**Lactococcus lactis** Dihydroorotate Dehydrogenase A Mutants Reveal Important Facets of the Enzymatic Function*

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Dihydroorotate dehydrogenases (DHODs) catalyze the oxidation of (S)-dihydroorotate to orotate in the biosynthesis of UMP, the precursor of all other pyrimidine nucleotides. On the basis of sequence, DHODs can be divided into two classes, class 1, further divided in subclasses 1A and 1B, and class 2. This division corresponds to differences in cellular location and the nature of the electron acceptor. Herein we report a study of *Lactococcus lactis* DHODA, a representative of the class 1A enzymes. Based on the DHODA structure we selected seven residues that are highly conserved between both main classes of DHOD as well as three residues representing surface charges close to the active site for site-directed mutagenesis. The availability of both kinetic and structural data on the mutant enzymes allowed us to define the roles individual structural segments play in catalysis. We have also structurally proven the presence of an open active site loop in DHODA and obtained information about the interactions that control movements of loops around the active site. Furthermore, in one mutant structure we observed differences between the two monomers of the dimer, confirming an apparent asymmetry between the two substrate binding sites that was indicated by the kinetic results.

Dihydroorotate dehydrogenases (DHODs) catalyze the stereospecific oxidation of (S)-dihydroorotate to orotate through reduction of their prosthetic FMN group. This is the only redox reaction in the de novo biosynthesis of pyrimidine nucleotides. In rapidly proliferating cells pyrimidine salvage pathways cannot compensate for the lack of UMP caused by inhibition of DHOD. This makes DHODs attractive targets for antiproliferative, antiparasitic, and immunosuppressive drugs used in organ transplantation and in treatment of inflammatory diseases (1–6).

Based on presently known sequences, DHODs can be divided into two main classes (7). Class 1 enzymes, which are subdivided into class 1A and 1B enzymes, are cytosolic proteins, whereas class 2 enzymes are membrane-associated. The bacterium *Lactococcus lactis* contains genes that encode DHODs representing subclass 1A and 1B, DHODA, and DHODB. Crystal structures have been determined for both of these without and in the presence of the product orotate (8–10). DHODA is a dimer formed by two identical PyrD subunits each containing an FMN group (11). The natural electron acceptor for DHODA is fumarate (12), but all DHODs can to some extent use a variety of other electron acceptors such as soluble quinones, dyes, and molecular oxygen (13, 14). For DHODA it has been suggested that the substrate and the natural electron acceptor use the same binding site. Kinetic investigations supported a one-site ping-pong mechanism and showed the second half-reaction to be the rate-limiting step for DHODA (13).

The class 1B enzyme is a heterotetramer consisting of two PyrDB subunits, homologous to the PyrDA subunits of DHODA, and two PyrK subunits (15). The PyrK subunits each contain FAD and an [2Fe-2S] cluster, and the presence of these subunits enables the tetrameric enzyme to use NAD+ as electron acceptor (10, 15). The class 2 DHODs are monomeric enzymes; there are structures known for the human and *Escherichia coli* DHOD (DHODC) (16, 17). Relative to the class 1 enzymes, they possess an extended N terminus, which plays a role in the membrane association of the enzyme and provides the binding site for the respiratory quinones that serve as physiological electron acceptors (16, 17). The earliest studies on DHOD from bovine liver (18) and *Crithidia fasciculata* (19) together with more recent studies on DHODB from *Enterococcus faecalis* (20) and on DHODC from *E. coli* (21) describe the stereospecific oxidation of (S)-dihydroorotate to orotate as mediated by an active site base. The active site base structures the CS-S proton of dihydroorotate (DHO) followed by hydride transfer from the C6 position of DHO to the N5 of FMN. Based on structural comparisons of class 1 and class 2 enzymes we have suggested that the mechanism for the first half-reaction is highly conserved, and two highly conserved lysines are essential for the catalytic function (17). In the class 1 enzymes the active site base is a cysteine residue (7) while in class 2 enzymes it is a serine (14).

In terms of structure DHODA represents the simplest type of DHOD, with a common binding site for the two substrates. At the same time DHODA exhibits properties that are common for DHODs in general, which makes the protein an excellent model.
system for detailed investigation of the function and structure of DHODs. The structure of the dimeric DHODA with orotate bound in the active site is shown in Fig. 1. Each subunit folds into a (β/α)8-barrel with the prosthetic FMN group situated at the C-terminal end of the β-strands at the top of the barrel and close to the dimer interface. The reaction product, orotate, stacks on the isoalloxazine ring of FMN in a favorable position for hydride transfer from the substrate to N5 the isoalloxazine ring. Cys-130, the active site base, is contained in a flexible and highly conserved loop (residues 129–138), which we refer to as the active site loop. This loop covers the active site, and it is obvious that it must undergo movements to let substrate in and product out of the active site (8–10). Cys-130 in the active site loop is only conserved in the class 1 DHODs, but the loop contains three residues that are totally conserved in both classes, Ser-129, Pro-131, and Asn-132. The role of the latter residue was assigned earlier (9), but from our structural investigations studies we were unable to assign any function to the two former residues. Another very highly conserved segment of amino acids, residues 50–57 in DHODA, found in the DHOD sequences, forms a fixed loop delineating the active site in the DHOD structures (8–10, 16, 17), which we refer to as the cis-proline loop. Two of the residues in this loop, Arg-57 and Pro-56 (forming a cis-peptide bond to the non-conserved Leu-55) are conserved in all DHODs. Their putative functions in catalysis have not been investigated. In addition to the two loop regions, the alignment of DHOD sequences and structures revealed a number of other residues conserved between the DHODs. Two of these (Asn-132 and Lys-43 in DHODA) are important for catalysis (7). Their side chains are hydrogen-bonded to the product orotate, and in addition Lys-43 interacts with O4 of FMN (9). Furthermore, in all the known structures...
the pyrimidine ring of orotate is held tightly by hydrogen bonds to three totally conserved asparagine residues (Asn-67, Asn-127, and Asn-193). Three basic residues, Arg-50, Lys-136, and Lys-213 are only conserved in class 1A. Their presence close to the active site (R50E, K136E, K213E) and proper orientation of the substrate. Arg-50 is located in the cis-proline loop, Lys-136 in the active site loop, and Lys-213 at the C-terminal end of a 310 helix, which we will refer to as the Lys-213 helix. The positions of these residues in the DHODA dimer are illustrated in Fig. 1.

