Active Site of Dihydroorotate Dehydrogenase A from *Lactococcus lactis* Investigated by Chemical Modification and Mutagenesis[†]

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ABSTRACT: The flavin-containing enzyme dihydroorotate dehydrogenase (DHOD) catalyzes the oxidation of dihydroorotate (DHO) to orotate, the first aromatic intermediate in pyrimidine biosynthesis. The first structure of a DHOD, the A form of the enzyme from *Lactococcus lactis*, has recently become known, and some conserved residues were suggested to have a role in the active site [Rowland *et al*. (1997) *Structure 2*, 239−252]. In particular, Cys 130 was hypothesized to work as a base, which activates dihydroorotate (DHO) for hydride transfer. By chemical modification and site-directed mutagenesis we have obtained results consistent with this proposal. Cys 130 was susceptible to alkylating reagents, and mutants of Cys 130 (C130A and C130S) showed hardly detectable enzyme activity at pH 8.0, while at pH 10 the C130S mutant enzyme had approximately 1% of wild-type activity. Mutants of Lys 43, Asn 132, and Lys 164 were also constructed. Exchange of Lys 43 to Ala or Glu (K43A and K43E) and of Asn 132 to Ala (N132A) affected both catalysis and substrate binding. Expressed as k_{cat}/K_M for DHO, the deterioration of these three mutant enzymes was 10^3-10^4-fold. Flavin spectra of the mutant enzymes were not, like the wild-type enzyme, bleached by DHO in stopped-flow experiments, showing that they were deficient with respect to the first half-reaction, namely reduction of FMN by DHO, which was not rate limiting for the wild-type enzyme. The binding interaction between flavin and the reaction product, orotate, could be monitored by a red shift of the flavin absorbance in the wild-type enzyme. The C130A, C130S, and N132A mutant enzymes displayed similar capacity to bind orotate. In contrast, orotate did not change the absorption spectra of the K43 mutant enzymes, although it did inhibit their activity. All of the mutant enzymes, except K164A, contained normal levels of flavin. The results are discussed in relation to the structures of DHODA and other flavoenzymes. The possible acid−base chemistry of Cys 130 is compared to previous work on mammalian dihydropyrimidine dehydrogenases, flavoenzymes, which catalyze the reversed reaction, namely the reduction of pyrimidine bases.

The flavin mononucleotide (FMN[1]) containing enzyme dihydroorotate dehydrogenase (DHOD) catalyzes the oxidation of (*S*)-dihydroorotate (DHO) to orotate. It is the fourth sequential step and the only redox reaction in the *de novo* pathway to uridine 5′-monophosphate (UMP). The reducing equivalents from the oxidation are transferred to the cofactor (FMN) which, in the second half-reaction, becomes reoxidized by an electron acceptor. On the basis of sequence homology, DHODs can, at present, be separated into two major families with less than 20% sequence identity between the families (see Figure 1 and ref 1). Enzymes of family 1 seem to be cytosolic (*2*−*4*), whereas several enzymes of

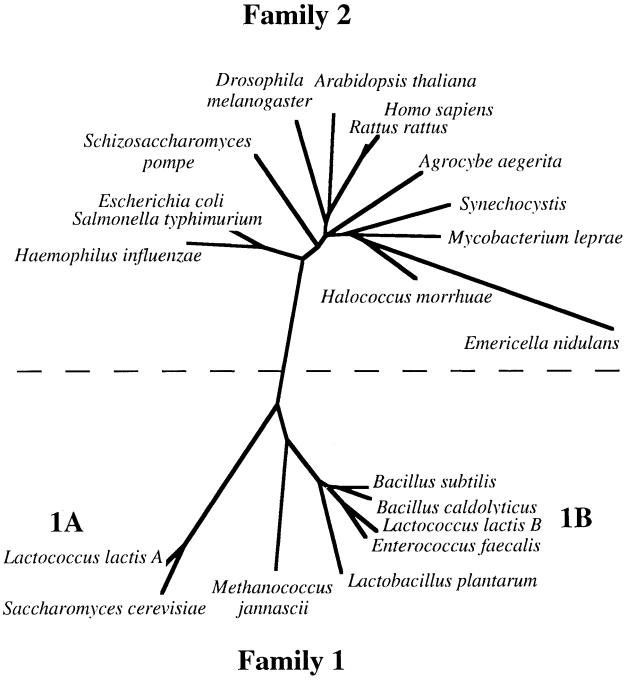

FIGURE 1: Evolutionary tree depicting the relationship between 21 different DHOD sequences. It is clear that the DHOD sequences fall into two major families, families 1 and 2. Furthermore, we have subdivided family 1 into two subgroups, 1A and 1B.

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[1] Abbreviations: DCIP, 2,6-dichloroindophenol; DHO, (*S*)-dihydroorotate; DHODA and DHODB, the A and B forms of dihydroorotate dehydrogenase from *L. lactis*; DPD, dihydropyrimidine dehydrogenase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; FMN, flavin mononucleotide; IAAM, iodoacetamide; Q_0, 2,3-dimethoxy-5-methyl-1,4-benzoquinone.

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family 2 were shown to be associated with membranes (*4*−*6*).

The Gram-positive bacterium *Lactococcus lactis* is the only organism, so far known, which produces two forms of DHOD (*7*). These two forms, called DHODA and DHODB, are different enough to represent two subgroups of family 1, i.e. families 1A and 1B. While DHODA is a homodimer containing FMN as the only prosthetic group, DHODB is a tetramer composed of two different types of polypeptides, termed the PyrDB and PyrK subunits, and contains FMN, flavin adenine dinucleotide (FAD), and Fe_2S_2 clusters as redox centers (*2, 3*). Although the polypeptide chains of DHODA and the larger subunit of DHODB are of the same length (311 residues), sequence identity between the two is only about 30%. Alone, the larger PyrDB subunit forms a homodimer and can bind FMN, but the dimer is both unstable and inefficient as a catalyst. On the other hand, the tetrameric holoenzyme, containing both PyrDB and PyrK subunits and all three cofactors, is stable and uses nicotinamide adenine dinucleotide efficiently as electron acceptor *(3)*. Enzymes of the DHODB type seem to be widespread among Gram-positive bacteria (e.g. *Bacillus subtilis*). In contrast, DHODA of *L. lactis* has only one identified close relative, the enzyme from baker's yeast (*Saccharomyces cerevisiae*) with which it shares 71% sequence identity and the capability to use fumarate as electron acceptor (*4, 8, 9*).

