Biochemical Pharmacology, Vol. 54, pp. 459–465, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(97)00197-4

Histidine to Alanine Mutants of Human Dihydroorotate Dehydrogenase

IDENTIFICATION OF A BREQUINAR-RESISTANT MUTANT ENZYME

*June P. Davis and Robert A. Copeland**

INFLAMMATORY DISEASES RESEARCH, THE DUPONT MERCK RESEARCH LABORATORIES, WILMINGTON, DE 19880-0400, U.S.A.

ABSTRACT. Dihydroorotate dehydrogenase (DHODase) is the rate-limiting enzyme of the mammalian *de novo* pyrimidine biosynthesis pathway, and is the molecular target of the antiproliferative, immunosuppressive compound brequinar sodium (BQR). We have shown previously that the activity of the recombinant human enzyme displays pH and diethylpyrocarbonate sensitivities that implicate a critical role for one or more histidine residues in catalysis [Copeland *et al.*, *Arch Biochem Biophys* **323:** 79–86, 1995.]. Here we report the results of alanine scanning mutagenesis for each of the 8 histidine residues of the recombinant human enzyme. In most cases, the replacement of histidine by alanine had little effect on the K_m values of the two substrates, dihydroorotate and ubiquinone, or on the overall k_{cat} of the enzymatic reaction. Replacement of H71, H129, and H364 by alanine, however, completely abolished enzymatic activity. The loss of activity for the H71A mutant was unexpected, since this residue is not conserved in the homologous rat enzyme; in the rodent enzyme this residue is an asparagine. Replacement of H71 by asparagine in the human enzyme led to a full recovery of enzymatic activity, indicating that a histidine is not required at this position. Replacement of H26 by alanine led to about a 10-fold reduction in catalytic activity relative to the wild-type enzyme, with no significant perturbation of the substrate K_m values. This mutant was, however, at least 167-fold less sensitive to inhibition by the noncompetitive inhibitor BQR. While the wild-type and other mutant enzymes displayed IC_{50} values for BQR inhibition between 6 and 10 nM, the H26A mutant was inhibited less than 25% at concentrations of BQR as high as 150 nM. These data suggest that H26 plays an important role in BQR binding to the enzyme. BIOCHEM PHARMACOL **54**;4:459–465, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. dihydroorotate dehydrogenase; brequinar; pyrimidine biosynthesis; immunosuppression; antiproliferative; mutagenesis

Immunoregulatory therapies provide a means of treating a variety of autoimmune diseases as well as for controlling immune-based rejection after organ transplantation. Although several immunosuppressive agents are presently in clinical use, current therapies all suffer from severe side-effects associated with the molecular mechanisms of action for these drugs. Within the last 5 years, there has emerged a new class of immunosuppressive compounds that act by inhibiting immune cell proliferation. Two examples of this class of compounds are BQR† and leflunomide. Both of these compounds, and related analogs of each, have been shown to be effective in blocking organ rejection in animal models of allograft and xenograft transplantation, and in animal models of autoimmune diseases and arthritis [1–5]. Leflunomide also has been demonstrated to have clinical efficacy in treating human rheumatoid arthritis [3].

Biochemical studies from our laboratory and others [6–9] have demonstrated recently that both BQR and the active metabolite of leflunomide share a common molecular target, the enzyme DHODase. Inhibition of DHODase by these compounds blocks *de novo* pyrimidine biosynthesis. This, in turn, leads to inhibition of proliferation for cells, such as immune cells, in which the pyrimidine salvage pathway cannot compensate adequately for the loss of *de novo* synthesis. Thus, inhibition of pyrimidine biosynthesis, particularly through targeting of DHODase, is a novel and effective mechanism for immunosuppression.

To better exploit DHODase as a target for immunoregulatory therapy, it is important to characterize the structural determinants of catalytic activity and inhibitor binding for this enzyme. Towards this end, our laboratory has reported the first expression and purification of an active recombinant form of human DHODase [10]. In the course of that work, we demonstrated that the recombinant human en-

* Corresponding author: Dr. Robert A. Copeland, Inflammatory Diseases Research, The DuPont Merck Research Laboratories, P.O. Box 80400, Wilmington, DE 19880-0400. Tel. (302) 695-7173; FAX (302) 695-7873; E-mail: Copelara@lldmpc.dnet.dupont.com

† *Abbreviations:* BQR, brequinar sodium; DCIP, 2,6-dichlorophenolindophenol; DHO, L-dihydroorotic acid; DHODase, dihydroorotate dehydrogenase (L-5,6-dihydroorotate:ubiquinone oxidoreductase; EC 1.3.3.1); dNTPS, deoxyribonucleotides; IPTG, isopropyl-β-D-thiogalactoside; NBT, nitroblue tetrazolium; PCR, polymerase chain reaction; and Q_6, ubiquinone-30.