We report here the kinetic characterization of ten mutant enzymes. K136E of these represent changes to alanine of residues totally conserved among all DHODs (N67A, N127A, N193A, P56A, R577, S129A, P131A) and three were constructed to change the surface charge at or near the active site (R50E, K136E, K213E). In addition structural investigations at low temperature were made for five of them in the presence or absence of the product orotate (N67A(Oro), P56A(Oro), R57A(Oro), K136E(Oro), K213E(Oro)).

A low temperature structure determination was also performed for the uncomplexed DHODA, as our initial structure determination for the enzyme was a room temperature study and contained Cys-130 in an oxidized form (8). These results from the kinetic and structural investigations have revealed several new characteristics of the enzymatic function of DHODA that have implications for the function of dihydroorotate dehydrogenases in general.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis and Purification of Mutant Enzymes**—The wild-type DHODA was prepared according to the procedure of Nielsen et al. (11). Mutations in the pyrDa gene were introduced by the polymerase chain reaction (PCR) as described by Björnberg et al. (7) using the plasmid pFN1 and mutant primers. The PCR products were digested with BamHI and HindIII and cloned into BamHI- and HindIII-digested pUE23–2 to generate plasmids similar to pFN1, but with a mutation in the pyrD gene. To ensure the presence of only the desired mutation the entire pyrDa gene of each clone was sequenced. The modified enzymes were purified as described for the wild-type (11) enzyme, although the different enzymes needed different volumes of isocratic washing with the 1M NaCl buffer to be eluted from the hydroxyl apatite column.

Enzyme concentrations were determined from the flavin content (7) after denaturation of the protein in 1% sodium dodecyl sulfate (SDS) for a few minutes at room temperature. The absorbance measured to calculate the enzyme concentration was based on the extinction coefficient of 12.5 nmol−1 cm−1 for free FMN at 445 nm.

**Steady State Kinetics**—Steady state kinetic measurements of the enzyme activity with different electron acceptors were carried out in 0.1 M Tris-HCl, pH 8.0 with 1 mM DHO as substrate under normal aerobic conditions (Table I). A Zeiss Specord S10 diode array instrument thermostatted at 25 °C was used to record the spectrum as a function of time. When DCIP served as electron acceptor the absorbance at 600 nm with an extinction coefficient ε = 20 nmol−1 cm−1 was used to estimate the orotate concentration. With oxygen as electron acceptor the concentration of orotate was estimated from the absorbance at 278 nm using an extinction coefficient ε = 7.7 nmol−1 cm−1, and with fumarate as electron acceptor the orotate absorbance at 300 nm (ε = 3.05 nmol−1 cm−1) was used to estimate its concentration.

**Redox Potentials**—Redox potentials (Table III) were determined by using the xanthine-xanthine oxidase method described by Massey (22) with phenosafranin as reference dye, E0(pH 7.0) = −252 mV. Enzyme concentrations were near 1 mg/ml in 0.05 mM sodium phosphate, pH 7.0.

**Crystallization**—Prior to crystallization the proteins were dialyzed against a 25 mM NaH2PO4 buffer, pH 6.0, containing 10% glycerol. The P56A, R57A, N67A, K136E, and K213E mutant enzymes were crystal-
The binding of orotate was determined by the red shift assay as described in "Experimental Procedures." The apparent $K_D$ values ($K_{app}$) for DHO were determined from the reaction velocities at five concentrations of DHO (0.05-1 mM) at fixed concentration of orotate (5 mM), while the apparent $K_D$ values ($K_{app}$) for furamate were estimated from measurements of the reaction velocities at five concentrations of furamate (0.25-5 mM) at fixed concentration of DHO (1 mM). The apparent $V_{max}$ ($V_{app}$) was estimated by varying the concentration of a 1:2 mixture of DHO and furamate from 0.65 to 1.1 mM DHO (0.1-2 mM furamate) and extrapolating to infinite substrate concentration. Structures have been determined for the enzymes shown in bold.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_D$ for orotate</th>
<th>$K_{app}$ for DHO</th>
<th>$K_{app}$ for furamate</th>
<th>$V_{app}$</th>
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<tbody>
<tr>
<td>Native</td>
<td>29 ± 2</td>
<td>18 ± 4</td>
<td>250 ± 50</td>
<td>0.18 ± 0.1</td>
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<tr>
<td>N67A</td>
<td>44 ± 2</td>
<td>1,758 ± 197</td>
<td>50 ± 10</td>
<td>0.38 ± 0.04</td>
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<tr>
<td>N127A</td>
<td>394 ± 0.214</td>
<td>1,240 ± 10</td>
<td>Inhibited*</td>
<td>3.3 ± 0.3</td>
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<tr>
<td>N193A</td>
<td>11,600 ± 1,150</td>
<td>9,300 ± 5600</td>
<td>Inhibited*</td>
<td>1.7 ± 1.0</td>
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<tr>
<td>P56A</td>
<td>1,450 ± 70</td>
<td>56 ± 15</td>
<td>70 ± 20</td>
<td>0.39 ± 0.02</td>
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<td>R57A</td>
<td>95 ± 3</td>
<td>51 ± 4</td>
<td>1,200 ± 200</td>
<td>32.8 ± 0.6</td>
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<tr>
<td>S129A</td>
<td>91 ± 6</td>
<td>34 ± 4</td>
<td>30 ± 20</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>P131A</td>
<td>40 ± 4</td>
<td>56 ± 9</td>
<td>60 ± 20</td>
<td>3.7 ± 1.4</td>
</tr>
<tr>
<td>R50E</td>
<td>92 ± 4</td>
<td>47 ± 5</td>
<td>540 ± 60</td>
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<tr>
<td>K136E</td>
<td>7.6 ± 1.2</td>
<td>14 ± 3</td>
<td>90 ± 40</td>
<td>13.7 ± 0.4</td>
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<tr>
<td>K213E</td>
<td>117 ± 3</td>
<td>127 ± 12</td>
<td>40 ± 20</td>
<td>6.3 ± 0.2</td>
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</table>