The enzymes from Gram-negative bacteria (e.g. *Escherichia coli*) and eukaryotic organisms are related by approximately 40% sequence identity and form the second family. The *E.coli* enzyme is attached to the cell membrane (*10*), while at least some of the eukaryotic enzymes are localized in the inner mitochondrial membrane (*4, 6*). These enzymes seem to use long-chain ubiquinones (Q_6 and Q_{10}) as electron acceptors, and the electrons generated are delivered to the respiratory chain (*6*). We study the two proteins found in *L. lactis*, i.e. DHODA and DHODB, as representatives for each of the subgroups 1A and 1B of family 1 and the *E. coli* enzyme as a representative of family 2.

The first structural information on a DHOD became available recently, as the crystal structure for DHODA from *L. lactis* was determined to 2.0 Å resolution (*1*). A noncrystallographic twofold axis relates the two subunits in the dimeric protein. Each subunit folds into an α/β-barrel with the flavin mononucleotide (FMN) sitting between the top of the barrel and a subdomain formed by several barrel inserts. The two FMN molecules are well separated in the dimer. A small cavity above the isoalloxazine ring was suggested as the substrate binding site (*1*). The shape of the cavity is well defined and perfectly suited to fit in DHO. On this basis, we selected the following four residues as good candidates for mutational studies that could contribute to the understanding of the catalytic function of the DHODs: (a) Cys 130 and Lys 43 were selected because they are conserved in all sequences of DHODs belonging to family 1. Cys 130 is the only residue with possible acid−base properties within 8 Å from the substrate cavity in DHODA, while Lys 43 is involved in unique interactions with the isoalloxazine ring of FMN and could contribute to substrate binding by its positive charge. (b) Asn 132 and Lys 164 were selected, because they are conserved in the sequences of all DHODs. Asn 132 is close to the substrate cavity, and Lys 164 makes a hydrogen bonding pattern to the FMN group, which is similar to that of other FMN binding proteins.

EXPERIMENTAL PROCEDURES

Evolutionary Analysis of DHOD Sequences. A multiple alignment of 22 currently known DHOD sequences was made using the program PileUp of the GCG program package (*11*). The distances obtained from this alignment were converted into the tree, shown in Figure 1, by use of the program TreeView (R. D. M. Page, IBLS, University of Glasgow, U.K.). The sequences of the different DHOD are given below by their accession numbers in the Swiss-Prot database or GenBank: the mushroom *Agrocybe aegerita* (P28294), the green plant *Arabidopsis thaliana* (P32746), the insect *Drosophila melanogaster* (P32748), the mammals *Homo sapiens* (Q02127) and *Rattus rattus* (X80778), the fungus *Emericella nidulans* (U47318), the parasite *Plasmodium falciparum* (Q08210), the Gram negative eubacteria *Escherichia coli* (P05021), *Salmonella typhimurium* (P25468), *Haemophilus influenzae* (P45477), *Mycobacterium leprae* (P46727), the cyanobacteria *Synechocystis* sp. PCC9603 (D90916), the yeasts *Saccharomyces cerevisiae* (P28272) and *Schizosaccharomyces pombe* (P32747), the Gram positive bacteria *Bacillus subtilis* (P25996), *Bacillus caldolyticus* (P46539), *Lactococcus lactis* DHODA (P54321), *Lactococcus lactis* DHODB (P54322), *Enterococcus faecalis* (U24692) and *Lactobacillus plantarum* (Z54240), and the archae *Halococcus morrhua* (X72588, partial sequence) and *Methanococcus jannascii* (MJ0654, Tigr Organization). The DHOD sequence from *P. falciparum* was deduced from a chromosomal DNA sequence. In the tree, it branched away from the path leading to *E. nidulans*, but appeared quite distantly related due to two major inserts in the deduced protein sequence without similarity to the other DHODs. For that reason it was omitted from the presentation in Figure 1. The sequence of the DHOD from *Dictyostelium discoideum* (P07670) has been left out from the alignment, because it shows very little similarity to any other DHODs and would fall into a family entirely of its own.

Materials. Materials used for DNA manipulation and protein purification were obtained from the same sources as described previously (*2, 3*). Iodoacetamide (IAAM), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), (*S*)-dihydroorotate (DHO), orotate, and 2,3-dimethoxy-5-methyl-1,4-benzoquinone (Q_0) were purchased from Sigma and used without additional purification.

General Instrumentation. Absorption spectra were recorded in a Zeiss Specord S10 diode-array photometer. Enzyme assays were also run on this instrument or on a UV−vis SPEKOL photometer from Zeiss. The stopped-flow equipment (SX-18MV) was from Applied Photophysics.

Inactivation of the Enzyme by Alkylation. Alkylation reactions were performed in 500 μL portions on ice bath (0 °C) for DTNB and at 25 °C for IAAM. The buffer concentration was 0.1 M in the presence of 1.0 mM EDTA and 10% glycerol. The buffers used were MES (pH 5.5−6.25), HEPES (6.5−7.25), and Tris-HCl (pH 7.5−9.5). The concentrations of enzyme and alkylator were 1.0 and 500 μM, respectively. Reactions were started by the addition of alkylator from stock solutions of either 20 mM of DTNB (in ethanol) or of 100 mM of IAAM. Samples (20 μL) were collected at different time points and analyzed directly in assay 1.

Construction of Active Site Mutations and Expression in E. coli. The *pyrDa* gene, encoding DHODA of *L. lactis*, was previously cloned in the expression vector pUHE23-2, which carries a strong synthetic LacI repressible promoter, to give pFN1 (*2*). Using pFN1 as template, mutagenesis was carried out by "overlap extension" with four primers (*12*). For each mutant a pair of complementary oligonucleotides were synthesized. They were all 33-mers extending five codons on either side of the exchanged codon and, to facilitate screening, designed to change the restriction enzyme pattern. Two flanking primers previously used to position the coding region in the expression vector (*2*) were used together with the mutant primers to amplify the *pyrDa* gene as two separate fragments. The resulting two "megaprimers" were used as templates in a second round of PCR (together with the flanking primers) to obtain a continuous copy of the gene which was ligated into the expression vector pUHE23-2. The mutant constructs were first transferred to the strain NF1830 (*araD139* Δ(ara-leu)7697Δ lacX74 galUK rpsL recA [F′*lacI*^{q1}*Z::Tn5*]). DNA sequencing (Sequenase version 2 kit) was used to confirm the entire coding region of the inserts. As described for the wild-type enzyme, the strain SØ6645 (*araD139*Δ(ara-leu)7679 galU galK Δ(lac)-174 ΔpyrD(MluI-BssHII::Km^r) [F′ *proAB lacI*^{q}*ZΔM15 Tn10*]), partially deleted for the chromosomal *pyrD* gene, was used for expression. The bacteria were grown with shaking at 37 °C in LB broth (*13*) supplemented with uracil (20 μg/mL).