Received 6 January 1997; accepted 26 February 1997.

zyme, and the natural enzyme isolated from human liver mitochondria, display pH–activity profiles that suggest the participation of one or more histidine residues in the mechanism of catalytic turnover. We further demonstrated that the enzyme could be inhibited by the histidine-selective covalent modifier diethylpyrocarbonate. Taken together, these results imply an important role for histidine in catalysis by human DHODase.

To investigate further the role of histidine residues in DHODase function, we mutated each of the 8 histidines of the recombinant human enzyme individually to alanine and assessed the effects of these mutations on enzyme activity, substrate binding, and BQR inhibition. We report the results of these studies here. In the course of these studies, we have identified three mutants that effectively eliminate enzymatic activity. Additionally, we have identified an enzymatically active mutant that is resistant to inhibition by BQR. This unexpected result provides the first structural insights into the location of the BQR binding site on the human enzyme.

MATERIALS AND METHODS

Materials

Oligonucleotides were purchased from OligoTherapeutics Inc. (Wilsonville, OR) and BioSynthesis (Lewisville, TX). PCR products were purified using Qiaquick-spin PCR purification columns from Qiagen (Santa Clarita, CA) and a GeneClean Kit from Bio101, Inc. (La Jolla, CA). The pET11a vector was from Novagen (Madison, WI). Plasmids were purified using either the Qiaprep-8 mini-prep kit or Qiatip-100 Midi prep columns by Qiagen. Ultma DNA polymerase was purchased from Perkin–Elmer (Foster City, CA). The enzymes *Bam*HI, *Nhe*I, *Sal*I, and T4 DNA ligase were from Gibco (Gaithersburg, MD); *Nde*I and *Sac*II were from New England Biolabs (Beverly, MA). Luria Bertani broth (LB) was from Gibco. L-Dihydroorotic acid, coenzyme Q_6, NBT, DCIP, Tris, Triton X-100, ampicillin, IPTG, dNTPS, and uridine were purchased from Sigma (St. Louis, MO). BQR ([6-fluoro-2-(2′-fluoro-1,1′-biphenyl-4-yl)-3-methyl-4-quinoline carboxylic acid sodium salt]; NSC 368390; DuP785) was synthesized at DuPont Merck.

Construction of Mutant DHODases

An N-terminally truncated cDNA for human DHODase (Fig. 1; Ref. 10) was PCR amplified from the "PYRDH" clone [11], which was provided by Dr. Michele Minet (Centre de Genetique Moleculaire, Gif sur Yvette, France). Primers 1 and 6 (Table 1), containing initiation and termination codons, respectively, were used to provide *Nde*I and *Bam*HI restriction sites for subcloning the gene for DHODase into the Novagen pET11a plasmid. This plasmid contains the inducible T7 promoter and *lac* operator to permit high level protein expression. Plasmids were maintained in the DH5a strain of *Escherichia coli*.

Mutations in the gene for DHODase were made using

```
  1 MATGDERFYA EHLMPTLQGL LDPESAHRLA VRFTSLGLLP
 41 RARFQDSDML EVRVLGHKFR NPVGIAAGFD KHGEAVDGLY
 81 KMGFGFVEIG SVTPKPQEGN PRPRVFRLPE DQAVINRYGF
121 NSHGLSVVEH RLRARQQKQA KLTEDGLPLG VNLGKNKTSV
161 DAAEDYAEGV RVLGPLADYL VVNVSSPNTA GLRSLQGKAD
201 VRRLLTKVLQ ERDGLRRVHR PAVLVKIAPD LTSQDKEDIA
241 SVVKELGIDG LIVTNTTVSR PAGLQGALRS ETGGLSGKPL
281 RDLSTQTIRE MYALTQGRVP IIGVGGVSSG QNALEKIRAG
321 ASLVQLYTAL PFWGPPVVGK VKRELEALLK EQGFGGVTDA
361 IGADHRR
```

FIG. 1. Amino acid sequence of recombinant human DHODase, as described in Copeland *et al.* [10]. The M at position 1 represents the initiating methionine in the truncated 39 kDa construct. The 8 histidine residues of the enzyme are highlighted in boldface.

the PCR mutagenesis technique described by Innis *et al.* [12]. This technique requires two rounds of PCR amplification and four oligonucleotide primers. Two inside primers, of opposite orientation, in the region of the desired mutation are designed to contain a region of complementarity (≥13 bases), with at least one primer incorporating the desired mutation. Two additional "outside" primers are complementary to the template and define the 5′ and 3′ limits of the fragment to be amplified. As indicated in Table 1, primers designated "F" are forward primers corresponding to the coding DNA strand and "R," reverse primers corresponding to the noncoding strand. In the first round of PCR, the DHODase plasmid was used as a template for two separate reactions in which (a) a forward outside primer was combined with a reverse inside primer, and (b) a reverse outside primer was combined with a forward inside primer. Following purification, the two PCR products were combined in the second round of PCR. Since the fragments contain a region of complementarity as defined by the original inside primer pair, they serve as the template, and the outside primers are used to amplify the full-length fragment.

