Role of Lys100 in Human Dihydroorotate Dehydrogenase: Mutagenesis Studies and Chemical Rescue by External Amines

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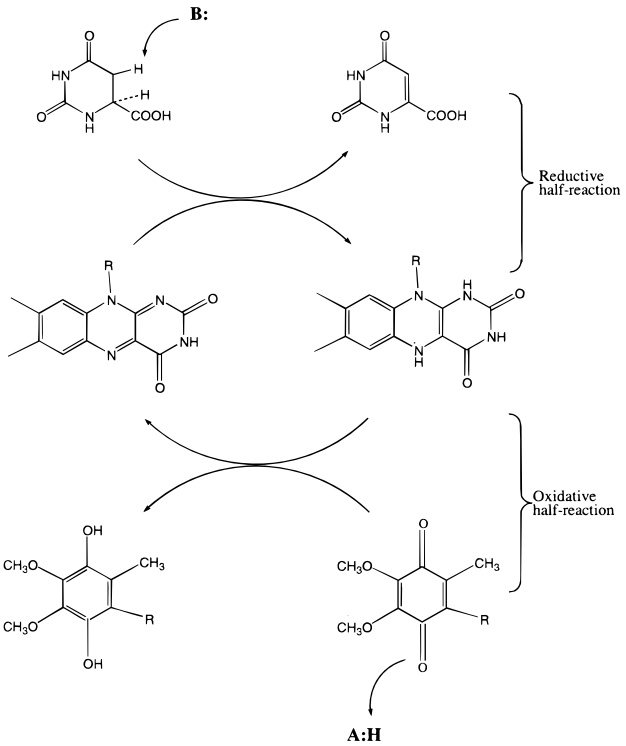
ABSTRACT: Chemical modification, mutagenesis, chemical rescue, and isotope effect studies are used to identify and probe the roles of several conserved amino acid groups in catalysis by human dihydroorotate dehydrogenase. Time- and pH-dependent inactivation of human dihydroorotate dehydrogenase by trinitrobenzenesulfonate implicates at least one critical lysyl residue in catalysis. Of four highly conserved lysines, only the cognate of Lys255 was previously suggested to have catalytic functionality. We now show that replacement of either Lys184 or Lys186 by mutagenesis does not impact, whereas substitution of Lys100 abolishes, enzymatic activity. However, activity is partially restored to K100C (or K100A) by inclusion of exogenous primary amines in reaction mixtures. This rescued activity saturates with respect to numerous amines and exhibits a steric discrimination reflected in $K_{d,(amine)}$ values. For all amines, rescued k_{cat} values were only ~10% of wild type and independent of amine basicity. K_M values for dihydroorotate and coenzyme Q_0 were similar to wild type. Thus, exogenous amines (as surrogates for Lys100) apparently complement a chemical, not binding, step(s) of catalysis, which does not entail proton transfer. In support of this postulate, solvent kinetic isotope effect analysis indicates that Lys100 stabilizes developing negative charge on the isoalloxazine ring of flavin mononucleotide during hydride transfer, as has been observed for a number of flavoprotein oxidoreductases. Ser215 of human dihydroarotate dehydrogenase (DHODase) was also studied because of its alignment with the putative active-site base Cys130 of *Lactococcus lactis* DHODase. Substantial retention of activity by S215C, yet complete loss of activity for S215A, is consistent with Ser215 serving as the active-site base in the human enzyme.

DHODase[1] (E. C.1.3.99.11) serves an essential function in nucleic acid synthesis by catalyzing the rate-limiting step in the biosynthesis of pyrimidine nucleotides, the coenzyme Q-dependent, flavin-mediated, oxidation of DHO (Scheme 1). Recent efforts at developing "antipyrimidine" drugs with antiinflammatory, immunosuppressive, and antiproliferative efficacy profiles have focused on human DHODase as a promising target enzyme for the treatment of diseases involving aberrant cell proliferation (*2*, *3*).

Although less is known concerning the molecular mechanism of the human enzyme, mechanistic studies on bovine liver DHODase have suggested that substrate oxidation involves active-site base-assisted proton abstraction of the C5-*pro-S* proton of DHO with concomitant (or subsequent) hydride transfer from C6 of substrate to the flavin (*4*) in the reductive half-reaction (Scheme 1). Crystallographic (*5*) and mutagenesis studies (*6*) of *Lactococcus lactis* DHODase suggested a potential role for Cys130 as an active-site base in this reaction. Interestingly, the cognate residue in mammalian DHODase and DHODase from Gram-negative bacteria is a conserved serine (Ser215 in human and Ser175 in *Escherichia coli*), whose significance in catalysis was demonstrated for the *E. coli* enzyme through site-directed mutagenesis. If true, the fact that two residues (cysteine and serine) with different physicochemical properties are most

Scheme 1: Reactions Catalyzed by Human DHODase



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[1] Abbreviations: DHODase, dihydroorotate dehydrogenase; DHO, dihydroorotate; Q_0, coenzyme Q_0; Ni−NTA, nickel−nitrilotriacetic acid; TNBS, trinitrobenzenesulfonate; DCIP, dichloroindophenol.

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likely involved in the same chemical step in evolutionarily divergent proteins suggested that the basicity of these residues must be modulated by their immediate microenvironment at the active site.