* Raising the furamate concentration from 0.25 to 5 mM systematically reduced the activity of these enzymes. The activity of the N193A enzyme was halved, while the activity of the N127A enzyme was reduced by 20 percent.

The rate constants ($k_{bm}$) were determined by 3-6 shots at 6-9 concentrations of DHO ranging from 0.025 to 2 mM (in a few cases 8 mM) and the saturation of DHO was determined by use of the equation ($k_{bs} = k_{b} / (1 + K_{D}(DHO))$). In bold the data for the enzymes of which the structure was solved.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Bleaching rate $k_{b}$</th>
<th>Saturation constant $K_D$</th>
<th>Redox potential (mV)</th>
</tr>
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<tbody>
<tr>
<td>Native</td>
<td>187 ± 2</td>
<td>169 ± 6</td>
<td>-245</td>
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<tr>
<td>R50E</td>
<td>43.0 ± 0.5</td>
<td>226 ± 8</td>
<td>-245</td>
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<td>K136E</td>
<td>86 ± 2</td>
<td>107 ± 6</td>
<td>-245</td>
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<td>R213E</td>
<td>3.47 ± 0.02</td>
<td>295 ± 12</td>
<td>-251</td>
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<td>S129A</td>
<td>30.7 ± 1.2</td>
<td>369 ± 47</td>
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<tr>
<td>P131A</td>
<td>6.00 ± 0.06</td>
<td>86 ± 3</td>
<td>-263</td>
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Table III

**Stopped flow determinations of the saturation of the bleaching of wild type and mutant DHODA under anaerobic conditions**

The majority of the mutant enzymes, albeit at different concentrations of orotate due to their different affinities for the product. A noticeable exception was the N67A mutant enzyme for which orotate caused a normal red shift of the 456-nm absorbance peak, while the ligand did not depress and red shift the absorbance peak at 372 nm. Instead this developed a characteristic shape (Fig. 2B) indicating unusual surroundings of the flavin group in the N67A protein enzyme after orotate binding or an unusual binding mode for orotate. Titration of the enzymes with orotate could be used to determine the $K_D$ values for the enzyme-orotate complexes. Examples of the saturation curves are shown in Fig. 3 and the resulting $K_D$ values for all the enzymes are given in Table II. The binding data did not fully fit the shape of a hyperbolic saturation curve (Equation 1 and see Fig. 3A). Instead, the red shift continued to develop at high concentrations of orotate when it was expected to be complete from the estimated $K_D$ values, indicating either a negative cooperativity in the binding of orotate or a different affinity for orotate at the two sites of the dimeric DHODA. Only a few of the enzymes bound orotate strongly enough to attempt a fit of the data to a two-site saturation model (Equation 2) without constraints. As shown in Fig. 3A for the K136E enzyme, this analysis indicated that 70% of the sites bound orotate with high affinity ($K_D = 3 \mu M$) and 30% had low affinity for orotate, $K_D = 86 \mu M$. However, as the $K_D$ values of the high affinity site generally correspond well with the $K_D$ values determined by fitting the data to the one site saturation model (Equation 1) these $K_D$ values are given in Table II.

**RESULTS**

**Kinetic Data and Redox Potentials**

Interaction with the Product; Orotate Binding—The binding of orotate to wild-type and mutant enzymes was assessed by measuring the red shift of the flavin absorption spectrum produced by addition of orotate. Examples of the spectrum and spectral changes are shown in Fig. 2. For the wild-type enzyme the addition of orotate caused a 15-nm red shift of the long wavelength absorbance peak at 456 nm and an 8-nm red shift, a suppression, and a characteristic sharpening of the absorbance peak at 372 nm. Similar spectral changes were seen for the majority of the mutant enzymes, albeit at different concentrations of orotate due to their different affinities for the product.

