Crystal Structure of the Productive Ternary Complex of Dihydropyrimidine Dehydrogenase with NADPH and 5-Iodouracil

IMPLICATIONS FOR MECHANISM OF INHIBITION AND ELECTRON TRANSFER*

Received for publication, December 13, 2001, and in revised form, January 16, 2002
Published, JBC Papers in Press, January 16, 2002, DOI 10.1074/jbc.M111877200

Doreen Dobritzsch§§, Stefano Ricagno¶¶, Gunter Schneider‡, Klaus D. Schnackerz, and Ylva Lindqvist†††

From the §Division of Molecular Structural Biology, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-17177 Stockholm, Sweden and ¶Theodor-Boveri-Institut für Biowissenschaften, Physiologische Chemie I, Am Hubland, D-97074 Würzburg, Germany

Dihydropyrimidine dehydrogenase catalyzes the first and rate-limiting step in pyrimidine degradation by converting pyrimidines to the corresponding 5,6-dihydro compounds. The three-dimensional structures of a binary complex with the inhibitor 5-iodouracil and two ternary complexes with NADPH and the inhibitors 5-iodouracil and uracil-4-acetic acid were determined by x-ray crystallography. In the ternary complexes, NADPH is bound in a catalytically competent fashion, with the nicotinamide ring in a position suitable for hydride transfer to FAD. The structures provide a complete picture of the electron transfer chain from NADPH to the substrate, 5-iodouracil, spanning a distance of 56 Å and involving FAD, four [Fe-S] clusters, and FMN as cofactors. The crystallographic analysis further reveals that pyrimidine binding triggers a conformational change of a flexible active-site loop in the α/β-barrel domain, resulting in placement of a catalytically crucial cysteine close to the bound substrate. Loop closure requires physiological pH, which is also necessary for correct binding of NADPH. Binding of the voluminous competitive inhibitor uracil-4-acetic acid prevents loop closure due to sterical hindrance. The three-dimensional structure of the ternary complex enzyme-NADPH-5-iodouracil supports the proposal that this compound acts as a mechanism-based inhibitor, covalently modifying the active-site residue Cys-671, resulting in S-(hexahydro-2,4-dioxo-5-pyrimidinyl)cysteine.

Dihydropyrimidine dehydrogenase (DPD,1 EC 1.3.1.2) is a cytosolic enzyme catalyzing the NADPH-dependent reduction of uracil and thymine to the corresponding 5,6-dihydropyrimidine, the first and rate-limiting reaction in the three-step pathway of pyrimidine degradation (1). In mammals, this is the only pathway leading to the synthesis of the putative neurotransmitter β-alanine (2).

The human enzyme has recently evolved as an adjunct target for anticancer drug design because it also degrades 5-fluorouracil (5FU), one of the most widely prescribed chemotherapeutic agents for treatment of many common malignancies (3). 5FU primarily targets the enzyme thymidylate synthase and thereby interferes with DNA synthesis (4). Approximately 85% of the administered dose is rapidly degraded by DPD to therapeutically inactive but toxic fluorinated products (5, 6). Furthermore, accurate determination of an optimal drug dose proved to be very difficult due to substantial differences in the activity of DPD among individuals (7, 8). Severe life-threatening toxicities have been reported after treatment of cancer patients with inherited DPD deficiency with standard doses of 5FU (9–11). The catalobism of 5FU via DPD and the other two enzymes of the pyrimidine degradation pathway thus represents a major determinant of the pharmacokinetics of this anticancer drug. Its inhibition may result in increased 5FU efficacy, administration of lower doses, and diminished drug side effects. To date, several DPD inhibitors (eniluracil, 5-chloro-2,4-dihydroxypyridine, and 3-cyano-2,6-dihydropyridine) are utilized or under evaluation as modulators of 5FU treatment (12).

The high sequence identities (>90%) between human (13) and other mammalian DPD, such as bovine (14) and pig (15), suggest very similar reaction mechanisms and three-dimensional structures. Recently, the x-ray structure of the best-characterized mammalian enzyme, recombinant pig liver DPD, has been determined to a resolution of 1.9 Å (15). The enzyme is a homodimer of 2 × 111 kDa. Each subunit of 1025 amino acids carries one FAD, one FMN, and four [4Fe-4S] clusters (16). According to the non-classical two-site ping-pong kinetic mechanism, the enzyme contains separate binding sites for the electron-donating cosubstrate NADPH and the electron-accepting pyrimidines, respectively (17).