To gain deeper insight into the chemical mechanism of human DHODase, we have extended these initial studies to identify other catalytically important residues through a combined approach of chemical modification, site-directed mutagenesis, and validation of essentiality by recovery of impaired mutant activity by external compounds. Detailed kinetic analysis places these alterations within the context of specific facets of the DHODase catalytic mechanism. In the absence of structural data[2] we focused on Lys100,[3] Lys184, and Lys186, as well as Ser215. The lysyl residues are highly conserved in the primary amino acid sequence among family 2 DHODases (coenzyme Q reducing enzymes) and could fulfill a number of essential catalytic roles, including activation of the putative active-site serine, general acid catalysis required for the oxidative half-reaction (*4*), or electrostatic stabilization of reaction intermediate(s). Although our results demonstrate that Lys184 and Lys186 are inessential for catalysis, we show that Lys100 is critical for hydride transfer in the reductive half-reaction. Finally, results of replacing Ser215 by site-directed mutagenesis are consistent with this residue fulfilling the catalytically important role played by the Cys130 cognate residue in *L. lactis* DHODase.

MATERIALS AND METHODS

Site-Directed Mutagenesis. Site-directed mutagenesis was performed with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. Plasmid pKm, a derivative of pBR322 that contains the full-length human DHODase-encoding gene under the control of a *lac* promoter and in C-terminal linkage with a His6-encoding sequence, was used as template. All primers used for generating mutants were synthesized by Sigma Genosys. Mutations were confirmed by DNA sequencing at the DNA sequencing core facility of DuPont Pharmaceuticals Company.

Purification of Wild-Type and Mutant Enzymes. *E. coli* TAP330, a *pyrD*$^-$ derivative of BL21(DE3) (Novagen), was transformed with the plasmid encoding either wild-type or mutated DHODase, selected for kanamycin resistance, and cultured in LB broth containing 50 μg/mL kanamycin. Cultures were harvested after overnight growth in the presence of 0.1 mM isopropyl β-D-thiogalactoside, and the respective DHODase variants were purified on Ni−NTA affinity resin (Qiagen). All purification steps were carried out at 4° C. Cells were resuspended at a 1:1 (w/v) ratio of cells to lysis buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, and 10 mM imidazole), disrupted by sonication, and centrifuged at 10000*g* for 45 min. The supernatant was then treated with DNase (1 μg/mL) at 4° C for 30 min and loaded onto a 10 mL Ni−NTA column that had been preequilibrated with lysis buffer. After the column was washed with 100 mL of wash buffer (50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl, and 20 mM imidazole), the protein was eluted with a linear gradient (20−250 mM) of imidazole in the same buffer. The resultant wild-type enzyme was ~98% pure, as judged by Coomassie Blue staining of SDS−PAGE gels. However, mutant enzymes contained minor contaminants, which generally reduced their purity to 70−80% (based on visual inspection of the gels). Therefore, initial estimates of protein concentrations, as determined from a Bradford dye-binding assay (BioRad), were subsequently adjusted by quantitative Western blot analysis with primary antibody against His-tag epitope and known amounts of wild-type protein for calibration. Band intensities were determined by scanning densitometry (Molecular Dynamics, Sunnyvale, CA) and quantitated with ImageQuant 5.0 software (Molecular Dynamics).

CD Spectral Measurements. Wild-type and mutant enzymes were prepared in 20 mM potassium phosphate buffer, pH 7.0, at a final concentration of 0.65 mg/mL (wild type), 0.55 mg/mL (K100A), and 0.12 mg/mL (K100C). Room-temperature spectra were obtained in a 0.1-cm light path cuvette, on a Model 62DS Circular Dichroism Spectrometer (Aviv, Lakewood, NJ).

Activity Assay and Steady-State Kinetics. DHODase activity was measured by following DCIP reduction at 610 nm (ϵ = 20 mM^{-1} cm^{-1}), as a result of Q_0 reduction, in the presence of DHO. Standard assays contained 0.03 mM Q_0, 0.2 mM DHO, and 50 μM DCIP in 0.1 M Tris, pH 7.5; Q_0 and DHO concentrations were varied, where indicated. K_M and V_{max} values were calculated by fitting the data to a rectangular hyperbola with SigmaPlot software:

$$v = VA/(K + A) \quad (1)$$

where A is the variable substrate concentration.

Inactivation of the Wild-Type Human DHODase by TNBS. Concentrated enzyme (1.6 μM) was preincubated at room temperature with 0.2 mM TNBS. Aliquots were periodically removed and diluted (50-fold) into a standard activity assay mixture to monitor residual enzyme activity. The sensitivity of inactivation rate to pH was studied by preincubating the enzyme in a similar manner in buffers of various pH values (0.1 M Tris, pH 7.5−9.5; 0.1 M CAPS, pH 10−12). Identical reaction mixtures that lacked TNBS served as controls.

Rescue of the Lys100 Mutant Activity by External Amines. The effect of added primary amines upon catalytic rate was studied in the standard activity assay by adding different amounts of amines (from 2 M stocks adjusted to pH 7.5) and maintaining a constant ionic strength of 0.2 M by addition of NaCl (or tetramethylammonium chloride). Because the rescue of activity in K100C by external amines obeyed saturation kinetics with respect to the amine rescuers, eq 1 was used to determine k_B, the second-order rate constant for rescue, as $k_{cat}/K_{d(amine)}$ (*7*). To consider the effect of amine basicity on rescued activity, calculated k_B values for each amine were corrected for the free amine concentration at a given pH (pH 7.5 in all assays):

$$k_B = k_B' [\text{amine}]_{total}/(1 + (H^+/K_a)) \quad (2)$$

where k_B is the experimentally determined bimolecular rate

[2] Crystal structures of *E. coli* (Sine Larsen, personal communication) and human DHODases (*1*) were reported during the preparation of this manuscript that have subsequently provided insight into the spatial disposition of specific amino acids within the enzyme's active sites.