**Data Collection and Processing**—All data sets were collected under cryogenic conditions (120 K). Mother liquor containing 10% glycerol was used as cryoprotectant. The data sets were integrated and scaled using DENZO and SCALEPACK (23). Structure factors were derived from the reflection intensities using TRUNCATE (24). The main statistics for data collection and integration are given in Table IV. The crystals of the mutated proteins belong to the same space group (P2_1) as the native enzyme. The unit cell parameters are similar and slightly smaller than those obtained earlier for the structures at room temperature (8, 9). The average of the cell parameters were $a = 53.04 ± 0.22 \AA$, $b = 108.36 ± 0.24 \AA$, $c = 66.01 ± 0.26 \AA$, and $\beta = 103.8 ± 0.11^\circ$.

**Refinement**—AMoRe (25) was used to place the existing model of the native enzyme (8) in the unit cell. To generate the P56A(Oro) and N67A(Oro) structures the refinement was then carried out using REFMAC (26) and ARP (27) applying tight restraints between the two NCS-related molecules of the dimer. For all the other structures the refinement was carried out with CNS (28) using the automatic procedure for water insertion and restrained CNS. In two structures, K136E and native, CNS was released for parts of the structure, while in R57A(Oro) the CNS restraints were totally released in the final refinement steps. O (29) was used to introduce the mutations and for manual rebuilding. The final refinement statistics are given in Table IV. In all structures more than 90% of the residues are found in the allowed regions of the Ramachandran plot (30), with no residues in the disallowed regions. The illustrations of the molecular structures were prepared using RASMOL (31), MOLSCRIPT (32), and RASTER 3D (33).
TABLE IV
Data collection and refinement statistics

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<tr>
<th></th>
<th>Native + DTT</th>
<th>N67A + orotate</th>
<th>K213E + orotate</th>
<th>P56A + orotate</th>
<th>R57A + orotate</th>
<th>K136E + orotate + DTT</th>
<th>K136E + DTT</th>
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<td>1.80 (1.84-1.80)</td>
<td>1.84 (1.87-1.84)</td>
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<td>1.93 (1.97-1.93)</td>
<td>1.70 (1.73-1.70)</td>
<td>2.25 (2.29-2.25)</td>
<td>1.41 (1.43-1.41)</td>
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<td>I/</td>
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<td>(%)</td>
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<td>22.6</td>
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FIG. 2. Absorption spectra and orotate-induced red-shifted spectra normalized to equal enzyme concentration of the native and three DHOD mutants. A: spectrum without orotate; B: spectrum after addition of 0.5 mM orotate corrected for dilution; C: difference spectrum between A and B. Symbols: solid line, native enzyme; thick line, N67A; thin line, N127A; broken line, N193A.
ing these data, it should be kept in mind that the first half-reaction for the wild-type enzyme, i.e. the reduction of the flavin by DHO, is much faster (>50-fold) than the second half-reaction, which is the reoxidation of the flavin by the electron acceptor (7). This means that changes of the amino acid sequence that affect the first half-reaction may not necessarily impair the steady state activity of a mutant enzyme. A few features of the steady state activities are worth noticing. First and as expected, major effects on the activities were produced by alanine mutations of the asparagines (Asn-67, Asn-127, and Asn-193), which form hydrogen bonds to substrate and product in the active site (9). Second, and more surprising, it turned out that a change of the conserved Pro-56 in the cis-proline loop to alanine strongly reduced the steady state activity with all three electron acceptors, while a change of the conserved Arg-57 to alanine increased the steady state activity of DHO with all three electron acceptors, indicating an important and complex role of the cis-proline loop for optimal function of the active site. Third, it appeared that the changes of the two fully conserved residues (Ser-129 and Pro-131) that flank the active site basic residue (Cys-130) to alanines only moderately impaired the steady state activity of DHODA; while the P131A mutation makes the steady state activity independent of electron acceptor choice and may thus have a strong effect on the rate of the first half-reaction the S129A substitution primarily reduces the activity with fumarate as electron acceptor. The fourth feature of interest in the steady state data in Table I is that of mutations R50E, R136E, and K213E that influence the surface charge of the enzyme, only the K213E mutation interferes significantly with the steady state activity.

Table II shows the $K_D$ values for binding of orotate to wild type and DHODA enzymes and the apparent $K_m$ and $V_{max}$ values for the saturation of the enzymes with DHO and fumarate. Generally, there is a good correlation between the dissociation constants ($K_D$) for the orotate enzyme complex and the $K_m$ for DHO as the mutant enzymes that bind orotate poorly generally are difficult to saturate with DHO. However, the mutant enzyme N67A, which displayed an unusual absorption spectrum in the presence of orotate appears to bind orotate almost as strongly as wild-type DHODA, while it has a very high $K_m$ for saturation with DHO. It is also remarkable that a change of the residue Lys-136, which we originally expected to be involved in attracting DHO to the active site, produces an enzyme that binds orotate more strongly than the wild-type enzyme does.

The R57A mutant enzyme is unusual since it has increased activity with all electron acceptors, (especially DCIP) and the $K_m$ values for saturation with DHO and fumarate are increased 3–5-fold. It is also remarkable that two mutant enzymes, N193A and N127A, which have lost hydrogen bonds to the substrate (DHO), although stimulated by low concentrations of fumarate (0.25 mM), are inhibited by higher concentrations of fumarate (Tables I and II). This may indicate a competition between the substrate (DHO) and the electron acceptor (fumarate) for binding in the active site of DHODA in line with the observations of Björnberg et al. (13).
In general there is a large variation of the steady state parameters for the different electron acceptors among the mutant enzymes. Fumarate is the natural substrate for DHODA, and it has been supported by kinetic studies that it binds at the site as orotate. DCIP is a much larger molecule and the electron transfer to this molecule cannot be envisioned to take place by direct interaction with the flavin group but rather through more complex electron transfer pathways. Conversely oxygen is a very small molecule and a much poorer substrate that can bind at numerous places at the protein, which opens the possibility for a variety of different electron transfer pathways.