Complementation of Strain SØ6645. The uracil requirement of SØ6645 cells is supressed by the presence of the plasmid pFN1 even without induction of gene expression. The ability of the mutants to complement the strain was tested by plating transformed cells on defined agar medium. The (A + B) medium of Clark and Maaløe (*14*) supplemented with glucose (0.4%), casamino acids (0.4%), and thiamine (1 μg/mL) was employed. Plates contained ampicillin (100 μg/mL) and three levels of isopropyl thiogalactoside (IPTG), 0, 0.05, and 0.5 mM.

Preparation of Enzymes. The mutant enzymes were purified essentially according to the procedure of Nielsen *et al*. (*2*), and the final yields were comparable to that of wild-type enzyme (20−30 mg/L bacterial culture). Cell pellets as well as purified enzyme preparations were distinctly yellow, with exception of the K164A mutant enzyme. Purified enzyme (5 −10 mg/mL) was stored at −20 °C in 50 mM sodium phosphate pH 6.0, 1 mM EDTA, and 50% glycerol. *L. lactis* DHODB and the *E. coli* enzyme were purified as described by Nielsen *et al*. (*3*) and Jensen and Larsen (*5*), respectively.

Determination of Enzyme Concentration. Extinction coefficients for wild-type and mutant forms of the enzyme were determined after release of free flavin. Samples of enzyme in 50 mM sodium phosphate, pH 7.0, were incubated for 5 min at 100 °C and cleared by centrifugation. Spectra of these samples were recorded before and after this procedure. The concentration of free FMN was calculated using an extinction coefficient of 12 500 M^{-1} cm^{-1} at 445 nm. Protein concentrations were analyzed by the Lowry method (*15*) using bovine serum albumin as standard.

Assay of Dihydroorotate Dehydrogenase Activity. Assay 1. The standard assay, in which the oxidation of DHO is coupled to the reduction of DCIP, was performed essentially according to Karibian (*10*). DCIP loses absorbance at 600 nm upon reduction ($\epsilon = 20$ mM^{-1} cm^{-1}). The assay mixture (1.0 mL) contained 0.1 M Tris-HCl, pH 8.0, 1.0 mM EDTA, 1.0 mM DHO, and 50 μM DCIP. Triton X-100 and KCN were not included. Assay 1 was also employed without addition of the electron acceptor DCIP. The production of orotate was then monitored at it's maximal absorbance at 278 nm ($\epsilon = 6.7$ mM^{-1} cm^{-1}).

Assay 2. In this assay 2,3-dimethoxy-5-methyl-1,4-benzoquinone (Q_0) was the electron acceptor, and the reaction was monitored by measuring the increase in absorbance at 300 nm. Both reaction products, orotate and reduced Q_0, absorb at this wavelength whereas the substrates have negligible (DHO) or very low absorbance ($Q_0 < 0.4$ mM^{-1} cm^{-1}). We used the isosbestic point, at 287 nm, between oxidized and reduced Q_0 and where orotate displays high absorbance (6.08 mM^{-1} cm^{-1} (*16*)) to determine a combined extinction coefficient at 300 nm at pH 8.0 ($\epsilon = 3.6$ mM^{-1} cm^{-1}). Assay 2 was used to determine the influence of pH on enzyme activity, since the combined extinction coefficient was considered as valid over a wide pH range, while the absorbance of DCIP changed as a function of pH. Absorption spectra of orotate and DTT-reduced Q_0 were inspected from pH 3.5 to 10.5, and they appeared to be identical up to pH 8.5 where the absorbance of reduced Q_0 at 300 nm began to decrease. The orotate spectra was also altered above pH 9.0, where the absorbance at 300 nm was increased, and both these spectral changes were consistent with expected deprotonation reactions.

The kinetic parameters, V_{max} and K_M for Q_0, were determined for the wild-type enzyme in assay 2 in 0.1 M Tris-HCl, pH 8.0, and 1.0 mM EDTA. These parameters were also determined at pH 6.0 in 0.1 M MES and 1 mM EDTA. The enzyme showed hyperbolic saturation kinetics when both substrates, DHO and Q_0, were varied at equal concentration (20−1000 μM), as expected for a ping-pong mechanism (*17*). V_{max} could thus be determined from such saturation curves. The K_M values for Q_0 were then calculated from V_{max} and the V_{max}/K_M obtained from the saturation curve for Q_0 at a fixed concentration of DHO, as described by Cleland (*17*).

Assay 3. A combination of assays 1 and 2, assay 3 was used to determine the K_M values for DHO. Both electron acceptors were used, DCIP at 50 and Q_0 at 200 μM in 0.1 M Tris-HCl, pH 8.0, and 1.0 mM EDTA. The absorbance decrease at 600 nm was monitored. Inhibition of the wild-type enzyme by orotate was analyzed in Assay 3 by varying the DHO concentration (20, 50, 100, 200 and 300 μM) at five fixed concentrations of orotate (0, 10, 20, 40, and 80 μM). In the corresponding analysis of the three mutant enzymes K43E, K43A, and N132A, the substrate and inhibitor concentrations were varied over a wider range (DHO, 100, 250, 500, 1000, and 2000 μM; orotate, 0, 25, 50, 100, 250, and 500 μM). Although the absorption of DCIP is perturbed at low pH, the general ability of bleaching upon reduction is retained and assay 3 was used to determine the K_M value for DHO in 0.1 M MES, pH 6.0, and 1 mM EDTA. At pH 8.0 the concentration of wild-type enzyme was typically 2.5 nM while the mutant enzymes were used at a 1000-fold higher concentration. At pH 6.0, the concentration of wild-type enzyme was increased 7-fold. Reactions were linear with respect to time for at least 0.5 min at the lowest substrate concentrations.