[3] Unless otherwise indicated, residue numbers refer to the sequence of full-length human DHODase.

constant and k_B' is corrected for free amine concentration at a given pH. Analysis of molecular volume effects were as described by Toney and Kirsch (*8*).

Solvent Kinetic Isotope Effects. Reaction mixtures containing 0.1 M Tris buffer (pH 7.5), fixed-saturating substrate (0.03 mM Q_0 when DHO was varied; 0.2 mM DHO when Q_0 was varied), and DCIP (50 μM) were prepared as 10× stock solutions and then diluted with H_2O or D_2O. Variable substrates were separately prepared as 10× stock solutions and likewise diluted with H_2O or D_2O. Solvent kinetic isotope effects were calculated with the programs of Cleland (*9*). Data from the experiments that yielded solvent kinetic isotope effect on *V* only or on both *V* and *V*/*K* were fit to eqs 3 and 4, respectively, where *A* is the substrate concentration, F_i is fraction of the label, and E_V and $E_{V/K}$ are the isotope effect minus 1 on *V* and *V*/*K*, respectively.

$$v = VA/[K + A(1.0 + F_i E_v)] \quad (1)$$

$$v = VA/[K\,(1.0 + F_i\,E_{V/K}) + (1.0 + F_i\,E_v)] \quad (4)$$

RESULTS

Identification of Essential Lysine Residue(s). Human DHODase is inactivated by the lysine-specific agent TNBS (*10*) in a time- and pH-dependent manner (data not shown). Loss of enzyme activity was below detection limits during preincubation with TNBS at neutral pH (<1% loss of activity over a 30-min incubation period). However, inactivation rates increased significantly with increasing pH (20-fold increase in $k_{inact.}$ from pH 9.5 to 12) and demonstrated complex kinetics, consistent with the enzyme containing at least one catalytically important lysine residue with a basic pK_a and nucleophilicity normally expected for a lysyl ϵ-amine (*11*).

To further investigate the potential roles of lysine residues in the catalytic turnover of human DHODase, we compared the amino acid sequences of several Gram-negative bacteria (since they have the highest degree of homology) and mammalian DHODases (Figure 1). In addition to Lys255, which was proposed through structural considerations to be requisite for flavin binding (*12*), we noted three other highly conserved lysine residues (Lys100, Lys184, and Lys186) and subsequently mutated them individually to alanine or cysteine for functional analysis. Lysates of cells expressing mutated or wild-type DHODases were analyzed by SDS−PAGE to assess the expression level of each protein. All of the mutant constructs yielded comparable amounts of recombinant proteins, as judged by Western blot analysis. Thus, relative activities were determined in crude extracts and expressed as percentages of the wild-type enzyme activity corrected for the total protein concentration. As seen in Table 1, Lys184 and Lys186 replacements had minimal effect on the activity of the enzyme. However, substitutions at position 100 resulted in complete loss of enzymatic activity.

Purification, Activity, and Structural Integrity of K100A and K100C. To further assess the levels of catalytic impairment effected by replacement of the lysyl side chain at position 100, K100A and K100C variants (as well as wild type) were purified by a single-column protocol that exploited the introduced C-terminal His-tag sequence. Since both of the mutated proteins were devoid of enzymatic activity, detection was based on SDS−PAGE analysis. Bands having the same mobility as wild-type DHODase were pooled and subjected to N-terminal amino acid sequence analysis, which confirmed their identities as DHODases.

Even following purification, no activity was observed for either mutant enzyme when assayed at substrate levels up to 50 times the K_M values exhibited by wild-type enzyme for DHO and Q_0 and at protein concentrations at which ~0.1% of activity should have been detectable. To address whether the impaired activities of the mutants resulted from improper folding or global conformational changes introduced by the amino acid substitutions, we compared the circular dichroic (CD) spectra of the mutant versus wild-type enzymes. Virtually identical spectra were obtained for WT and mutant proteins (data not shown). Thus, substitution of Lys100 does not appear to affect the global secondary structure of the enzyme, and therefore conformational changes caused by the mutation, if any, are minor.

Chemical Rescue of K100A and K100C Activity by External Amines. Noncovalent chemical rescue (*8*) of inactive K100A and K100C DHODases was employed to validate the integrity of mutant proteins and gauge the essentiality and catalytic enhancement provided by the affected side chains. Although substantial activity (up to 10%) was restored by inclusion of primary amines in assay mixtures containing K100C or K100A, we focused our further studies on the former mutant. Diagnostic of enzymatic catalysis, the rescued activity was proportional to protein concentration (data not shown) and exhibited Michaelis−Menten kinetics with respect to substrates. In the presence of 0.2 M methylamine, the k_{cat} for rescued K100C was about 8% that of wild type, while K_m values for DHO and Q_0 were similar to that of wild type (Table 2). Values obtained for wild-type enzyme in the presence of amine increased moderately (7-fold or less) relative to those measured in the absence of external amine (K_{DHO} = 12 μM, K_{Q0} = 5 μM, and k_{cat} = 8.5 s^{-1}). No substantial conversion of substrate was observed in control reactions containing amine but lacking enzyme.