The pH dependence of the kinetic parameters suggests the following reaction mechanism for the reduction of uracil/thymine by pig liver DPD (17). DPD binds to site 1 with the pro-S side of the nicotinamide facing toward the FAD-isalloxazine ring. Hydride is transferred from the C-4 of NADPH to N-5 of FAD, leaving NADP⁺ and FADH². There is evidence from kinetic isotope effects that electron transfer to site 2 via the [4Fe-4S] clusters does not occur until NADP⁺ is released. At site 2 reduced FMN is formed. The pyrimidine substrate is bound with the si-face at the C-6 atom directed toward the N-5
of FMN and with the si-face of C-5 directed toward an enzyme general acid, which has been identified as the thiol group of cysteine 671 (16). The transfer of hydride from reduced FMN and the proton from Cys-671 is proposed to occur in a concerted anti-addition reaction.

The three-dimensional structure of pig liver DPD (15) revealed a highly modular organization of the subunit. It consists of five distinct domains, each carrying a subset of the various prosthetic groups. The α-helical domain I (residues 27–172) contains two of the iron-sulfur clusters, nFeS1 and nFeS2, of which the latter shows an unusual cluster coordination comprising one glutamine and three cysteine residues. The Rossman-type nucleotide binding folds of domains II (residues 173–286, 441) bind FAD and NADPH, respectively. The interface between these domains forms site 1 of DPD, whereas the pyrimidine binding site 2, containing FMN, is located on top of the αβ/γ-sarc domain IV (residues 525–847). The remaining iron-sulfur clusters cFeS1–cFeS2 are bound to the core of the C-terminal domain V (residues 1–26 and 848–1025). The three-dimensional arrangement of the distinct domains in the homodimeric enzyme leads to the formation of two electron-transfer chains, in which the electrons are transferred from the FAD to nFeS2 and subsequently to nFeS1. The remaining gap between nFeS1 and the FMN is closed by the clusters of the C-terminal domain V of the other subunit in the dimer. This domain swapping makes the dimer the minimal catalytic unit of pig liver DPD.

In addition to the x-ray structure of ligand-free DPD, that of a catalytically inactive mutant (C671A) in complex with NADPH and the anti-cancer drug 5FU was determined to 2.0-Å resolution (15). The binary complex of DPD was obtained by crystallization of wild-type DPD in the presence of 1 mM NADPH and 5 mM 5FU. A change of pH in the crystals was achieved by soaking crystals of the complex DPD/NADPH in 100 mM Hepes (pH 7.5), 22% polyethylene glycol 6000 (w/v), 0.8 mM NADPH, for 20 min before data collection. The complex DPD/UAADPD was prepared by soaking wild-type DPD crystals for 2.5 h in a solution containing 100 mM Hepes (pH 7.5), 22% polyethylene glycol 6000 (w/v), 1 mM UAA, and 2.5 mM NADPH. The crystals were stable under these conditions and did not change their appearance. All DPD complexes crystallized in space group P2₁, with four molecules (two homodimers) in the asymmetric unit.

In addition to the x-ray structure of ligand-free DPD, that of a catalytically inactive mutant (C671A) in complex with NADPH and the anti-cancer drug 5FU was determined to 2.0-Å resolution (15). The observation led to the conclusion that this ternary complex does not represent a productive enzyme-substrate complex. Instead, the nicotinamide ring of NADPH was not properly oriented to the FAD N-5 atom. Second, the loop comprising residues 525–847 in an open and partially disordered conformation. Hence, the assumption that pH might influence the loop conformation is based on the presence of a histidine residue at position 673, which most likely participates in substrate binding. This histidine residue should be positively charged at pH 4.7 but neutral at the pH optimal for catalytic activity.

To address this question, we prepared crystals of wild-type pig liver DPD with NADPH and two different inhibitors, 5-iodouracil (5IU) and uracil-4-acyclic acid (UAA), respectively. While UAA is a competitive inhibitor of DPD (apparent Kₐ = 78.6 ± 20.2 μM) (18), 5IU serves as a substrate and is converted to 5-iodo-5,6-dihydrooracil, which has been shown to be a potent alkylating agent able to covalently modify the Cys-671-thiol group (19). This may lead to trapping of the active-site loop in its closed conformation. Additionally and for purposes of comparison, the structure of the binary DPD-5IU complex was determined. In this report, we describe the three-dimensional structures of these inhibitor–enzyme complexes and discuss their implication for the mechanism of DPD.

**MATERIALS AND METHODS**

**Protein Purification and Crystallization**

Pig liver DPD was produced as recombinant protein in *Escherichia coli*, and has been purified and crystallized as described previously (20, 21). The binary complex of DPD was obtained by crystallization of wild-type DPD in the presence of 1 mM 5IU, the ternary DPD-5IU/NADPH complex, by soaking of DPD with 1 mM 5IU and 5 mM NADPH. A change of pH in the crystals was achieved by soaking crystals of the complex DPD-5IU/NADPH in 100 mM Hepes (pH 7.5), 22% polyethylene glycol 6000 (w/v), 0.8 mM NADPH, for 20 min before data collection. The complex DPD-UAADPD was prepared by soaking wild-type DPD crystals for 2.5 h in a solution containing 100 mM Hepes (pH 7.5), 22% polyethylene glycol 6000 (w/v), 1 mM UAA, 2.5 mM NADPH. The crystals were stable under these conditions and did not change their appearance. All DPD complexes crystallized in space group P2₁, with four molecules (two homodimers) in the asymmetric unit.