The kinetic data referring to the first half-reaction of some of the DHODA enzymes (including the wild type) are shown in Table III together with the redox potentials of the enzyme-bound flavin. The $k_p$ values for saturation of the enzymes with DHO in the first half-reaction varied only by a factor of 3 between the different enzymes, but several of the mutations created significant variations in the maximal bleaching rate ($k_b$) at saturating concentrations of DHO. Most hampered was the K213E mutant enzyme for which $k_b$ was reduced about 60-fold, resulting from an unexpected influence of Lys-213 on positioning of the active site flexible loop (see below), and the P131A enzyme for which $k_b$ was reduced by about 30-fold, indicating also a crucial role for residues that flank the catalytic Cys-130 in the flexible loop. We find it however, amazing that the mutation S129A only gives a 6-fold reduction of the maximal bleaching rate ($k_b$), since Ser-129 is conserved in all DHODs as well as in the dihydropyrimidine dehydrogenases, enzymes that reduce pyrimidine bases as the first step in pyrimidine breakdown.

The flavin group shows a remarkable constant redox potential in the mutant enzymes close to the $-245$ mV seen for the wild-type enzyme, the most negative potential being $-263$ mV displayed by the P131A mutant. Thus changes of redox potential do not explain the observed changes of kinetic parameters of the mutant enzymes.

Crystal Structures

Cryogenic techniques have allowed us obtain a higher resolution structure of the uncomplexed native enzyme. This structure was solved from crystals grown in the presence of DTT to avoid oxidation of the active site Cys-130 that was observed previously in the room temperature structure (8). We wanted to exclude the possibility that the closed conformation of the active site loop observed in the uncomplexed native DHODA structure is due to an oxidized active site cysteine, preventing the loop opening. This new structure showed unambiguously that no oxidation had taken place and that the active site loop adopts the same conformation as in the previously reported structure (8). The higher resolution enabled us to identify an octahedrally coordinated Mg$^{2+}$ ion at the C-terminal end of helix a1 in each of the subunits. The Mg$^{2+}$ ions were located in electron densities that previously had been refined as water molecules. The presence of the Mg$^{2+}$ ions cannot be related to the function of DHODA, their role is rather to neutralize the helix dipole.

The structures of the mutant enzymes containing orotate share the common feature that they maintain most of the direct interactions with orotate seen in the native structure (9) with K213E(Oro) as the only exception (see below). The new high resolution structure of the native DHODA is shown in Fig. 1 together with the structures determined for two of the mutant enzymes having orotate bound in the active site K213E(Oro), P56A(Oro), and the structure of the unliganded K136E enzyme. N67A(Oro), R57A(Oro), and K136E(Oro) are not shown as they appear essentially identical to the native in this representation. Though the structures were determined at essentially the same temperature, they show significant variations in their mobility as indicated by the variations in B-factors and conformations of the active site loop. The most remarkable result is displayed by K213E(Oro) structure, which shows the active site loop in an open conformation similar to the one previously seen in the structure of native DHODB (10). The active site loop in the K136E structure was modeled in two different conformations, one corresponding to the closed form of the native DHODA and another to a partially open form. In the following a description will be given of the structures of the mutant enzymes according to their position in the structure and to their order of appearance to the approaching substrate (Fig. 1).

Mutations That Affect the Surface Charges: R50E, K136E, and K213E—The surface area of DHODA close to the active site contains three positively charged residues, Arg-50, Lys-136, and Lys-213, which are conserved among the class 1A DHODs. These residues were genetically changed to glutamate residues in order to probe the effect of changing charges adjacent to the entrance of the active site. The stopped flow kinetic experiments showed that the modified enzymes all exhibit decreased bleaching rates of the flavin cofactor compared with the native enzyme, and that the effect is most pronounced for the K213E enzyme (Table III). This may be taken as a support of our original assumption that these residues conserved in class 1A play a role in the first step of the reaction through attraction of the substrate; however, the structure of the K213E mutant protein provided the unexpected result that the active site loop is most stable in an open conformation even with orotate bound in the active site (Figs. 1 and 4a). This dramatic structural change is associated with alterations in the hydrogen-bonding system around the active site as illustrated in Fig. 4b. In the native structure the ammonium group of Lys-213 is hydrogen-bonded to Asn-197 O61 and to the carbonyl group of Pro-131, which is conserved among all DHODs. The side chain of Lys-213 is also interacting through water molecules with the backbone atoms of Phe-216 and Val-133, and with the side chains of Asn-132 and Ser-68. These interactions seem to maintain a closed active site loop. The change of the side chain from a hydrogen-bond donor to a hydrogen-bond acceptor in the K213E mutant enzyme leads to a loss of the hydrogen bonds that connects the active site loop to the Lys-213 helix in the native structure, which explains why the active site loop adopts an open conformation (Fig. 4a).