A variety of primary amines were tested for their ability to restore activity to K100C through noncovalent complementation (Table 3). The rescued mutant activities obeyed saturation kinetics with respect to all amines tested, whereas amines very modestly impacted the catalytic capacity of wild-type enzyme (Figure 2). Rescue was therefore analyzed according to k_{cat} (maximal turnover at saturating amine), K_d (half-saturating level of amine), and k_B' [corresponding to $k_{cat}/K_{d(free\ amine)}$, the bimolecular rate constant, or efficiency of rescue] values obtained under these conditions (Table 3). Of note, no significant differences in k_{cat} were observed with amines of different pK_a values (Table 3). Furthermore, reactions containing 2-fluoroethylamine (pK_a = 9.0) at pH 7.5 (3% free base) and 8.5 (32% free base) provided nearly identical kinetic parameters (data not shown). These results demonstrate that deprotonation of the amine itself does not accelerate the reaction, thus excluding a direct role of amine in acid−base catalysis.[4]

Hence, differences in k_B' were primarily due to different K_d values for the various amines, suggesting that complementation of the catalytic defect by external amines is limited by amine binding and proper positioning at the site voided by the mutation. Accordingly, the rescue efficiency was sensitive to steric factors, with a good linear relationship observed between amine molecular volume and either k_B' [$k_{cat}/K_{d(free\ amine)}$] for amines of similar pK_a value (slope *V* =

```
          1                                                  50
 Ecoli    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~MYYPF VRKALFQ..L
  Salm    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~MYYPF VRKALFQ..L
Haemop    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~MYQL FRHGIFQ..M
Helico    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~MLYSL VKKYLFS..L
 Human    KLPWRHLQKR AQDAVIILGG GGLLFASYLM ATGDERFYAE HLMPTLQGLL

          51                                                 100
 Ecoli    DPERAHEFTF QQLRRITGTP .FEALVRQKV PAK...PVN. CMGLTFKNPL
  Salm    DPERAHEFTF QQLRRITGTP .LEALVRQKV PTK...PVT. CMGLTFKNPL
Haemop    DAEKAHNFTI QCL.KLAGNP LFQPILKSLI HAPKGFPKT. VMGVNFPNPI
Helico    DAEDAHEKVC KILRMLSSSP FLCNLIDSQW GYQNPKLENE ILGLHFPNPL
 Human    DPESAHRLAV ....RFTSL. ...GLLPRAR FQDSDMLEVR VLGHKFRNPV

          101                                                150
 Ecoli    GLAAGLDKDG ECIDALGAMG FGSIEIGTVT PRPQPGNDKP RLFRLVDAEG
  Salm    GLAAGLDKDG ECIDALGAMG FGSLEIGTVT PRPQPGNDKP RLFRLVDAEG
Haemop    GLAAGADKNG DAIDGFGALG FGFLELGTVT PVAQDGNAKP RQFRLIEAEG
Helico    GLAAGFDKNA SMLRALMAFG FGYLEAGTLT NEAQVGNERP RLFRHIEEES
 Human    GIAAGFDKHG EAVDGLYKMG FGFVEIGSVT PKPQEGNPRP RVFRLPEDQA

          151                                                200
 Ecoli    LINRMGFNNL GVDNLVENVK ....K.AHY. .DGV.LGINI GKNKDTPVEQ
  Salm    LINRMGFNNL GVDNLVENVK ....K.AHF. .DGI.LGINI GKNKDTPVEN
Haemop    IINRNGFNNN GIDYLIENVK ....N.ARY. .KGV.IGINI GKNKFTSLEQ
Helico    LQNAMGFNNY GAILGVRSFK ....RFAPY. .KTP.IGINL GKNKHIEQAH
 Human    VINRYGFNSH GLSVVEHRLR ARQQKQAKLT EDGLPLGVNL GKNK.TSVD.

          201                                                250
 Ecoli    GKDDYLICME KIYAYAGYIA INISSPNTPG LRTLQYGEAL DDLLTAIKNK
  Salm    GKDDYLICME KVYAYAGYIA INISSPNTPG LRTLQYGDAL DDLLTAIKNK
Haemop    GKDDYIFCLN KAYNYAGYIT VNISSPNTPD LRQLQYGDYF DDLLRSIKDR
Helico    ALEDYKAVLS KCLNIGDYYT FNLSSPNTPN LRDLQNKAFV HELFCMAKE.
 Human    AAEDYAEGVR VLGPLADYLV VNVSSPNTAG LRSLQGKAEL RRLLTKVLQE

          251                                                300
 Ecoli    QNDLQAMHHK YVPIAVKIAP DLSEEELIQV ADSLVRHNID GVIATNTTLD
  Salm    QNDLQAIHHK YVPVAVKIAP DLCEEELIQV ADSLLRHNID GVIATNTTLD
Haemop    QAILANQYNK YVPIAVKIAP DLTESELVQI ADTLVRHKMD GVIATNTTIS
Helico    ......MTHK ..PLFLKIAP DLETDDMLEI VNSAIGAGAH GIIATNTTID
 Human    RDGLRRVHRP ..AVLVKIAP DLTSQDKEDI ASVVKELGID GLIVTNTTVS

          301                                                350
 Ecoli    RSLVQGMKNC DQTGGLSGRP LQLKSTEIIR RLSLELNGRL PIIGVGGIDS
  Salm    RSLVQGMKNC QQTGGLSGRP LQLKSTEIIR RLSQELKGQL PIIGVGGIDS
Haemop    RDTVTGMKNA EQQGGLSGKP LQHKSTEIIK RLHQELKGQI PIIGSGGIDG
Helico    KSLVFAPK.. .EMGGLSGKC LTKKSREIFK ELAKAFFNKS VLVSVGGISD
 Human    RPAGLQGALR SETGGLSGKP LRDLSTQTIR EMYALTQGRV PIIGVGGVSS

          351                                                400
 Ecoli    VIAAREKIAA GASLVQIYSG FIFKGPPLIK EIVTHI~~~~ ~~~~~~~~~~
  Salm    VIAAREKIAA GATLVQIYSG FIFKGPPLIK EIVTHI~~~~ ~~~~~~~~~~
Haemop    LQNAQEKIEA GAELLQVYSG LIYHGPKLVK ELVKNIK~~~ ~~~~~~~~~~
Helico    AKEAYERIKM GASLLQIYSA FIYNGPNLCQ NILKDLVKLL QKDGFLSVKE
 Human    GQDALEKIRA GASLVQLYTA LTFWGPPVVG KVKRELEALL KEQGFGGVTD

          401
 Ecoli    ~~~~~~~~
  Salm    ~~~~~~~~
Haemop    ~~~~~~~~
Helico    AIGADLR~
 Human    AIGADHRR
```