**Data Collection**

Before data collection, DPD-5IU crystals were transferred for 5 min into a cryo solution containing 100 mM sodium citrate (pH 4.7), 22% (w/v) polyethylene glycol 6000, and 20% (v/v) glycerol. For the DPD-5IU/NADPH crystal, the buffer component of this cryo solution was replaced by 100 mM Hepes (pH 7.5). The crystal of the DPD-UAADPD complex was soaked in a cryo solution consistent with that used for ligand soak/pH change, but 20% (v/v) glycerol were added. All crystals were flash-frozen in a nitrogen gas stream.

X-ray diffraction data were collected at 100 K at beam line ID-EH3 of the European Synchrotron Radiation Facility (European Synchrotron Radiation Facility, Grenoble, France) with a MAR CCD detector for the two ternary complexes and at the EMBL beam line BW7 at the DORIS storage ring, Deutsches Elektronen-synchrotron (Hamburg/Germany) with a MARResearch Imaging Plate for the binary complex. Data were indexed and integrated using DENZO (22) and scaled with the CCP4 suite of programs (23). Table I gives the details of the data collection statistics.

**Structure Determination and Refinement**

**DPD-5IU (pH 4.7)—Initial rigid body refinement using the refined structure of the ligand-free enzyme (without water molecules) as a model and data to 2.5-Å resolution resulted in values of 27.9% for Rcryst and 28.8% for Rasym, respectively.** The |Fᵣ| − |Fᵣ| map computed from this initial model showed clear electron density for 5IU bound close to the cofactor FMN. Models for the inhibitor and water molecules were added. The high resolution of the data allowed the identification of several alternative conformations of amino acid side chains (residues

---

**Ternary Complex of DPD with NADPH and 5-Iodouracil**

<table>
<thead>
<tr>
<th>TABLE I</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DPD-5IU</strong></td>
</tr>
<tr>
<td>Cell</td>
</tr>
<tr>
<td>a (Å)</td>
</tr>
<tr>
<td>b (Å)</td>
</tr>
<tr>
<td>c (Å)</td>
</tr>
<tr>
<td>β (°)</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
</tr>
<tr>
<td>Unique reflections</td>
</tr>
<tr>
<td>Multiplicity*</td>
</tr>
<tr>
<td>Completeness* (%)</td>
</tr>
<tr>
<td>Rint (%)</td>
</tr>
<tr>
<td>Iσo (%)</td>
</tr>
</tbody>
</table>

* Values for the last resolution shell are given in parentheses.
The ternary complex of DPD with NADPH and 5-iodouracil was studied. The crystallographic data have been deposited in the Protein Data Bank, with accession codes 1gte (DPD-5IU), 1gtg (DPD-5IU-NADPH), and 1gt8 (DPD-UAANADPH).

**RESULTS**

**Effects of pH Change on Crystal Packing**—The pH change from 4.7 to 7.5 did not have any obvious effects on the appearance of the DPD crystals. Nevertheless, it caused significant changes in unit cell dimensions and packing of the molecules within the crystal. Both ternary complexes obtained at pH 7.5 showed an elongation of unit cell dimension c by ~4 Å. This was accompanied by a change in packing of the two tightly associated homodimers within the asymmetric unit, leading to substantial alterations in dimer-dimer and crystal contacts. The majority of the contacts observed in unliganded DPD are absent in DPD-5IU-NADPH (pH 7.5) and DPD-UAANADPH (pH 7.5), and new but fewer contacts are formed.

**Overall Structure of the Complexes**—The binding of the pyrimidine analogs at pH 4.7 did not introduce major changes in the protein backbone structure, as reflected by a root mean square deviation of 0.24 Å, measured for all Ca atoms of the subunits of ligand-free DPD and the binary complex DPD-5IU. For the ternary complexes obtained at pH 7.5, the superposition with ligand-free DPD yielded root mean square deviations of 0.40 Å for all Ca atoms.