At the orotate binding site the hydrogen bond between Asn-132 and orotate is missing in the K213E(Oro) structure, as it cannot be formed with the active site loop in the open conformation. The open loop conformation explains why both dihydroorotate and orotate are poorly bound in the K213E mutant, as shown by the higher dissociation constants in Table II. Furthermore with the active site loop in the open conformation the catalytic Cys-130 is in a position that is far from optimal for catalysis, explaining the strongly reduced bleaching rate of the K213E mutant enzyme in the first half-reaction (Table III). The side chain of Lys-136 displays a variety of conformations in the different structures, where its only hydrogen-bond partners are water molecules. It is either oriented toward the solvent or points toward Asn-127 in the orotate binding site, which originally gave us reasons to believe that it could be important for substrate guidance and delivery to the active site. A hydrogen bond formed between the Lys (or Glu)-136 backbone NH and the carbonyl group from Val-133 is conserved in all the DHODA structures. In the K136E structure the active site loop in one of the subunits could be refined in two conformations (Fig. 1). One of these (70% populated) corre-
sponds to an intermediate conformation between open and closed, and the other (30% populated) to the closed conformation found in the other subunit. The small decrease in the dissociation constant for orotate and a $K_m$ for dihydroorotate equivalent to the native enzyme suggests that the role of Lys-136 is not to attract the substrate as we originally proposed (9). The presence of the extra half-open loop conformation could imply that substitution of a Lys with a shorter and negatively charged side chain may interfere with the mechanism of loop opening and provides an alternative explanation for the decreased bleaching rate. The increase in the orotate affinity suggests that it has become more difficult for orotate to leave the active site in the K136E mutant, but it is hard to say whether this is related to the intermediate conformation of the active site loop in the K136E structure.

Arg-50 is in the same position and makes similar interactions in all the DHODA structures, where it faces the active site loop from the opposite side relative to Lys-213 (Fig. 1). This arginine residue is conserved in DHODB (Arg-55), while in DHODC it is replaced by Gln-92. Close to Gln-92 are both Lys-144 and Arg-90 whose positively charged side chains are located in the same area as Arg-50 in DHODA, suggesting that the presence of a positive charge is important. This is to some degree supported by the kinetics of the R50E mutant. A negative charge in this region may induce repulsion of the substrate and affect formation of the Michaelis-Menten complex, resulting in decreased affinity for substrate and product, whereas the other kinetic parameters are only affected marginally.

**Mutations in the cis-Proline Loop: P56A and R57A**—Two adjacent residues in the cis-proline loop (Pro-56, Arg-57) are conserved among all DHODs, and the cis-peptide bond connecting Pro-56 to the preceding residue is also fully conserved in all known DHOD structures. Considering that the backbone fold in the P56A(Oro) and R57A(Oro) structures is virtually identical to the structure of the native enzyme, it was surprising that the mutant enzyme P56A has lost most of its activity, whereas the R57A protein appears more active than the native enzyme in particular with DCIP as the electron acceptor (Tables I and II). Orotate binds very poorly to the P56A mutant enzyme, but this is reasonably easily saturated with dihydroorotate ($K_m$ is increased by about 3-fold), because the enzyme also has a very low (50-fold decreased) catalytic activity. Re-
placement of Pro-56 with an alanine residue does not seem to affect the cis-peptide bond or the direct interactions between DHODA and orotate.

The active site loop has a poorly defined electron density in the P56A(Oro) structure, which made it impossible to identify the side chains of residues 133 to 136. The temperature factors, in the active site loop, and in general are particularly high in this structure (Fig. 1). The b and c cell dimensions are significantly longer (1.8 standard deviations) than the calculated average for the low temperature structures, indicating a slightly looser packing of the crystal. Furthermore though the P56A mutant shows the same degradation pattern with trypsin as the native enzyme, it is degraded faster (data not shown).

Prolines are known to exert a rigidifying effect on loop structures, so an increase in mobility caused by a substitution of a proline with an alanine is not unusual. It is difficult to explain however how the higher flexibility is transmitted from the cis-proline loop to the active site loop and the rest of the structure, given that the cis-proline loop itself is not particularly disordered in P56A(Oro). The increased mobility in particular of the active site loop, seen in relation to the reduced activity of the P56A mutant could imply that the active site base is not in its optimal position for catalysis. In the native structure the Arg-57 side chain interacts with the carbonyl groups of Glu-51 and the totally conserved Asn-53 via its NH1. The carbonyl group from Arg-57 is hydrogen-bonded to the backbone of Ser-68, which interacts with the side chain of Asn-53. Asn-53 is hydrogen-bonded to the carbonyl group of Asn-132, another totally conserved residue in the active site loop. We originally thought that these interactions could contribute to stabilization of the cis-proline loop and ensure correct orientation of residues in the active site loop. The kinetic results showed on the contrary an increased catalytic activity of the R57A mutant enzyme. There are however no significant differences between the coordinates or thermal mobility of the native and the R57A(Oro) structures so the structural basis for the increased catalytic rate of the R57A mutant enzyme remains elusive.

Mutations Flanking the Catalytic Base Cys-130: S129A and P131A—Two residues, Ser-129 and Pro-131, which flank the catalytic base, Cys-130, were also selected for mutation and changed to alanines. Both of the residues are conserved in all DHODs and in the dihydroprymidinid dehydrogenases, which carry out an inverse reaction, namely the reduction of uracil and thymine during breakdown of pyrimidine bases. Although there is no structural information about these two mutant enzymes (S129A and P131A) information about the structural role of the residues and their significance for catalysis can be inferred from the structural investigations of the other mutants.