FIGURE 1: Amino acid sequence alignment of family 2 DHODases. Lys100, Lys184, Lys186, Lys225 and S215 are shown in boldface type. Sequence abbreviations: Ecoli = *Escherichia coli* DHODase; Salm = *Salmonella typhimurium* DHODase; Haemop = *Haemophilus influenzae* DHODase; Helico = *Helicobacter pylori* DHODase; Human = full-length human DHODase. Note that the numbering scheme is relative to the composite sequence.

-0.009 ± 0.001) (Figure 3) or k_B [$k_{cat}/K_{d(total\ amine)}$] for all amines tested (slope $V = -0.0087 \pm 0.002$) (inset to Figure 3).

Solvent Kinetic Isotope Effects. The catalytic cycle of DHODase involves at least one proton abstraction in the reductive half-reaction (base-assisted deprotonation of C5

Table 1: Comparison of Activities for Wild-Type and Mutant Human DHODases

enzyme	relative activity[a] (%)
WT	100
K100A	<0.1
K100C	<0.1
K184A	104
K184C	85
K186A	95
K186C	90
S215A	<0.1
S215C	25

[a] Values for cell-free extracts were determined in parallel reactions containing saturating concentrations of substrates (0.2 mM DHO, 0.03 mM Q_0) relative to values for wild-type DHODase in the absence of amines. All values were normalized to the level of DHODase present in the sample, as described in under Materials and Methods.

Table 2: Kinetic Parameters for the Reactions Catalyzed by Wild-Type and K100C Human DHODases in the Presence of 0.2 M Methylamine[a]

enzyme	K_{DHO} (μM)	K_{Q0} (μM)	k_{cat}, s^{-1}
WT	30.0 ± 2.1	36.0 ± 6.0	6.4 ± 0.1
K100C	41.5 ± 3.3	36.7 ± 5.5	0.54 ± 0.08

[a] Standard errors represent the values obtained by fitting the data to a rectangular hyperbola (SigmaPlot).

of DHO) and most likely acid-assisted protonation of reduced quinone in the oxidative half-reaction (*4*) (Scheme 1). To study the rate-limiting proton transfers in the reactions catalyzed by wild-type and rescued mutant DHODase, we measured kinetic parameters in H_2O and D_2O solutions (Figure 4). A large solvent kinetic isotope effect ($D_2OV = 3.2 \pm 0.1$) was observed on V_{max} when DHO was varied, with no effect on V/K_{DHO} (Table 4). On the other hand, a solvent kinetic isotope effect of 2.7 ± 0.3 was observed for V/K_{Q0}, indicating that a proton transfer in the oxidative half-reaction is mostly rate-limiting. Similarly, reduction of the quinone substrate (via oxidation of DHO) was found to be rate-limiting for bovine liver DHODase (*4*). No solvent kinetic isotope effect was observed on either V or V/K_{DHO} when K100C was used as a catalyst in the presence of 0.2 M methylamine, suggesting that the rate-limiting step(s) is not the same as for the reaction catalyzed by wild-type enzyme. Nevertheless, a remaining small solvent isotope effect on V/K_{Q0} (1.9 ± 0.2) suggests that the rate limitation of the oxidative half-reaction was not completely overcome by mutation of Lys100. Identical solvent kinetic isotope effects were measured with wild-type enzyme in the presence and absence of methylamine, demonstrating that the altered solvent kinetic isotope effects with rescued K100C did not derive artifactually from external amine.

[4] However, we could still construct a classic Brønsted plot ($\log k_B'$ versus pK_a) that exhibited a well-correlated positive slope of $\beta \sim 0.7$. Although this cannot be explained by traditional acid/base interpretations, it nonetheless suggests an apparent free energy relationship. The reasons for this relationship are currently unclear, but again it is solely reflected in the varied K_d values for the amines and may be unique to the rescue of position-100 mutants of DHODase. Of note, this result clearly suggests a potential source of misinterpretation in the application of chemical rescue to enzymatic systems (especially those that exhibit rate saturation with respect to amine) and demonstrates the need for careful discrimination of the impact of individual kinetic constants upon observed trends.

Analysis of Ser215-Replaced Mutants. Mutation of Ser215 to alanine resulted in catalytically inactive protein. However, the S215C mutant displayed about 25% of wild type activity (Table 1), which is in good agreement with results previously obtained for *E. coli* DHODase (*13*).