In complex DPD-5IU-NADPH (pH 7.5), but not in complex DPD-UAANADPH (pH 7.5), the active-site loop was observed in its closed conformation. Differences to ligand-free DPD occurring in both ternary complexes are located in the NADPH-binding site, where several amino acids adopt different conformations to allow proper NADPH binding, as described below. In addition, a slight movement of the NADPH binding domain with respect to the domain arrangement for unliganded DPD was observed. Superposition of the α5/βc-barrel domain IV of the corresponding structures reveals that the NADPH binding domain III changes its position by a modest movement (maximum difference in Ca-coordinates is 2.7 Å for residue 415) with respect to the FAD binding domain II, resulting in a slight widening of the NADPH binding cleft (Fig. 1). The displacement corresponds to a rotation by approximately 2° and a translation along the rotation axis by 0.7 Å.

**NADPH-binding Site**—Binding of NADPH in the cleft formed between domains II and III was accompanied by only local changes of amino acid conformations within domain II, whereas it involved both local and more global rigid body movements of residues originating from domain III (Fig. 2). A reorientation of the side chains of Phe-438 and Arg-364 resulted in stacking interactions of these residues with the NADPH adenine moiety. Residues 485–488, comprising a loop and the first residue of the following α-helix of domain II, moved in all NADPH-containing complexes with
respect to their position in ligand-free DPD. The observed displacement of Co atoms was maximal for Ala-486 (−1.7 Å). The position of the side chain of Asn-487 as observed in ligand-

domains III and III

domains III

domains III

domain II

domain II

free DPD would cause steric clashes with a bound NADPH molecule. By rotation around the N-Co and Co-C bonds of Ala-486, a shift of the Asn-487 side chain by 5.6 Å measured for
the carboxamide nitrogen is achieved, which then interacted with the 3-hydroxyl of the nicotinamide-ribose via a hydrogen bond. The new backbone conformation of stretch 485–488 is stabilized by two new hydrogen bonds formed between the main chain nitrogen atoms of Asn-487 and Thr-488 to the carboxyl-group of Glu-491. The most significant conformational change necessary for the proper positioning of the nicotinamide moiety of NADPH involves Asp-342. In all complexes obtained at pH 4.7, its side chain partially occupies the space for the nicotinamide of NADPH when bound in productive fashion. In ligand-free DPD a carboxylate oxygen is at a 3.6-Å distance from the FAD N5 at a position very close to the C4 atom of NADPH in complex DPD-5IU-NADPH (pH 7.5). After NADPH binding the Co of Asp-342 is displaced by 1.9 Å, the NADPH-nicotinamide moiety is stacked between the FAD isoalloxazine ring and the side chain of Asp-342, and one of the carboxyl oxygens of Asp-342 is hydrogen-bonded to the backbone amide of Val-373. Additionally, this movement of Asp-342 causes a displacement of residues 340–344, resulting in disruption of the hydrogen bonds between the backbone oxygen of Ala-340 and the backbone nitrogen atoms of Arg-371 and Ala-372, which are found in ligand-free DPD, the DPD-5IU (pH 4.7) complex, and also in the previously reported complex DPD-5FU-NADPH (pH 4.7) (15).

In the latter, the mode of NADPH binding was non-productive, because the nicotinamide ring was partially disordered and flipped away from the FAD isoalloxazine ring onto the protein surface. In that complex no domain rearrangement could be observed. Nevertheless, most of the more local alterations in the conformation of residues lining the walls of the NADPH binding pocket are seen in DPD-5FU-NADPH (pH 4.7).

Ligands in the Pyrimidine-binding Site—For all complexes of DPD, electron density shows the inhibitor molecules bound almost parallel to the FMN isoalloxazine ring plane, replacing several water molecules found in the active site of the unliganded enzyme (Fig. 3A).

In DPD-5IU (pH 4.7), the electron density is well defined for all atoms of 5-iodouracil (Fig. 3C). The binding geometry corresponds to that observed for 5-fluorouracil in the DPD-5FU-NADPH structure (15) (Fig. 3B), with the C6 atom of the inhibitor closest to the N5 of FMN (3.8 Å), the O2 atom in a 3.7-Å distance to both, the N10 and C9A of FMN, and the iodine located 3.7 Å from the FMN O4. As already noted for 5FU, there are no hydrogen bonds between amino acid side chains and the 5-halogen atom.

The ligand-associated electron density seen in DPD-UAA-NADPH (pH 7.5) is large enough to fit an uracil-4-acetic acid molecule, but due to the low resolution of the data, the binding geometry of the inhibitor cannot unambiguously be determined and will therefore not be discussed in further detail (Fig. 3D).

The electron density observed for inhibitor molecules bound in the active sites of the DPD-5IU-NADPH (pH 7.5) complex is heterogeneous and differs between each of the four molecules in the asymmetric unit (Fig. 4, A–D). Furthermore, there are ambiguities in assignment of the observed electron density to distinct inhibitor derivatives for these active sites. In the following, we will therefore describe only the features of the most prominent inhibitor derivative species (with the highest occupancy) for each active site.