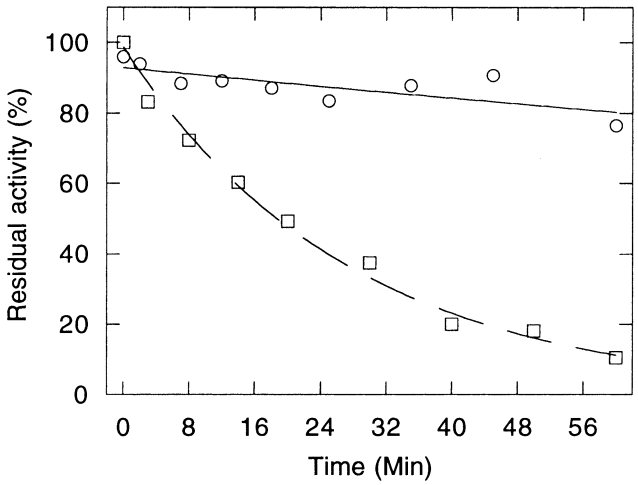

FIGURE 2: Protection against DTNB alkylation by orotate. Enzyme (1.0 μM) and DTNB (500 μM) were incubated on an ice bath in 0.1 M Hepes, pH 7.1, 1 mM EDTA, and 10% glycerol. The reaction was started by the addition of DTNB and followed for 1 h by measuring the residual activity of the enzyme in assay 1 (squares). To a parallel reaction (circles), orotate (100 μM) was included before the addition of DTNB. The activity of the unprotected sample at time zero was defined as 100% activity. From the data, the calculated half-life of the enzyme activity was about 300 min with and 19 min without the presence of orotate.

In all three assays, one unit is defined as the amount of enzyme that produces 1 μmol of orotate/min at 37 °C.

Stopped-Flow Measurements. Bleaching of the flavin absorbance of wild-type enzyme upon reduction with DHO under aerobic conditions was followed at 457 nm at 6 °C with a stopped-flow spectrophotometer. After mixing (1:1), the concentration of enzyme was 9 μM (to obtain an initial absorbance of 0.1) in the presence of 300 μM DHO. The buffer used, 0.1 M Tris-HCl and 1 mM EDTA, was adjusted to pH 8.0 at room temperature. After calibration of the pH-meter on an ice bath, the pH of this buffer was 8.5.

Data Treatment. Hyperbolic saturation curves from kinetic experiments (varying the concentrations of DHO and Q_0) and binding of orotate to the enzyme as monitored by red-shift were fitted by using the BIOSOFT program UltraFit for Macintosh. Standard errors were also calculated by this program. Inhibition by orotate was also analyzed by the fitting program of Cleland (*18*). K_{ii} and K_{is} are the inhibitor constants obtained from the effect of orotate on intercepts and slopes of double reciprocal plots (*19*). First-order rates obtained on the stopped-flow were analyzed by the software provided by the manufacturer (Applied Biophysics). Figures were generated by the program Kaleidagraph (Abelbeck Software).

RESULTS

Susceptibility Toward Alkylating Reagents. In preliminary experiments, we observed that both DHODA and DHODB of *L. lactis* were inactivated by the alkylating reagents, DTNB and IAAM. The rate of inactivation was higher with DTNB than with IAAM. The presence of the product, orotate, protected both the DHODA (Figure 2) and DHODB (data not shown) against DTNB inactivation, suggesting the occurence of a cysteine residue in the active sites of these enzymes. A likely candidate is Cys 130, since it is the only conserved cysteine between the two DHODs from *L. lactis*. It is the deprotonated thiol group of cysteine which is the reactive species in alkylation, and the rate would therefore be expected to increase at higher pH values. The pK_a values of cysteine residues in proteins are typically above 8.5 (*20*). Our preliminary experiments at neutral pH made us suspect that the reactive cysteine residue would display a low pK_a value, and we started to investigate its ionization properties. Rates of alkylation were determined at increasing pH values with IAAM, from 5.5 to 9.5 (Figure 3) and with DTNB, from 5.1 to 8.3 (data not shown). Inactivation with DTNB is a thiol−disulfide exchange reaction and above pH 8.3, the rate became too fast to measure ($t_{1/2}$ less than 1 min). Alkylation reactions were followed to higher pH with IAAM, but no plateau was reached under the conditions used (Figure 3). These experiments exclude an unusually low pK_a value for the reactive cysteine in the free form of DHODA.

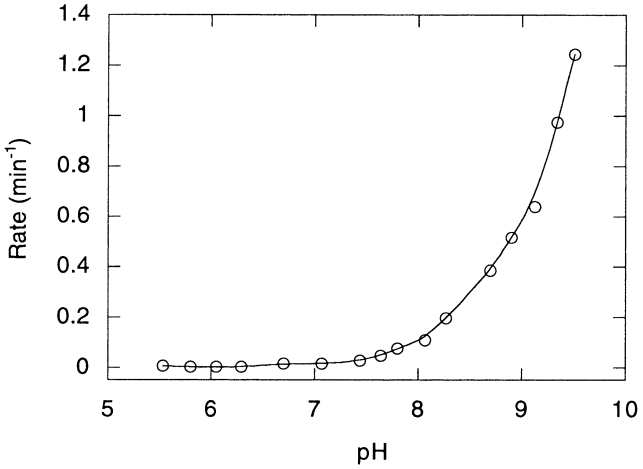

FIGURE 3: Dependence of the rate of inactivation by IAAM on the pH. First-order rates of inactivation by IAAM were measured as described in Experimental Procedures. The lowest rates from pH 5.5 to 6.3 correspond to half-lives of the enzyme of about 2 h. The highest rate measured, 1.2 min^{-1} at pH 9.5, corresponds to a half life of about 30 s.

In sequence alignments, the *E. coli* enzyme has a serine residue at the position corresponding to Cys 130. The activity of that enzyme was resistant to both alkylators (data not shown).

Activity of Mutant and Wild-Type Enzyme. In spite of the fact that the proteins were made in substantial amounts under inducing conditions (0.5 mM isopropyl thiogalactoside), none of the mutant forms of DHODA were able to complement the pyrimidine requirement of the host strain (SØ6645). Activities of the mutant enzymes relative to the wild-type were assessed by assay 1 in crude extracts of induced cells, and except for the K43E mutant enzyme, there was no increase above the background level from equally treated cells carrying the pUHE23-2 vector without inserted *pyrDa* sequences.

Specific activities of the purified enzymes are presented in Table 1. The K43E mutant enzyme was the most active one, but the decrease of specific activity was still more than 100-fold. The K43A and N132A mutant enzymes showed a further decrease in specific activity while the C130A, C130S, and K164A mutant enzymes formed another category with hardly detectable activity. Addition of FMN to the assay mixture did not restore any activity of the K164A mutant enzyme. When no DCIP is included in assay 1, molecular oxygen serves as electron acceptor. The turnover for the wild-type enzyme under these conditions was 6 ± 1