DISCUSSION

The pyrimidine requirement of rapidly proliferating cells usually exceeds their salvage capacity. Hence, de novo pyrimidine biosynthesis is of critical importance to cell growth. Oxidation of DHO by DHODase is a rate-limiting step in UMP biosynthesis, and this enzyme has been demonstrated to be a molecular target for the active metabolite of leflunomide (A771726) (*14*). Despite intensive interest in mammalian DHODase, a limited amount of detailed mechanistic information, in particular the identification of active-site amino acid residues that are important for catalysis, is available for use in the discovery of novel therapeutic reagents targeted toward this enzyme. Kinetic isotope and pH studies on bovine liver mitochondrial DHODase indicate that both general acid- and general base-assisted catalysis occurs during the enzyme's distinct half-reactions (*4*). Alanine scanning mutagenesis identified several histidine residues that are critical for enzymatic activity, but the functional role(s) of these residues was were not revealed by these studies (*15*). Meanwhile, solution of the three-dimensional structure of bacterial DHODase A identified a cysteine (Cys130) as the residue most properly positioned to function as the general base, due to its proximity and interactions with DHO (*5*). Even though the homology between enzymes from Gram-positive bacteria and mammals is extremely modest, the active site region is highly conserved, with the cysteine substituted by serine (Ser215 in human DHODase).

As suggested from an earlier study of *E. coli* DHODase (*13*), our current results with the human enzyme are more consistent (though not definitive) with Ser215 acting as an active-site base rather than solely as a partner in hydrogen-bonding interactions. In particular, retention of significant (25%) activity upon cysteinyl replacement (with complete loss of activity upon alanyl replacement) may reflect retention of potential general-base character, whereas the same replacement might be expected to have a greater impact upon hydrogen-bonding interactions.

Basicity and nucleophilicity of catalytic serine residues in a number of other proteins are augmented by an adjacent, positively charged histidine or lysine [or arginine, in the case of pig heart aconitase (*16*)]. There is only one histidine in the amino acid sequence of human DHODase (His40) which is also conserved among other serine-containing DHODases. However, mutation of this residue to alanine (His11 in the numbering system used for the truncated version of the enzyme) does not change the enzyme activity (*15*). In addition, our data and subsequent crystallographic results[2] are inconsistent with lysyl activation of the serine. In particular, the three-dimensional crystal structure of human DHODase with bound inhibitor shows that Ser215 is properly oriented to abstract the C5-*pro*-S proton of DHO and may be activated as a general base due to an adjacent phenylalanine and a hydrogen-bond relay consisting of water and a conserved threonine residue (*1*). The functionality of

Table 3: Kinetic Constants for the Amine-Assisted Catalysis of Human DHODase K100C Mutant[a]

amine	pK_a[b]	molecular volume[b] (Å^3)	k_{cat}[c] (s^{-1})	K_d[c,d] (mM)	k_B'[c,e] (10^3, M^{-1} s^{-1})
methyl-	10.6	42.1	0.24 ± 0.02	37.9 ± 5.1	7.91 ± 0.30
ethyl-	10.6	60.9	0.20 ± 0.03	39.7 ± 2.2	6.43 ± 1.00
propyl-	10.5	79.8	0.28 ± 0.02	82.5 ± 6.9	3.38 ± 0.20
butyl-	10.6	98.7	0.27 ± 0.03	136 ± 10	2.47± 0.10
ammonia	9.2	23.2	0.22 ± 0.02	65.8 ± 8.1	0.17 ± 0.01
2-fluoroethyl-	9.0	64.4	0.25 ± 0.01	83.4 ± 7.7	0.097 ± 0.001
2,2,2-trifluoroethyl-	5.7	71.6	0.26 ± 0.02	105 ± 10	0.0025 ± 0.0005

[a] Assays were carried out with 0.2 mM DHO and 0.03 mM Q_0 as described under Materials and Methods. [b] Values from ref *8*. [c] Standard errors represent the values obtained by fitting the data to a rectangular hyperbola (SigmaPlot). [d] Value for total amine concentration. [e] Value corrected for free amine concentration.

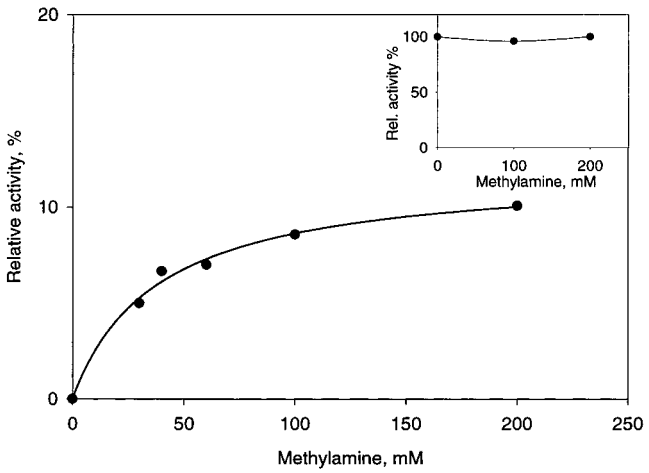


FIGURE 2: Recovery of K100C mutant activity by inclusion of methylamine. The solid line represents the best fit obtained by nonlinear regression to the Michaelis−Menten equation. (Inset) Effect of the added methylamine on the wild-type enzyme activity. Ordinate values are relative to the k_{cat} for wild-type enzyme measured in the absence of methylamine. Methylamine concentrations are shown as total (free base plus protonated species). Reaction conditions were as described in under Materials and Methods.