In chain A, no density was observed for the iodine atom, but there was continuous density between the sulphydryl group of Cys-671 and the C5 atom of the pyrimidine ring. This electron density suggests that in subunit A the expected enzymatic reduction of 5-iodouracil occurred, and the reduction product, 5-iodo-5,6-dihydrouracil, subsequently attacked Cys-671, leading to its covalent modification to S-(hexahydro-2,4-dioxo-5-pyrimidinyl) cysteine.

The active sites of molecules B and C contain mainly uracil and 5,6-dihydrouracil, respectively, but also some 5-iodouracil (chain C) or 5-iodo-5,6-dihydrouracil (chain B). It is possible to distinguish between the substrate (5IU) and the product of the enzymatic reduction (5-iodo-5,6-dihydrouracil) by analyzing the position of the 5-substituent. In the fully oxidized substrate 5-iodouracil, the iodine is placed in-plane with the pyrimidine ring. For 5-iodo-5,6-dihydrouracil with its sp3-hybridized C5, it is positioned out of the ring plane, with the iodine surprisingly pointing toward Cys-671. Because the C5 atom receives its proton from Cys-671 (Scheme 1), one would expect to find the opposite enantiomer. However, the direct neighborhood of the C4 carbonyl group allows an inter-conversion between both enantiomers via a keto-enol mechanism, although it is not clear from the structure whether the reduction product can undergo this conversion within the active site or is first released and then rebound in the observed enantiomeric form.

There is almost no density for a 5-substituent of the inhibitor derivative bound in the vicinity of the FMN cofactor in molecule D (Fig. 4D). Because a distinction between uracil and 5,6-dihydrouracil is not possible at the current resolution of 2.25 Å for the DPD-5IU-NADPH data, the decision to interpret and refine the ligand as uracil rather then 5,6-dihydrouracil was arbitrarily made.

Conformational Changes within the Active-site Pocket—Amino acid side chains involved in substrate/inhibitor binding in all DPD complex structures are asparagines 609, 668, and 736 as well as Thr-737 (Scheme 1). These primary binding partners interact via hydrogen bonds with both pyrimidine ring nitrogens and carbonyl groups. The only changes occurring in the pyrimidine binding pocket apply to residues of the so-called active-site loop (residues 670–682) as well as to the two adjacent amino acids Leu-669 and Ala-683 (Fig. 5). In the ligand-free enzyme and in all complexes except DPD-5IU-NADPH (pH 7.5), this loop adopts an open conformation, leaving the pyrimidine-binding site solvent-accessible. The hydroxyl group of Ser-670 is then involved in a weak hydrogen bond (3.3 Å) to the O4 oxygen of the pyrimidine, whereas the general acid Cys-671 is located >10 Å distant from the ligand C5 atom. Residues 675–679 are fully solvent-exposed, and in three of the four protein chains in the asymmetric unit disordered, as indicated by a lack of electron density.

DPD-5IU-NADPH (pH 7.5) is the only complex in which the active-site loop has been observed in the closed state. Residues 674–678 here form a new short 310 helical turn, and residue 682 prolongs the 310 helix after the active-site loop. Superposition of the DPD-5IU-NADPH (pH 7.5) structure with DPD-5IU (pH 4.7) or ligand-free DPD (Fig. 5) shows that residue Ser-670 changes its position significantly by 3.6 Å, as measured for the Cα atoms. Its hydroxyl group is no longer involved in substrate/inhibitor binding but in two new strong hydrogen bonds to the backbone carbonyl of Lys-709 and to the carboxamide nitrogen of Asn-736. Hence, it now bridges two residues involved in either FMN binding (Lys-709) or substrate/inhibitor-binding (Asn-736).

As a direct consequence, Cys-671 is moved closer to the ligand, allowing the formation of the covalent bond (in chain A) or van der Waals interactions (chains B, C, D) between its thiol group and the C5 atom of the ligand. In the active sites of molecules C-D, the distance between Cys-671-5y and the ligand C5 is ~3.3 Å, suitable for proton transfer. His-673 is also involved in ligand binding, although by van der Waals rather than polar interactions. The His-673 Cα is, compared with its position in the open loop conformation, now located 12.6 Å...
closer to the active site. Of the remaining active-site loop residues none is directly involved in substrate/inhibitor binding. The electron density observed for residues 676–682 is not equally well defined in all 4 molecules in the asymmetric unit. In molecule A containing the covalent modification electron density is continuous for these residues but weaker than for the preceding amino acids 669–675. Considering also the observed higher temperature factors (average B-value is 69.6 Å² for residues 676–683 and 39.4 Å² for residues 669–675), it can be concluded that this stretch of amino acids still shows higher mobility than surrounding parts of the protein. In chains B-D the density is more diffuse, and the temperature factors are even higher (average B-value for 669–683 is 55.6 Å² in chain B, 61.1 Å² in chain C, and 58.8 Å² in chain D), indicative of that these residues might be partly disordered. The maintenance of a certain degree of active-site-loop mobility can be of advantage