Table 1: Properties of the Mutant Enzymes[a]

enzyme	specific activity with DCIP (units/mg)[b]	specific activity with O_2 (units/mg)[b]	V_{max} ((μmol/min)/mg)[c]	K_M for DHO (μM)[c]	K_{is} for orotate (μM)[c]	K_D for orotate (μM)[d]
wild-type	44	13	150 ± 5	35 ± 2	31 ± 5.5	13.1 ± 0.7
K43E	0.36	0.31	0.58 ± 0.02	470 ± 30	105 ± 10	(no red-shift)
K43A	0.079	0.024	0.17 ± 0.008	890 ± 90	185 ± 24	(no red-shift)
C130A	<0.005	<0.001	–[e]	–[e]	–[e]	6.7 ± 0.6
C130S	<0.008	<0.009	–[e]	–[e]	–[e]	14.5 ± 0.6
N132A	0.17	0.24	0.24 ± 0.005	110 ± 10	22.7 ± 2.6	11.1 ± 0.3
K164A	<0.005	<0.001	–[e]	–[e]	–[e]	(no flavin)

[a] Standard deviations are those given by the fitting program. [b] Determined by assay 1. [c] Determined by assay 3. [d] Estimated from the red-shift as described under Experimental Procedures. [e] The activities of the enzymes were too low to give meaningful estimates of these parameters.

s^{-1} (or 13 units mg^{-1}) at 37 °C (Table 1). When the mutant enzymes were assayed with O_2 as the only electron acceptor, their activity seemed less affected than when other electron acceptors (DCIP or Q_0) were present. Notably, the activities of the K43E and N132A mutant enzymes were only decreased by factors of 40 and 50, respectively (Table 1).

The large extinction coefficient of (oxidized) DCIP gives a good sensitivity of assay 1, but the high initial absorbance ($1.0A_{600}$ at 50 μM) and a limited solubility do not allow concentrations of the electron acceptor which saturates the wild-type enzyme. In the presence of 2 mM DHO, the apparent K_M value for DCIP was 0.21 ± 0.07 mM at pH 8.0. In contrast, the corresponding apparent K_M values for the K43 and N132 mutant enzymes were less than 10 μM, indicating that they were defective in a reaction step different from electron acceptor binding. The K_M value for Q_0 was determined to be 75 ± 5 μM for the wild-type enzyme (assay 2, pH 8.0).

Assay 3, which includes both Q_0 (200 μM) and DCIP (50 μM) as electron acceptors, was used to determine K_M values for DHO. Owing to the strong positive redox potential, all reducing equivalents are caught directly or indirectly by DCIP and rates can be followed by the change of DCIP absorbance at 600 nm. Such mixed systems with DCIP and another quinone have been used before in studies of DHODs, e.g. recently by Knecht *et al.* (*6*). The K_M values for wild-type and mutant enzymes are presented in Table 1. The apparent V_{max} value for the wild-type enzyme (150 μmol min^{-1} mg^{-1}) is lower than the V_{max} seen with assay 2 (200 μmol min^{-1} mg^{-1}), because the concentration of electron acceptor (200 μM Q_0 and 50 μM DCIP) is not saturating in assay 3.

Spectral Properties of Mutant Enzymes. Oxidized wild-type enzyme exhibits a typical flavin spectrum with two broad peaks, the first one at 370−375 and the second one at 455−457 nm (Figure 4A). The mutants C130A, C130S, and N132A were similar to the wild-type enzyme with respect to flavin spectra and extinction coefficients at 457 nm. Both flavin peaks of the K43E and K43A mutant enzymes were slightly moved toward shorter wavelengths (360−373 and 450−452 nm), consistent with an important role of the Lys 43−flavin interaction seen in the structure (*1*). The extinction coefficients of enzyme-bound flavin for the wild-type enzyme, the C130 and N132 mutant enzymes at 457 nm and the K43 mutant enzymes at 450 nm, were between 11 and 12 mM^{-1} cm^{-1}, well within the range determined for other flavoenzymes. Enzyme concentrations determined from the flavin absorption were in good agreement (±10%) with the protein concentrations determined by the Lowry method, indicating that these mutant proteins and the wild-type enzyme were saturated with FMN. The K164A mutant enzyme appeared colorless and did not display absorbance above 300 nm, showing that it had lost the flavin cofactor.

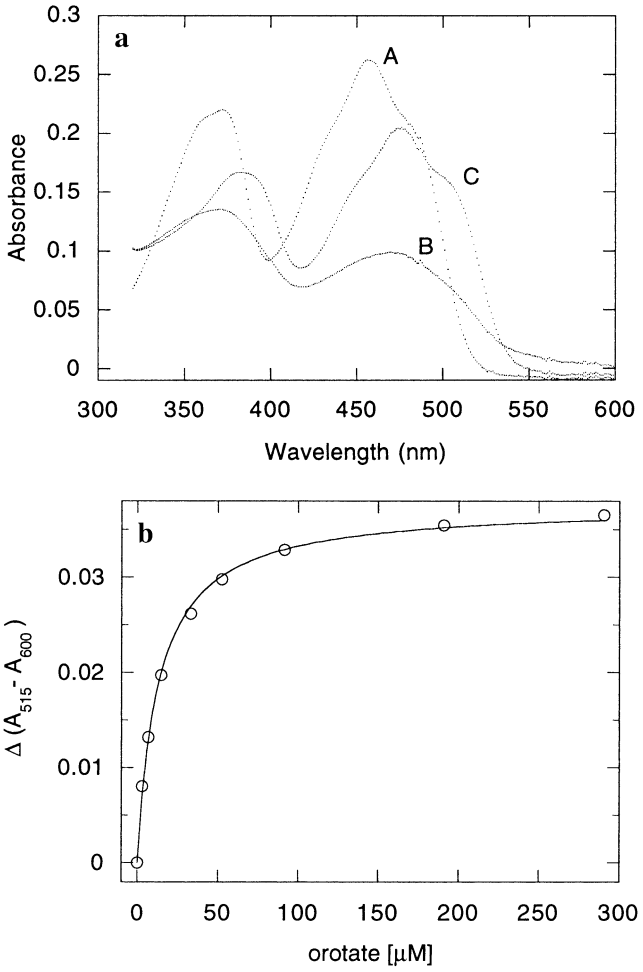

FIGURE 4: (a) Absorption spectra of DHOD. Enzyme was diluted in 50 mM sodium phosphate (pH 7.0) to a concentration of 23 μM in 1.0 mL. The sample was centrifuged and degassed before the first spectrum (A) of the oxidized enzyme was recorded at 6 °C. The next spectrum (B) of the reduced enzyme was recorded a few seconds after the addition of 20 μL of 20 mM DHO. The last spectrum (C) was recorded 5 min after the addition of DHO. At that time, the enzyme had generated orotate which shifts the two flavin peaks to higher wavelength. (b) Binding of orotate as monitored by red-shift. Wild-type DHODA was diluted in 50 mM sodium phosphate pH 7.0 to a concentration of 10 μM and spectrum was recorded. Spectra were then recorded after eight successive additions of orotate. The red-shift, quantified as $\Delta(A_{515}-A_{600})$, was plotted against the free concentration of orotate. The resulting curve indicates an apparent dissociation constant of 13.1 ± 0.7 μM orotate for the wild-type DHODA.