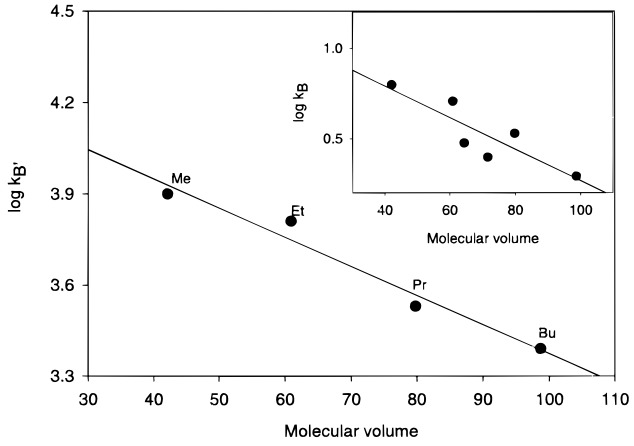


FIGURE 3: Effects of amine molecular volume on rescue of K100C with amines of similar pK_a values (K_d corrected for free amine). (Inset) Correlation between the rescue efficiency and molecular volume, K_d for the total amine. Data were taken from Table 1, including 2-fluoroethyl- (pK_a 9.0) and 2,2,2-trifluoroethyl- (pK_a 5.7) amines. Ammonia was excluded from the analysis because of deviation from the trend attributable to solvation effects.

this potential proton-transfer chain awaits further experimental validation.

Although far from conclusive, our initial chemical modification results with TNBS suggested that one or more lysines may be critical for catalysis in DHODase. Amino acid alignments of DHODases from *Salmonella typhimurium*, *E. coli*, *Haemophilus influenzae*, *Helicobacter pylori*, and human indicate four conserved Lys residues. One of these (Lys255) is also conserved among DHODases from Gram-positive bacteria where it forms a hydrogen bond with N(1) of the isoalloxazine ring of flavin mononucleotide (FMN) and is important in flavin binding (*12*). We have extended this analysis to the remaining three conserved lysyl residues in human DHODase (Lys100, Lys184, and Lys186), to find that only Lys100-replaced mutants exhibit a substantial loss of activity, suggestive of its essentiality for catalysis.

The contribution of the ϵ-amine side chain of Lys100 to catalysis was further delineated by the restoration of activity conferred upon the K100C mutant by exogenously supplied amines. As first applied by Toney and Kirsch (*8*) in studies of Lys258 of aspartate aminotransferase and since extended to a number of enzymes (for examples see refs *7* and *17*−*20* and citations therein), functional complementation of impaired mutant enzymes ("chemical rescue") allows physicochemical analysis of the catalytic impact of an amino acid residue through more subtle alteration of functional properties than is attainable by site-directed mutagenesis alone. For DHODase, the substantial, yet incomplete, restoration of activity to K100C clearly denotes the essentiality of a properly oriented ϵ-amine at position 100 for catalytic efficiency and supports the spectroscopic evidence for structural integrity of the purified mutants. Importantly, the Michaelis constants for DHO and Q_0 in the rescued mutant approximate wild-type values, despite the ~10-fold lower k_{cat}. Therefore, the primary effect of Lys100 on the impacted rate-determining step must derive from transition-state (i.e., catalytic) interactions rather than ground-state (substrate) stabilization. Nevertheless, the maximal extent of rescued activity was independent of amine pK_a, invariant between a pH range sufficient for 10-fold difference in ionization state of 2-fluoroethylamine, but clearly dependent upon amine molecular volume. Together these results suggest that the amine (and by extension Lys100 in wild-type enzyme) does not promote a kinetically significant direct proton transfer but rather effects catalysis through sterically restricted hydrogen-bonding interactions related to transition-state stabilization.

During the course of our study, a three-dimensional structure of the *E. coli* DHODase has been solved (Sine Larsen, personal communication) that is consistent with a role for Lys100 in transition-state stabilization. In this structure Lys66 (corresponding to Lys100 in human enzyme) occupies the same space as Lys43 in *L. lactis* DHODase A