FIG. 3. The pyrimidine binding site.
Stereo view of the pyrimidine binding sites of ligand-free DPD (A), DPD-5FU-NADPH (pH 4.7) (B), DPD-5IU (pH 4.7) (C), and DPD-UAA-NADPH (pH 7.5) (D). The cofactor FMN, the inhibitor molecules, ligand-replaced water molecules, and residues involved in ligand binding are given as ball-and-stick models with oxygens in light gray, nitrogens in black, and all other atoms in gray. The 2Fo − |Fc| map is contoured at a level of 1σ for A–C. In D, the |Fo| − |Fc| map for the ligand is contoured at a level of 3σ; the ligand itself is shown at a position best fitting the electron density.
taking into consideration that the loop has to switch between open and closed conformation within each catalytic cycle for substrate binding/product release. The movements should therefore require only small activation energy.

Nevertheless, the closed loop conformation is stabilized by a number of new interactions not observed in the open conformation. A ring nitrogen of His-673 is in hydrogen bond distance to the backbone oxygen of Glu-611. The side chains of Met-675 and Met-680 cluster together with the side chains of Met-642, Ile-613, Leu-612, and Val-583, forming a small hydrophobic core right beside the substrate binding pocket. These residues create a hydrophobic environment for the 5-methyl group of the thymine substrate but are located distant enough to accommodate also larger substituents at the pyrimidine C5. In molecule A, where electron density for the side chains of both Glu-677 and Arg-678 is observed, these residues are involved in altogether four new hydrogen bonds. The glutamate carboxyl group interacts with the main chain nitrogens of residues Phe-935 and Gly-936 of molecule B, thereby participating in the formation of the dimer interface. One of the guanidinyl nitrogens of Arg-678 interacts with the backbone oxygen atoms of Asp-581 and Ile-582.

**DISCUSSION**

*Active-site Loop Closure Is Substrate Binding and pH-dependent—* With the new DPD-complex structures available, several open questions regarding the reaction mechanism can finally be addressed. Until now, one obstacle of the interpretation has been that the flexible loop segment, which controls substrate entry and product release from the active site, was only observed in the open conformation. Loop closure is an absolute prerequisite for catalytic activity because it not only excludes the surrounding solvent from the active site, but most importantly, also places Cys-671 at the location required for proton transfer to the pyrimidine C5.

Our results suggest that substrate/inhibitor binding in the active-site pocket subsequently triggers closure of the active-site loop. The energy gained by exchanging the pyrimidine O4 as a partner of Ser-670 in a rather weak hydrogen bond against the backbone carbonyl of Lys-709 and the side chain amide of
Asn-736, resulting in two new much stronger hydrogen bond interactions, may account for part of the energy necessary for the loop conformational change.

The failure to trigger the active-site-loop closure in complex DPD-5FU-NADPH (15) can clearly be attributed to the non-physiological pH of the crystallization solution (an influence of the C671A exchange in this complex can be ruled out due to the observation of the open loop conformation in DPD-5IU (pH 4.7)). At pH 4.7, the loop residue His-673 carries a positive charge, which either hinders the loop to reach the closed conformation or which the substrate-binding site cannot accommodate in the closed state. His-673 may therefore account for the group with a pK~6.5, identified by studies on pH dependence of kinetic parameters, which is required to be in an unprotonated state for optimum activity but that is not essential for catalytic activity. In DPD-UAA-NADPH (pH 7.5), the closure of the active site is prevented due to steric clashes of loop residues with atoms of the rather voluminous inhibitor molecule.

Comparison of DPD domain IV with the structurally and functionally closely related *Lactococcus lactis* dihydroorotate dehydrogenase class 1A in complex with the reaction product orotate (32) allowed predictions of the positions of the catalytically crucial Cys-671 (the counterpart in dihydroorotate dehydrogenase class 1A is Cys-130) and the succeeding residues Pro-672 and His-673 (Pro-131 and Asn-132) in the closed state. Superposition of dihydroorotate dehydrogenase class 1A with domain IV of complex DPD-5IU-NADPH (pH 7.5) indeed reveals that residues 669–673 of DPD share comparable positions and side chain conformations with residues 128–132 of dihydroorotate dehydrogenase class 1A. However, none of the residues Gly-674 to Cys-684 (Val-133–Leu-139) are structurally equivalent. A major difference between the ligand-bound and ligand-free structures of both enzymes is that alterations in loop conformation are far more pronounced in DPD.