The comparison between the K213E(Oro) and the native structure revealed some important features for the conserved Ser-129. In the native structures Ser-129 Oy forms a hydrogen bond to the carbonyl group of Lys-164, one of the two totally conserved Lys residues that interact with the FMN group. Furthermore Ser-129 Oy is hydrogen-bonded to Asn-193, which interacts with orotate. With the active site loop in the open conformation these direct interactions are not possible, but they occur via a water molecule and Ser-129 Oy is instead hydrogen-bonded to Gln-138 Oe1. From the comparison with the DHODB and DHODC structures, we have proposed that this serine and Gln-138 in DHODA act as hinges of the active site loop (17). The exchange of the Ser-129 with alanine leads to the loss of the hydrogen bond formed by the side chain and must affect the loop movement and orotate binding site. The S129A mutant enzyme had a decreased binding affinity for orotate and DHO (Tables I and II) and the bleaching rate constant was 6-fold reduced (Table III). The specific activity was almost normal, except when fumarate served as electron acceptor, in which case it was 5-fold reduced.

Like the K213E mutation the P131A mutation generated a decreased affinity for both orotate and DHO and a drop in the reaction velocity (Table I and II). The K213E(Oro) structure shows that the carbonyl oxygen of Pro-131 is important in stabilizing the closed loop conformation. Although the backbone interactions should be maintained in the P131A mutant, prolines tend to stabilize loop structures and the substitution by an alanine residue might increase the flexibility of the active site loop, disrupting the interaction with Lys-213 and favoring the open loop conformation.

The Active Site Mutants N67A, N127A, and N193A—These mutant enzymes were constructed in order to examine the influence of the asparagine residues (Asn-67, Asn-127, Asn-193) interacting with the pyrimidine ring of the product/substrate as revealed from the structure of the DHODA-orotate complex. The side chains of two of these (Asn-67 and Asn-193) form cyclic hydrogen-bonded systems with pyrimidine atoms N1, O2, and N3, O4, respectively, whereas only the amido group of the side chain of Asn-127 is hydrogen-bonded to O4 of orotate. In accordance with these interactions the N127A mutant displays the smallest changes of kinetic parameters among the three mutant enzymes (Tables I and II). The Km values for saturation of the N67A and N193A enzymes are very high and the maximal reaction velocities are somewhat decreased, but not dramatically. A remarkable feature of the N67A mutant enzyme is its ability to bind orotate much better than the other two enzymes with a dissociation constant comparable to the native enzyme (Table II). The N67A(Oro) structure provides an explanation for this ability. Fig. 4c illustrates how the replacement of Asn-67 with an alanine residue leaves sufficient space for two water molecules to occupy the space in the active site otherwise taken by N82 and O82 of the side chain. The water molecule found close to the position of N62 is hydrogen-bonded to orotate O2 and to the carbonyl group of the substituted Ala-67, the other water molecule fulfills the same hydrogen bonds as O82 from Asn-67 in the native structure and is also hydrogen-bonded to the carbonyl group of orotate and the backbone NH group of Met-69. These interactions anchor orotate almost as well in the active site of the N67A mutant as in the native enzyme. The very unusual red shift in the FMN spectrum of the orotate-bound enzyme (Fig. 2) can be explained by the differences in the hydrogen bonds involving water molecules. The spectra for the N127A and N193A mutant enzymes upon addition of orotate (Fig. 2) showed a red shift similar to the one seen for the native enzyme, which suggests that it has not been possible to replace the side chains of the asparagines residue with water molecules in these structures. Consistent with the observation that Asn-127 and Asn-193 are contained in a more tightly packed part of the active site than Asn-67 (9).

**DISCUSSION**

Roles of Different Residues in DHODA—The powerful combination of site-directed mutagenesis, kinetic analysis, and x-ray crystallography has allowed us to investigate the role of different protein segments in the function of DHODA. The importance of the hydrogen bonds between Asn-67, Asn-127, and Asn-193 and DHO has been demonstrated by the decreased activities of the N67A, N127A, and N193A mutant enzymes. Furthermore a comparison of the N67A(Oro) structure with the native (Fig. 4, b and c) provides an explanation of the unusual red shift shown by this mutant on orotate binding (Fig. 2). The three conserved asparagines may also contribute...
to the catalytic efficiency of the enzyme by withdrawal of electrons from the C5 position of DHO and thus lowering the $pK_a$ of the C5-proS-proton by the catalytic base Cys-130, as proposed by Fraaije and Mattevi (34). The magnitude of the effects on catalysis is difficult to access. The very weak binding exhibited by these mutant enzymes makes it difficult to saturate the mutant enzymes with substrate and determine $V_{max}$ accurately.

The role of the conserved residues Pro-56 and Arg-57 in the cis-proline loop is harder to pinpoint precisely. Substitution of these residues conserved in all DHOD classes with alanine affects the catalytic activity, consistent with their high conservation and with the observation from the crystal structures that the active site loop and the cis-proline loop are intimately connected (Fig. 1). The effects on the activities of the two variants was however unexpected. While the activity of the P56A variant is impaired, the R57A variant shows an increase in activity despite reduced affinity for substrate and product. The structures of the two mutant enzymes are very similar to the native enzyme and do not provide a direct structural explanation for the effects of the mutations. Since they deviate in their mobility of the active site loop, which is less mobile in the R57A structure, it is tempting to attribute their enzymatic differences to the difference in mobility. The increased mobility in P56A may influence the position of the catalytic Cys-130, so it is not optimally positioned for catalysis causing the lower activity.