Binding of Orotate. Partial bleaching of the flavin absorbance upon addition of DHO was observed in the diode spectrophotometer (Figure 4A, spectrum B). The residual peaks are consistent with a fast reoxidation by molecular oxygen. When no more production of orotate was observed, the initial absorbance spectrum of the oxidized enzyme was not recovered (compare spectra A and C). The observed prominent red-shift of the flavin absorbance (spectrum C) is probably an effect of the expected close stacking of the two aromatic molecules, FMN and orotate (*21*). Both flavin peaks (at 370−375 and 455−457nm) were shifted toward higher wavelengths, and this was also observed when orotate was added to the enzyme. The change in the absorption spectrum, expressed arbitrarily as $\Delta(A_{515} - A_{600})$, showed saturation upon increasing concentration of orotate and was used to estimate the binding strength. The binding curve thus obtained for the wild-type enzyme (shown in Figure 4B) indicated an apparent dissociation constant (K_D) of 13 μM. We used the red-shift to analyze our inactive mutant enzymes with respect to product interaction. Red-shifts and similar K_D values were observed for the mutant enzymes C130A, C130S, and N132A (Table 1). In contrast, the absorption spectra of the mutant enzymes K43E and K43A were not altered by addition of orotate. For comparison with the K_D values calculated from the red-shift, we analyzed the inhibitory effects of orotate. DHODA catalyzes the interconversion of DHO and [^{14}C]orotate indicative of a classical ping-pong mechanism (*2*), implying that the product, orotate, can function as an electron acceptor for this enzyme. Mixed-type inhibition by orotate against DHO was observed in assay 3, but under the conditions used (i.e. a high and fixed concentration of the electron acceptors Q_0 and DCIP), the apparent K_M values for DHO were much more affected than the apparent V_{max} values. For the wild-type enzyme a K_{is} value (Table 1) of 31 ± 5.5 μM and a K_{ii} value of 250 ± 68 μM were obtained by analysis of 25 assays using different concentrations of DHO and orotate at fixed concentrations of Q_0 (200 μM) and DCIP (50 μM). The K_{is} value for orotate (30 μM) and the K_M value for the substrate DHO (35 μM, Table 1) are thus somewhat higher than the K_D values determined from the red-shift. The K_{is} values for the K43 and N132 mutant enzymes are also included in Table 1. The K_{ii} values, which are not shown, were very high for the mutant enzymes (≤2 mM) consistent with the very low K_M values observed for the electron acceptor (see above).

The inhibitiory effect of orotate on DHODA differs from those previously determined for the membrane bound enzymes of family 2 from bovine liver mitochondria (*22*) and *E. coli* (A. C. Grüner, Masters Thesis, University of Copenhagen, 1996). In these cases, clearcut competitive inhibition were observed.

Reactivity of Cys 130. Alkylation of a cysteine residue by DTNB involves release of a yellow TNB anion (ϵ_{412} = 13.6 mM^{-1} cm^{-1} (*23*)) and the subunit of the *L. lactis* A enzyme (wild-type enzyme) contains three cysteine residues. Under denaturing conditions (6.4 M urea), all three residues reacted with DTNB (a release of 2.8 TNB anion per subunit was observed), but only one (0.92) residue reacted under native conditions. When the corresponding titration was performed with the mutant enzyme C130S, two (2.0) residues reacted under denaturing conditions and none (0.05) under native conditions. Thus, Cys 130 was identified as the reactive cysteine residue. The alkylation, which renders the enzyme inactive, was reversible since more than half of the original enzyme activity was recovered when a sample of enzyme, alkylated under native conditions, was treated by DTT.

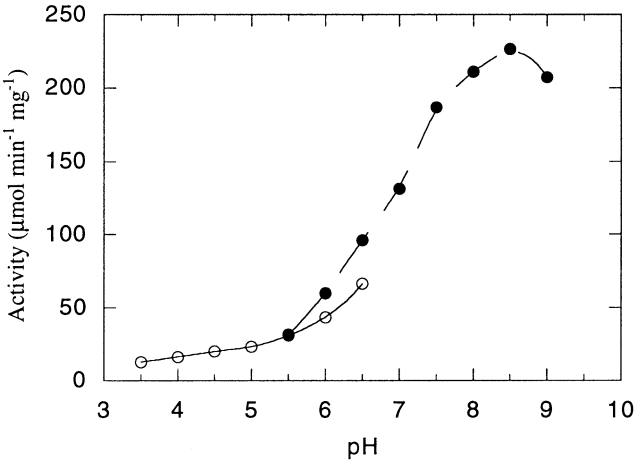

FIGURE 5: Steady state activity of DHODA as a function the pH. Assay 2 was employed and the concentration of both substrates, DHO and Q_0, was 1.0 mM. Assays were performed in a system containing two buffers, both at 50 mM, supplemented with 1 mM EDTA. The buffers used were acetate/MES (open circles) from pH 3.5 to 6.5, and MES/Tris-HCl (filled circles), from 5.5 to 9.0. The concentration of enzyme was 10 nM. To calculate the activity at pH 9.0 a combined extinction coefficient of 3.4 mM^{-1} cm^{-1} was used because of a reduction in the absorbance of reduced Q_0 at this pH.

In the structure of the native enzyme, Cys 130, appeared to be present in the form of a sulfenic acid (SOH) (*1*). Since sulphenic acids do not react with DTNB (*24*), the titration experiments described above indicate strongly that Cys 130 was oxidized during crystallization.

pH Dependence of Steady-State Enzyme Activity. Using a low concentration of DCIP as electron acceptor, Nielsen *et al*. (*2*) recently showed that DHODA has a rather broad pH optimum from 7.5 to 9.0 with maximal activity at pH 8.5. This means that the enzyme has considerable activity, when Cys 130 should be uncharged according to our alkylation studies. We decided to reinvestigate the activity profile (over the pH range 3.5−9.0) using a nearly saturating concentration of Q_0 (1 mM) as electron acceptor (Figure 5). A 4-fold rise in activity was seen by going from pH 6.0 to 8.0. The K_M values for DHO and Q_0 at pH 6.0, 50 ± 10 and 80 ± 10 μM, respectively, were not significantly different from the values obtained at pH 8.0 (35 μM for DHO and 75 μM for Q_0). Therefore, the increase in activity in the pH range, 6.0−8.0, must be ascribed to effects on V_{max}. At pH values above 9.5, we observed an increase in the apparent K_M for DHO and a decrease in the apparent V_{max} using DCIP (50 μM) as electron acceptor (data not shown).