**Reaction and Electron Transfer Mechanism**—The structure of DPD-5IU (pH 4.7) clearly shows that pyrimidine binding to domain IV is not dependent on the presence of NADPH in its binding cleft, which is in agreement with spectroscopic and kinetic data (16). Although the structure of a NADPH-DPD complex has not yet been determined, comparison of the available NADPH-free and NADPH-bound complex structures is consistent with the assumption that NADPH binding is also independent of the occupancy of the substrate binding pocket. Furthermore, proper NADPH binding (i.e., correct placement of the nicotinamide moiety close to the FAD isoalloxazine ring) does not seem to be coupled to loop-closure events in the pyrimidine-binding site. In both complexes, DPD-5IU-NADPH (pH 7.5) with
the active-site loop closed and DPD-UAA-NADPH (pH 7.5) with the loop open, NADPH is bound in an identical, “productive” manner.

Analysis of the structures of the DPD complexes reveals that no information transfer between NADPH and the substrate-binding site occurs other than via the electron transfer chain. NADPH binding domain III and FMN binding domain IV are located at opposite ends of the subunit and entirely separated from each other by domains I, II, and V (Fig. 1). The movement of domain III induced by NADPH binding does not cause significant conformational changes in the other four domains. The accelerating effect of uracil on the reduction of DPD by NADPH, which was revealed by monitoring absorbance changes during the reaction of DPD with NADPH in the absence and presence of uracil (16), is thus not coupled to conformational changes within the protein but is most likely exclusively based on the “sink function” of the pyrimidine substrate as final electron acceptor.

The catalytically competent binding of NADPH requires a slight widening of the cleft between domains II and III, which is achieved by a modest movement of domain III. A similar but more pronounced domain rearrangement as well as alterations in crystal packing contacts have been reported for adrenodoxin reductase upon binding of NADPH (33). Because no pH change is involved for adrenodoxin reductase, it can be argued that binding of NADPH in the conformation suitable for hydride transfer rather than the pH shift induces the domain movement observed for DPD.

However, there is an indirect pH effect. At a pH of 7.5, NADPH is bound to DPD in the proper conformation, whereas at pH 4.7 it is bound in a non-productive fashion. We examined the immediate surroundings of NADPH for amino acid residues, which could trigger the domain movement by adopting different conformations at the two pH values. The most obvious candidate for such a trigger function is Asp-342. Inevitably this residue has to move to make room for the nicotinamide moiety of NADPH. In ligand-free DPD and all complexes obtained at pH 4.7, the side chain of Asp-342 is not involved in hydrogen-bonding interactions, suggesting that Asp-342 is protonated under these conditions. At pH 7.5, the carboxyl group of Asp-342 needs to form a hydrogen bond interaction with the main chain amide of Val-373, the only candidate within an appropriate distance, to compensate for its negative charge. The formation of this hydrogen bond may require or induce the observed additional conformational changes in stretch 371–373, leading to the disruption of backbone interactions between this stretch and Ala-340 and subsequently to the movement of domain III.

Once NADPH is bound, hydride is transferred from C4 of the nicotinamide of NADPH to FAD N5. It appears that there is no compensation for the generated negative charge of reduced FAD (FADH−) localized at the N1 atom. Unlike other flavoenzymes (34), DPD provides no positively charged amino acid side chain or partially charged entity such as the N terminus of an α-helix or a cluster of backbone nitrogens in a reasonable distance to the FAD N1. The closest atoms are the side-chain hydroxyl group and the backbone amide of Thr-489, both located at a distance >3.4 Å to the N1 locus of FAD. Due to the missing charge compensation, the anionic form of the reduced flavin is not stabilized, and the redox potential of the cofactor is kept low by the protein environment. This might represent one of the driving forces for electron transfer to the nearest [4Fe-4S] cluster nFeS2.

The O2 hydroxyl of FAD is in hydrogen bond distance (2.6 Å) to a water molecule, which in turn is positioned at a distance of 3.0 Å to Sγ of Cys-130, a ligand of cluster nFeS2 (Fig. 6). This water molecule is present in all four polypeptide chains in the asymmetric unit and shows strong and well defined electron density. An additional hydrogen bond is formed with the backbone amide of Val-490 (2.8 Å). We propose that this is the most likely route for the transfer of two electrons per catalytic cycle between FAD and nFeS2. Concomitant with the electron transfer, the FAD-N5 proton must be released. An apparent route for proton release proceeds via Arg-235, which is located within hydrogen-bond distance to the N5 and O4 atoms of FAD. The proton can move to Arg-235 and from there either directly or via the carboxyl group of Glu-346 to the contacting water mol-
SCHEME 2. Proposed mechanisms of DPD inhibition by 5IU and inhibitor deactivation. a, 5IU is reduced to 5-iodo-5,6-dihydrouracil in a NADPH-dependent reaction. b, active-site loop opening is followed by fast release of the enzymatically generated enantiomer of 5-iodo-5,6-dihydrouracil, which can be transformed into the other enantiomeric form via keto-enol tautomerism and rebound to the enzyme. c, 5-iodo-5,6-dihydrouracil attacks the thiol group of Cys-671, leading to its covalent modification to S-(hexahydro-2,4-dioxo-5-pyrimidinyl)cysteine. d, 5-iodo-5,6-dihydrouracil can also be deactivated via elimination of HI, resulting in formation of uracil, which is a substrate of DPD. Its reduction generates 5,6-dihydrouracil. The boxed capital letters indicate the dominant species observed in the four subunits (A-D) of DPD in the asymmetric unit.
ecules and eventually be released to the bulk solvent. The electron transfer pathway between nFeS2 and cFeS2 (via nFeS1 and cFeS1) is shielded from solvent molecules through a number of hydrophobic residues, mostly isoleucines and leucines (Fig. 6).