It had been hypothesized that the positively charged residues Arg-50, Lys-136, and Lys-213 have a role in substrate guidance (9). The variant DHODAs where these residues are mutated show a decreased bleaching rate, indicating that the first half of the reaction has been affected. Though this could be interpreted as consistent with the substrate guidance role, the other kinetic and structural results show that this role is only likely for Arg-50. K136E has similar or increased affinity for DHO and orotate compared with native DHODA, which seems inconsistent with a role in substrate guidance. The open active site loop unexpectedly encountered in the K213E(Oro) mutant enzyme suggests an alternative explanation for the decreased bleaching rate, namely an impaired mechanism for controlling loop opening and closing. In the native enzyme the active site loop is closed, and the closed conformation depends on a hydrogen bond interaction of between Lys-213 and the carbonyl group 10 of the conserved Pro-131. Both the highest resolution native DHODA structure and R57A(Oro) contain some residual density too weak to be modeled, which could suggest that a very small fraction of the protein has the active site loop in the open conformation. The closed conformation of the active site loop appears to be the more stable but it is at the same time very mobile. As dihydroorotato approaches the active site, possibly guided by Arg-50, a transient interaction between Arg-50 and Lys-213 might favor temporary opening of the active site loop to let the substrate enter the catalytic site. In K213E the active site loop is most stable in an open conformation, which allows substrate to enter the active site but is not optimal for catalysis. Substitution of Pro-131 with Ala has similar kinetic consequences as substitution of Lys-213. So the open active site loop conformation might also be favored in this case because the hydrogen bond between Lys-213 and the backbone of residue 131 is destabilized by increased loop flexibility.

The active site loop moves around the hinges Ser-129 and Gln-138 in DHODA. In the closed loop conformation, the backbone NH group of Ser-129 is hydrogen-bonded to Asn-127 O61, and the Ser-129 Oy atom is hydrogen-bonded to Asn-93 N2, two residues involved in orotate binding. With the loop open Gln-138 Oe1 is hydrogen-bonded to Ser-129 Oy. The mutation of Ser-129 to alanine affects one of the hinges of the active site loop by destroying the side chain hydrogen bonds, which could be the explanation for the low reactivity of the S129A mutant. The active site loop moves around the hinges Ser-129 and Gln-138 in DHODA. In the closed loop conformation, the backbone NH group of Ser-129 is hydrogen-bonded to Asn-127 O61, and the Ser-129 Oy atom is hydrogen-bonded to Asn-93 N2, two residues involved in orotate binding. With the loop open Gln-138 Oe1 is hydrogen-bonded to Ser-129 Oy. The mutation

**Comparison between DHODs from Different Classes**—The structural alignment of representatives from the different classes of DHODs, i.e. DHODA, DHODB, and DHODC (17) showed that Lys-213 only is conserved among the class 1a enzymes and that the open conformation seen in the K213E(Oro) structure corresponds to the conformation of the loop observed in the structure of the uncomplexed class 1B enzyme, DHODB (10). In the DHODB structure in complex with orotate the loop is in a closed conformation, but could only be traced partially, while in the open conformation it could be traced completely, indicating that it moves to carry out the reaction but is more stable in the open conformation. The superposition of DHODA on the DHODB structure shows that Lys-213 in DHODA has no match in DHODB; however, the same space is occupied by Asn-215 and Ile-216. None of these two residues are able to form the same hydrogen bonds as the lysine suggesting that the absence of a lock system in DHODB to be the origin of the difference observed in stability of the loop conformations between DHODA and DHODB. In both DHODA and DHODB the active site loop alternates between an open and closed conformation due to dynamic motion. The loop is locked into the closed conformation in DHODA by Lys-213, but as DHODB lacks this residue its open loop conformation is the more stable. The importance of the closed loop in DHODB is likely to associated with the fact that the substrate DHO and the electron acceptor use the same binding site, meaning that the closed loop conformation is necessary for both half-reactions. In DHODB this is not the case as substrate and electron acceptor binding sites are widely separated and situated in different subunits. DHODC does also not contain a residue equivalent to Lys-213, and the corresponding protein segment is much shorter. Due to small structural differences in the domains surrounding the active sites of DHODA and DHODC and the presence of the extended N-terminal in DHODC, we would not expect the DHODC active site loop to open in the same way as in DHODB and DHODC (17). The structural differences around the active site loop and the monomeric state of the enzymes suggest that the class 2 DHODs have developed another mechanism for controlling the loop motion.

**Subunit Asymmetry**—Another result from the kinetic and structural studies is the apparent asymmetry in substrate binding at the two active sites of the DHODA dimer, resulting in an apparent negative cooperativity in ligand binding. The subunits in K136E differ, one subunit has a closed active site loop, while the other shows two conformations of the active site loop. It is noteworthy that the proportions of the two populations are the same as the proportions of low and high affinity sites derived from the kinetic data. These results provide the first structural indications for a non-equivalent behavior of the active sites in the two subunits. We favor the idea that the ligand binding to the first subunit alters the equilibrium between conformations of the active site loop in the second subunit.

**CONCLUSIONS**

The availability of both structural and kinetic data has allowed us to access the details of DHODA catalysis and assign roles for most of the conserved residues. These results demonstrated the great impact on the catalytic behavior exerted by the mobility of all the loops that delineate the active site. It has been shown that though all classes of DHOD carry out a similar reaction they have developed highly individual mechanisms for controlling the access to the active site and for regulation of
catalytic activity. Each class of DHODs appears to have developed a unique way of opening and closing of the active site loop that is a determinant of the catalytic function. We have also taken the first steps to reveal the functional asymmetry of the dimer.

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Lactococcus lactis Dihydroorotate Dehydrogenase A Mutants Reveal Important Facets of the Enzymatic Function

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