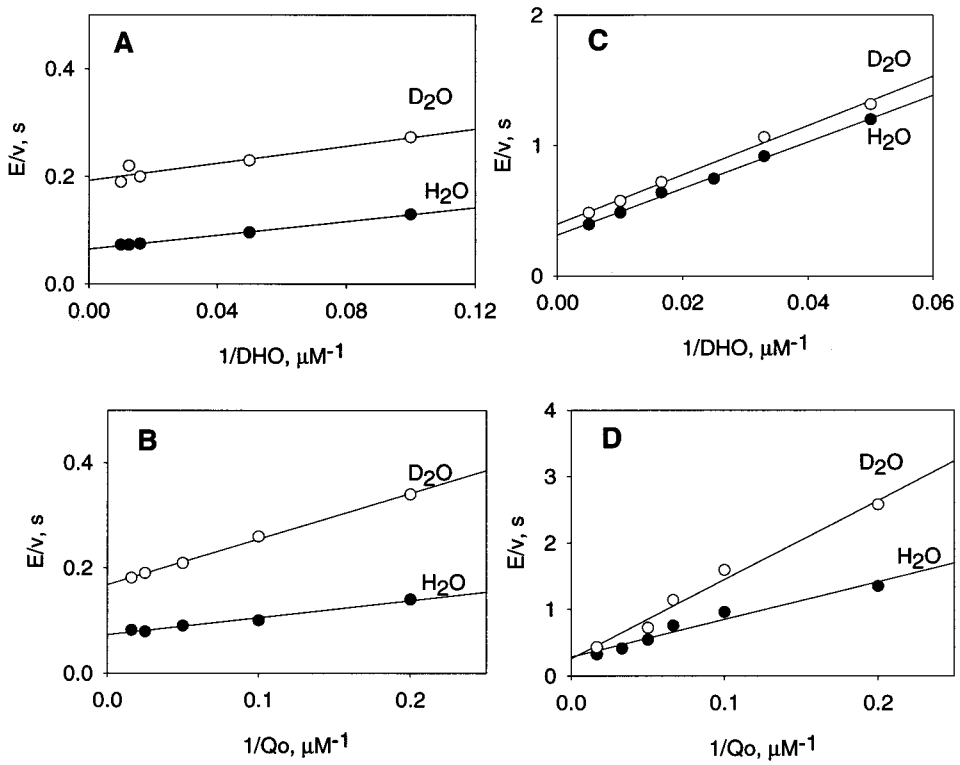
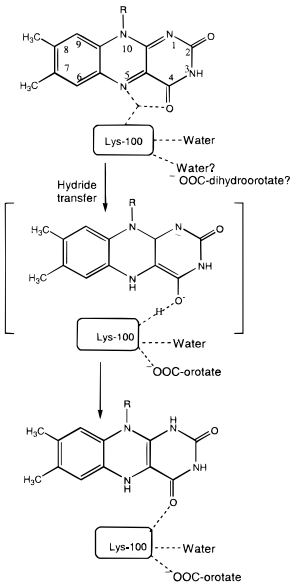


FIGURE 4: Solvent kinetic isotope effects for the reactions catalyzed by wild type (panels A and B) and K100C mutant (panels C and D) human DHODases in the presence of 0.2 M methylamine. Calculated values are presented in Table 4.

Scheme 2: Proposed Role of Lys100 in Charge Stabilization during the Reductive Half-Reaction[5]



(even though this functionality could not be predicted from amino acid alignment). As shown in the structure of the *L. lactis* DHODase, the ϵ-amine of Lys43 is involved in a bifurcated hydrogen bond with O4 and N5 of the isoalloxazine ring of the FMN and two linear hydrogen bonds to two water molecules (*12*). These hydrogen bonds could reflect the transition-state interactions suggested by the chemical rescue experiments. Upon orotate binding in the active site, one of the water molecules is displaced by the O8 atom of the orotate carboxylate (*5*). Interestingly, mutation of Lys43 in *L. lactis* DHODase diminished, but did not completely abolish, enzymatic activity (*6*). Our

Table 4: Solvent Kinetic Isotope Effects on the Reaction Catalyzed by Wild-Type and K100C Human DHODase in the Presence of 0.2 M Methylamine

enzyme	^{D2O}V	$^{D2O}V/K_{DHO}$	$^{D2O}V/K_{Q0}$
WT			
vary DHO	3.2± 0.1	1.0 ± 0.1	
vary Q_0	2.4 ± 0.2		3.1 ± 0.3
K100C			
vary DHO	1.2± 0.2	1.1 ± 0.2	
vary Q_0	0.98 ± 0.3		1.9 ± 0.2

inability to detect any activity with either the K100A or K100C mutant of human DHODase (in the absence of exogenous amines) suggests a more critical role for this residue in the human enzyme than it plays in the *L. lactis* enzyme.

Finally, loss of a solvent kinetic isotope effect on *V* in the reductive half-reaction catalyzed by mutant enzyme implies that hydride transfer is significantly slowed. A number of flavoprotein disulfide reductases have a conserved lysine residue hydrogen-bonded to O4 and N5 of the isoalloxazine

[5] The actual hydrogen-bonding pattern of Lys100 upon DHO binding is ambiguous. In the absence of bound substrate or product, Lys100 forms a bifurcated bond to the isoalloxazine ring and interacts with two well-ordered water molecules, as seen in *L. lactis* DHODase A crystal structure (*12*). On the other hand, in the enzyme−product complex (*1*), the carboxylate of orotate replaces one water molecule and is within hydrogen-bonding distance of Lys100. A different hydrogen-bonding pattern should be expected for bound substrate because of the axial carboxylate conformation of (*S*)-DHO (becoming too distant to form a hydrogen bond with Lys100), as has been suggested by molecular modeling of DHO in the active site of *L. lactis* DHODase A (*5*). Hence, we depict a conformation that still maintains the second bound water, but we acknowledge that an alternate constellation of hydrogen bonds may exist for Lys100 in the initial enzyme−DHO complex.

(*21*, *22*), and it has been suggested that such an interaction in glutathione reductase influences the redox potential of the flavin cofactor (*23*). Modulation of the physicochemical properties of the flavin in lipoamide dehydrogenase was studied by mutagenesis (*24*), leading to the conclusion that lysine (which is in an ion pair with glutamate) stabilizes the developing negative charge on the isoalloxazine ring, thus stabilizing the transition state for hydride transfer. Within this context, we propose that our chemical rescue analysis and solvent kinetic isotope data, in conjunction with information gained from emerging DHODase structures and structures of related enzymes, favor the hypothesis that the amino functionality is critical at position 100, most likely for hydrogen bonding and stabilization of the developing charge on the isoalloxazine ring during hydride transfer (Scheme 2),[5] but not as a direct mediator of proton transfer.

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