The two C130 mutant enzymes and the wild-type protein were analyzed at pH 10.0 (0.1 M glycine and 1 mM EDTA) with molecular oxygen as electron acceptor. Their activities were compared to those observed at pH 8.0 (assay 1, Table 1). The specific activity of the wild-type enzyme increased from 13 at pH 8.0 to 18 units/mg at pH 10.0, while the C130S and C130A mutant enzymes displayed increases in activity from <0.009 to 0.18 and from ≤0.001 to <0.003 units/mg, respectively. Thus the C130S mutant protein displayed approximately 1% of wild-type activity at pH 10.0, while it showed ≤0.01% activity at pH 8.0. These observations

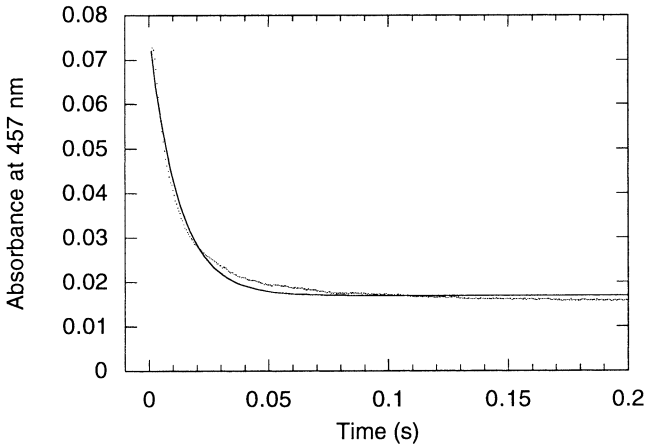

FIGURE 6: Reduction of the enzyme by DHO. Enzyme and DHO, 18 and 600 μM, respectively, were mixed (1:1) in the stopped-flow thermostated to 6 °C. The reaction was followed by the decrease in flavin absorbance at 457 nm and fitted to a first-order reaction. The rate from four consecutive shots was 83.2 ± 4.0. The experiment was repeated at another occasion, giving a rate of 86.0 ± 2.0 (n = 5). The steady state rate of orotate production was measured with the same reactants at 278 nm. After an initial burst, finished at 0.1 s, a constant increase of absorbance was observed up to 1.0 s.

support the notion that residue 130 (Cys or Ser) must be deprotonated to carry out its catalytic function.

Rate of the First Half-Reaction. The oxidation of DHO to orotate is accompanied by the corresponding reduction of the flavin group. This is the first reductive half-reaction and the bleaching of the flavin absorbance could be followed in a stopped-flow apparatus. The rate of reduction was too fast to measure at 37 °C. An experiment in 0.1 M Tris-HCl, pH 8.5, and 1 mM EDTA at 6 °C with a 30-fold excess of DHO over enzyme is shown in Figure 6. The apparently stable baseline observed represents a steady state situation where orotate is produced and the enzyme is reoxidized by molecular oxygen. The linear production of orotate, monitored at 278 nm between 0.1 and 0.4 s with the same reactants, was 0.9 s^{-1}. After 1.0 s, the rate of orotate production decreased and the flavin absorbance started to reappear. The rate of bleaching, fitted to the first-order rate equation as shown in Figure 6, was 82 s^{-1}. At the same temperature and buffer conditions, the V_{max} in assay 3 was 14 (μmol/min)/mg (8 s^{-1}). This strongly suggests that the first half-reaction is not rate limiting at pH 8.5. Bleaching of the flavin absorbance could not be detected with the mutant enzymes, not even with the least deficient enzyme, K43E at 37 °C. This indicated that the rate of the first half-reaction, i.e. reduction of the flavin, was more affected by the mutations than the second half-reaction, which is the reoxidation of FMN by oxygen.

As shown in Figure 6, the bleaching of the flavin in DHODA upon addition of substrate did not closely follow a single exponential decay function. A better fit was obtained by using the equation for a double exponential decay with rate constants of 150 s^{-1} and 20 s^{-1}, where the higher rate was related to the higher amplitude (0.05). This observation may indicate that the reduction of enzyme bound FMN occurs in two steps, but the biphasic appearence of the curve (Figure 6) may perhaps also be a consequence of the presence of oxygen.

DISCUSSION

Hines and Johnston (*25*) used DHO, deuterated at C5 and C6, as a substrate for mechanistic studies of DHOD from bovine liver mitochondria, which belongs to family 2 as the *E. coli* enzyme. They found that a general base is involved in the oxidation of DHO. This step, linked to the abstraction of the C5-*pro-S* proton (*26*), was rate limiting at low pH whereas reduction of the electron acceptor (Q_6) was rate limiting at high pH and required a general acid. Mechanisms for activation of a substrate for hydride delivery by proton abstraction have been outlined from studies of other flavoenzymes (*27*). In flavocytochrome *b2* (FCB), glycolate oxidase (GOX), and lactate monooxygenase, histidine residues close to the bound substrates are employed. In acyl-CoA dehydrogenase, a Glu residue serves as the active site base. Conserved in family 1 and situated in the substrate binding site of DHODA of *L. lactis*, Cys 130 was suggested as the catalytic base responsible for abstracting a proton from the C5 position of DHO (*1*). Activities of the C130 mutant enzymes near our detection limit at pH 8.0, and the dramatic recovery of activity of the C130S mutant protein at pH 10.0 is consistent with the proposed role of this residue. According to the undisturbed binding of orotate, the structural modifications of the Cys 130 mutant enzymes are minimal. Thus, the role of Cys 130 seems to be exclusively catalytic; the residue does not contribute to ligand binding in the ground state.

Cysteine residues as active site bases in flavoenzymes have not been described before, but a comparison to studies of dihyropyrimidine dehydrogenases (DPDs) is striking. Compared to the DHODs, DPDs catalyze the reverse reaction, reduction of pyrimidine bases (thymine and uracil) at the expense of NADPH. Since this reaction is believed to be rate limiting for the degradation of 5-fluorouracil, the mammalian DPDs have received considerable interest (*28*, *29*). Like DHODB, they contain FMN, FAD, and iron−sulfur clusters, but homodimers are formed by their much longer polypeptide chains (1025 residues in the human and pig enzyme (*28*)). 5-Iodouracil is a potent suicide inhibitor of DPD (*30*). After reduction to the alkylating product 5-iodo-5,6-dihydrouracil, the compound became covalently bound to the protein. A radiolabeled tryptic fragment was purified and identified as a derivative of the cysteine residue located in a sequence −NLSCP− (*30*), which corresponds to the region surrounding the highly reactive Cys 130 in DHODA. In DPD, the reactive cysteine residue was suggested to act as a general catalytic acid protonating the substrate at C5 (*29*). In contrast, in DHODA the cysteine residue should function as a general base abstracting a proton from C5. According to our alkylation studies, the pKa value of Cys 130 in the free form of DHODA is 9.0 or higher, i.e. rather normal (*20*). The sulfhydryl group is thus uncharged and probably by itself uncapable of acting as a base at physiological pH values. An interesting candidate for lowering the pK_a value in the enzyme−substrate complex is the carboxylate group attached to the C6 position of DHO.