Based on the positioning of cluster cFeS2 with respect to the FMN cofactor in the pyrimidine-binding site, we suggest the C7α methyl group of FMN as the site of electron entry into the flavin ring system. There is no obvious route for electron transfer between these two redox cofactors. The distances between Sγ δ of Cys-989, a cFeS2 ligand, to the closest amino acid residue (Ile-590 C6) and from there to the FMN-C7α are 4.4 and 3.6 Å, respectively. However, the simple positioning of the redox centers in close proximity (<14 Å) to each other might be effective enough to achieve electron tunneling (35).

The negative charge developing at FMN N1 upon full reduction of the cofactor to the hydrochinone form is compensated by a few surface-located loop regions, which are generally all solvent-exposed. This non-equivalency has, however, been observed in all DPD structures in so far as that part of the “proline-rich loop” (residues 899–910) is usually less disordered in subunits C and electron density for the active-site loop (in its open conformation) is usually best defined in subunit D. The features of the complex DPD+5IU-NADPH (pH 7.5) support the proposal that 5-iodouracil acts as a mechanism-based inhibitor covalently modifying the active-site residue Cys-671 and agree well with previously presented biochemical observations concerning catalytic and inhibitory mechanisms.

In conclusion, the interconversion between both enantiomers proceeds most likely via a keto-enol mechanism (Scheme 2b).

The further fate of 5-iodo-5,6-dihydropyrimidines in the pH range from 6.0 to 8.0, which gives raise to the conclusion that no enzymatic reduction or covalent modification of Cys-671 occurred before the transfer of the DPD+5IU-NADPH crystals to pH 7.5. Because substrates and products of the enzymatic reduction compete for the binding in the active site of DPD (19), it was unlikely to obtain complete inactivation of the enzyme in the crystals. We could not identify the source of the non-equivalency of the four molecules in the asymmetric unit of DPD crystals, which resulted in the accumulation of distinct inhibitor derivatives within the different active sites (A–D). Superposition of the subunits does not reveal other deviations in amino acid coordinates except in a few surface-located loop regions, which are generally all solvent-exposed. This non-equivalency has, however, been observed in all DPD structures in so far as that part of the “proline-rich loop” (residues 899–910) is usually less disordered in subunits C and electron density for the active-site loop (in its open conformation) is usually best defined in subunit D. The features of the complex DPD+5IU-NADPH (pH 7.5) support the proposal that 5-iodouracil acts as a mechanism-based inhibitor covalently modifying the active-site residue Cys-671 and agree well with previously presented biochemical observations concerning catalytic and inhibitory mechanisms.

The complex structures presented here provide for the first time a complete picture of the electron transfer chain in the productive enzyme-substrate complex. It was also shown that pyrimidine binding triggers a conformational change of a flexible active-site loop, resulting in placement of the catalytically crucial cysteine 671 close to the bound substrate. This loop closure as well as the correct binding of the coumarate NADPH requires physiological pH.

Acknowledgments—We thank A. Mozzarelli for advice regarding the soaking experiments. We thankfully acknowledge access to the synchrotron radiation at the European Synchrotron Radiation Facility and the Deutsches Elektronensynchrotron.

REFERENCES

Ternary Complex of DPD with NADPH and 5-Iodomucuric Acid

Crystal Structure of the Productive Ternary Complex of Dihydropyrimidine Dehydrogenase with NADPH and 5-Iodouracil: IMPLICATIONS FOR MECHANISM OF INHIBITION AND ELECTRON TRANSFER
Doreen Dobritzsch, Stefano Ricagno, Gunter Schneider, Klaus D. Schnackerz and Ylva Lindqvist


Access the most updated version of this article at doi: 10.1074/jbc.M111877200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 35 references, 7 of which can be accessed free at http://www.jbc.org/content/277/15/13155.full.html#ref-list-1