate the spatial relationship between the dimer subunits observed in the 3α-HSDH structure, the subunit arrangement of homotetrameric SDRs of known structure, has an important implication in the oligomerization properties of 3α-HSDH (see below).

Quaternary Structure—The quaternary structure of 3α-HSDH was composed by α-helical sections, is masking the Rossmann fold helices aE and aF, thus preventing the formation of a four-helix bundle. As a consequence, in 3α-HSDH dimerization takes place via a P-axis interface. The subunit contacts are formed by side chain-to-side chain interactions of two βG strands as well as by the parallel packing of two αG-helices against each other (Fig. 4C). In addition, the carboxyl-terminal helix aCT of each subunit is packing against the aF helix of the other subunit. Dimerization about a P-axis interface has been discussed as a possible mode of oligomerization in SDRs (28) but has never been observed in a crystal structure so far. The oligomerization contacts can clearly be distinguished from pure crystal contacts, since the contact surface involved in dimerization (about 1900 Å² per monomer) by far exceeds the total buried solvent-accessible surface area created by any of the three observed major crystal contacts (160 to 280 Å² per monomer) (Table IV).

Catalytic Center and Binding of NAD⁺—Three residues, namely Ser¹¹⁴, Tyr¹⁵⁵, and Lys¹⁵⁹ in 3α-HSDH, form a triad essential for catalysis and well conserved within the SDR family. The arrangement of these amino acids within 3α-HSDH is virtually identical to that in other SDRs of known structure emphasizing the probability of a common reaction mechanism within this protein family. Likewise, in the binary complex the NAD⁺ cofactor, which is bound at the carboxyl-terminal ends of the β-strands is well superimposable with the cofactors present in other binary or ternary complexes of SDRs. Accordingly, both ribose rings are present as 2⁺endo-conformers. The nicotinamide ring is bound in a syn and the adenine base in an anti-torsion angle to the respective ribose moiety. As expected, the orientation of the nicotinamide ring with respect to the catalytic triad is also very similar to what is observed in other SDR-NAD(P)(H) complexes. The structure suggests that 3α-HSDH depends on NAD(H) and will hardly accept NADP(H) as a cofactor. The side chain carboxylate of Asp³⁴, a residue highly conserved among enzymes preferring NAD(H) (29), forms hy-
hydrogen bonds to both the 2’ and the 3’ OH group of the adenosine ribose. Accordingly, a potential phosphate group linked to the 2’ position of the adenosine moiety would create unfavorable contacts in this area. In addition, in the observed conformation the side chain of the neighboring Ile^{33} would impede the presence of a phosphate for sterical reasons (Fig. 5). The preference of NAD(H) with respect to NADP(H), which can be inferred from the structure presented, supports the catabolic nature of 3α-HSDH.

**Substrate Binding Loop**—In the structures of many SDR enzymes, the so-called substrate binding loop (consisting of residues 188 to 213 in 3α-HSDH) is almost completely disordered in the absence of a bound cofactor and substrate. After binding of the dinucleotide cofactor a minor portion of this loop may become stabilized and only after binding of the hydroxyl/carbonyl substrate it adopts a defined, usually helical, conformation. Substrate specificity of SDRs is largely conveyed by

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**TABLE IV**

Contact areas of symmetry related molecules observed in 3α-HSDH crystals

| Symmetry operation | Contact area of the symmetry related molecules (Å^2) calculated for one monomer |
|--------------------|---------------------------------------------------------------------------------|
| Translation of the dimer along a | 158 |
| Translation of the dimer along b | 281 |
| Translation of the dimer along c | 266 |
| NCS of the two monomers | 1906 |
this loop. In many SDRs its flexibility and adaptability is assumed to be responsible for the broad spectrum of accepted substrates commonly observed for these enzymes. In the structure of apo-3α-HSDH alike no or only poorly defined electron density is detected for the major part of the substrate binding loop apart from residues 209 to 213 which are clearly defined. In the crystal structure of 3α-HSDH complexed to NAD⁺ residues 188 to 192 become visible in one of the two subunits. However, the better defined electron density in this region compared with the second subunit or the apo-form is very subtle and may not necessarily result from NAD⁺ binding.

To obtain some insight on how substrate recognition and thus specificity are achieved in 3α-HSDH, we tried to obtain crystals of a ternary complex. This was attempted by soaking several steroids or xenobiotics into freshly grown crystals of a ternary complex. This was attempted by soaking the oligomerization mode of 3α-HSDH, which is highly conserved among NAD(H) preferring SDRs, forms two hydrogen bonds to the 2' and 3' OH group of the adenosine moiety of the bound cofactor. Supposedly, it would form unacceptably short contacts to a potential phosphate group that would be linked to the 2' oxygen in a bound NADP(H) cofactor molecule. The superimposed 2Fobs − Fcalc electron density map is contoured at 1.5 σ.

Conclusions and Outlooks—The most remarkable feature of 3α-HSDH is an extended insertion within the Rossmann fold between strand βE and helix αF preventing the formation of a Q-axis type oligomerization interface which is present in all other homotetrameric and homodimeric SDRs of known structure. Since no residues of this insertion are either in contact with the catalytic center or with the putative substrate-binding site, the question about its function appears mandatory. It is tempting to speculate that the oligomerization mode of 3α-HSDH, which is likely to result from this insertion and which is so far unique among SDRs, might drive cooperativity among the subunits. The only presently available kinetic evidence for such cooperativity among SDR subunits was collected for two cis-retinol/3α-hydroxysterol short chain dehydrogenase isoenzymes, which reveal both sigmoidal and Michaelis-Menten kinetics depending on the substrate (30, 31). It must be noted, however, that the kinetic data for these isoenzymes have not been determined by means of the purified enzymes but by means of total cell extracts, thus suggesting not very conclusive results. A potential cooperativity among the subunits of an SDR has been discussed with respect to the crystal structure of the homotetrameric 3α,20β-HSD from Streptomyces hydrogenans (10, 32), which shows that residues from three subunits are lining up each substrate binding pocket. Cooperativity was proposed to be mediated by helix αF which harbors the catalytic Lys and Tyr and contributes in 3α,20β-HSDH to the formation of the substrate binding pocket of another, Q-axis related, subunit. In 3α-HSDH, however, subunits are not related by a Q-axis. No residues of the second subunit approach close enough to contribute to the formation of the substrate binding pocket of the first subunit, assuming that substrates are bound in 3α-HSDH at a site similar to other SDRs. A potential cooperativity in 3α-HSDH might be achieved via the substrate binding loop, whose carboxyl-terminal residues (209 to 213) contact in the presented structure the carboxyl-terminal parts of loops αF and αG of the second subunit. It thus may well transmit the information of substrate binding to one subunit via conformational changes to the second. It cannot be excluded, however, that the conformation of residues 209 to 213 as detected in the presented structure result from crystal packing and do not mirror the same situation in solution.

Elaborate and thoroughly collected kinetic data will have to be sampled in order to confirm or rule out the assumption of a possible cooperativity among the 3α-HSDH subunits. In addition, a mutated 3α-HSDH enzyme, where the insertion between strand βE and helix αF is deleted, may be conclusive to study its implication on the formation of the quaternary structure, substrate recognition, and a potential cooperativity. Furthermore, in case that no crystals can be grown of the wild type 3α-HSDH simultaneously accommodating the cofactor and substrate, such a ternary complex may be crystallized using the mutated enzyme depleted from the insertion between strand βE and helix αF found in the wild type.

Acknowledgment—We greatly thank Milton Stubbs (Institut für Pharmazeutische Chemie, Marburg) for help during data collection.

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