As the only positively charged residue in the predicted substrate binding cavity, Lys 43, which is conserved in family 1 of DHODs, was suggested to interact with the carboxylate group of DHO (*1*). Furthermore, the amino group of Lys 43 makes hydrogen bonds to the flavin N5 and O4 isoalloxazine ring atoms. Involvement in catalysis is implicated

by the very location of the residue, since N5 generally picks up one of the hydrogen atoms during reduction of FMN. A similar side chain interaction has not been observed in any of the other currently known structures of FMN-binding enzymes. The mutant enzymes (K43A and K43E) displayed increased K_M values for DHO (25- and 15-fold, respectively) and impaired binding of orotate as determined by increased inhibitor constants (K_{is}). The K43 mutant enzymes also had strongly reduced V_{max} values indicating that Lys 43 is at least as critical for catalysis, as it is for substrate binding. The absence of a red-shift of the flavin absorbance upon binding of orotate to the K43 mutant enzymes suggests that Lys 43 is very critical for stacking of orotate against FMN and, thus, perhaps also for the productive orientation of substrate (DHO) and flavin in the enzyme−substrate complex. Unexpectedly, the K43E mutant enzyme with a 300-fold reduction of V_{max} was more active than the K43A mutant enzyme, which showed a 900-fold reduction in V_{max}. Differences in redox potential, not investigated here, may be very important for the observed effects.

Four completely conserved Asn residues, Asn 67, Asn 127, Asn 132, and Asn 193, surround the substrate cavity in DHODA. Asn 132, as Cys 130 located in a flexible loop (connecting β4 and αA(*1*)) whose primary sequence is highly conserved, was selected for mutagenesis. Substitution of Asn 132 may indirectly affect Cys 130. Like the K43 mutant enzymes, the N132A mutant enzyme was deficient both with respect to catalysis and binding of DHO (Table 1). However, the increase in K_M was moderate (3-fold). Binding of orotate was intact according to the inhibitor constant (K_{is}) and the red-shift of flavin absorbance. Maybe the red-shift is primarily dependent on (a) the interactions of Lys 43 to the isoalloxazine ring and the carboxylate group of orotate and (b) hydrophobic interactions to the planar orotate molecule. This idea is supported by the different affinities for orotate shown by the Cys 130 mutant enzymes. Stronger binding was obtained by a more hydrophobic substitution (compare the data for the C130A and C130S mutant enzymes in Table 1).

In this investigation, we have not made attempts to directly study the oxidative half-reaction since anaerobic conditions are required. Reoxidation is probably driven by a large difference in redox potential between reduced FMN and the nonphysiological electron acceptors, Q_0, DCIP, and O_2. The K43 and N132 mutant enzymes were relatively less deficient with O_2 as electron-acceptor than with the other electron acceptors (Table 1, assay 1). They were not deficient with respect to binding of DCIP. In fact the apparent K_M values for DCIP, measured under conditions when the concentration of DHO was $\geq 2K_M$, were strongly reduced, consistent with the notion that the residues Lys 43 and Asn 132 are at least as important for catalysis as for substrate binding. Our studies indicate that Asn 132 and Lys 43 work in concert with Cys 130 to activate DHO, i.e. to initiate the first reductive half-reaction. A significantly different mechanism must be employed by the *E. coli* enzyme and other DHODs of family 2 since they do not possess the conserved cysteine and lysine residues of family 1.

The *E. coli* enzyme is resistant to alkylation. In sequence alignments (*1*), a conserved serine residue (−NIS**S**PN−) in the DHODs of family 2 occupies the position corresponding to the conserved and reactive cysteine residue (−NL/VS**C**PN−) in the enzymes of family 1. According to preliminary studies of mutants in the *E. coli* enzyme, this residue (Ser 175) is essential for activity (O. Björnberg, unpublished observations). A serine residue is conceivably more difficult to activate for a base function. If it works like Cys 130 in DHODA, it is presumably also highly dependent on other residues in the active site. Early work on DHODs from *Zymobacterium oroticum* and *Lactobacillus bulgaricus* indicated essential sulfhydryl groups (*21, 31*). The sequences of these enzymes are still unknown, but they are very likely to belong to family 1. Thus, in addition to sequence homology and subcellular location, susceptibility toward sulfhydryl group reagents seems to be another criterium for the division of DHODs in the two families shown in Figure 1.

The structure of DHODA has features in common with other well-characterized FMN binding enzymes. For example, FCB and GOX also form α/β-barrels, and some glycine residues involved in binding of the flavin phosphate group also seem to be conserved. Lys 164, which is conserved among the three DHOD families, has four possible H-bond interactions (<3.3 Å) with the FMN molecule. The ammonium group contacts N1 and O2 in the isoalloxazine ring and two of the ribityl oxygens (2′ and 3′). It is possible that Lys 164 by these interactions stabilizes the negative charge, which builds up on FMN during transfer of a hydride ion from the substrate, and it may eventually donate a proton to neutralize the reduced flavin. As previously discussed by Rowland *et al.* (*1*), other FMN binding enzymes also have positively charged residues (Arg or Lys) in a position similar to that of Lys 164 in DHODA. These residues have been suggested to increase the redox potential and thereby contribute to catalysis (*27*). A lysine residue in GOX (K230) makes four very similar H-bond interactions (<3.3 Å) with the FMN (*32*). Site-directed mutagenesis of the corresponding residue in FCB (K349R (*33*)) and in lactate monooxygenase (K266M (*34*)) resulted in inactive enzymes still capable of binding FMN. The K266M mutant enzyme of FCB was mainly produced in an insoluble form. In contrast, our K164A mutant enzyme in DHODA apparently lost its FMN binding capacity, but was expressed at apparently normal levels at 37 °C without an increased tendency to precipitate. One reason for the dramatic effect of this substitution on FMN binding may be that this enzyme, in contrast to the other proteins, has a phosphate binding site without positively charged residues.

Since the FMN molecule is so well buried in the native enzyme, the structure and stability of the K164A mutant protein devoid of FMN may be of interest.

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