A potential problem of PCR amplification is misincorporation of bases into the amplified fragment, which can lead to undesired mutations in the expressed protein. Since the risk of base misincorporation can be reduced by minimizing the size of the amplified DNA fragment, a new DHODase template was designed to include additional unique restriction sites. The parent template contained unique *Nde*I, *Sac*II and *Bam*HI sites. As shown in Fig. 2, the new DHODase template contained unique restriction sites at intervals of approximately 300 bp. The *Sal*I and *Nhe*I sites were introduced using the general PCR mutagenesis technique described above. In the first round of PCR, three reactions tubes each contained PYRDH plasmid plus one of the following pairs of primers: 1 + 2 or 3 + 4 or 5 + 6. For the second round of PCR, the three products were combined and amplified without the need for additional primer

TABLE 1. Oligonucleotide primers used in constructing DHODase mutants*

Primer	Site	Sequence
1F	(*Nde*I site)	5′-GGAGGATTCATATGGCTACCGGTGATGAGCG-3′
2R	(*Sal*I site)	5′-CCAAAGCCCATCTTATAAAGTCCGTCgACGGCTTCCC-3′
3F	(*Sal*I site)	5′-GGGAAGCCGTcGACGGACTTTATAAGATGGGCTTTGG-3′
4R	(*Nhe*I site)	5′-CTTTGACCACgCTaGCAATGTCCTC-3′
5F	(*Nhe* I site)	5′-GAGGACATTGCtAGcGTGGTCAAAG-3′
6R	(*Bam*HI site)	5′-CGGGGATCCTTACCTCCGATGATCAGCTC-3′
7F	(*Sac*II)	5′-CAGTGGACGCCGCGGAGGACTACG-3′
8R	(*Sac*II)	5′-CGTAGTCCTCCGCGGCGTCCACTG-3′
9R	(H11A)	5′-GAGTCGGCATCAGGgcTTCAGCATAG-3′
10F	(H11A)	5′-CTGATGCCGACTCTGCAGG-3′
11R	(H26A)	5′-CGAACAGCCAGTCTGgcGGCTGACTCC-3′
12F	(H26A)	5′-GACTGGCTGTTCGCTTCACC-3′
13R	(H56A)	5′-CCAGAACTCTCACTTCCAGC-3′
14F	(H56A)	5′-GTGAGAGTTCTGGGCgcTAAATTCCG-3′
15R	(H71A)	5′-GTCGACGGCTTCCCCAgcCTTGTCAAATCCTGC-3′
3F	(H71A)	5′-GGGAAGCCGTCGACGGACTTTATAAGATGGGCTTTGG-3′
16R	(H71N)	5′-GTCGACGGCTTCCCCATtCTTGTCAAATCCTGC-3′
3F	(H71N)	5′-GGGAAGCCGTCGACGGACTTTATAAGATGGGCTTTGG-3′
17R	(H122A)	5′-CCACTGAAAGCCCGgcACTGTTAAATCC-3′
18F	(H122A)	5′-GGATTTAACAGTgcCGGGCTTTCAGTGG-3′
19R	(H129A)	5′-CTGGCCCGTAACCTGgcTTCCACCACTG-3′
20F	(H129A)	5′-CAGTGGTGGAAgcCAGGTTACGGGCCAG-3′
21R	(H218A)	5′-CTGCCGGCCTGgcCACTCTCCGC-3′
22F	(H218A)	5′-GCGGAGAGTGgcCAGGCCGGCAG-3′
23R	(H364A)	5′-CGGGGATCCTTACCTCCGAgcATCAGCTCC-3′

* Lower case letters represent nucleotides that create the desired mutation. The letters "F" and "R" are used to designate forward and reverse primers, which correspond to the coding and noncoding DNA strand, respectively. Primers 1F–6R were used to make the 39 kDa DHODase construct and incorporate silent base changes that created unique restriction sites (underlined). Primers 7F and 8R are nonmutagenic primers used in PCR amplifications. Primers 9R–23R were used to incorporate histidine mutations.

(because the two outer fragments function as primers for the middle fragment). The resulting full-length DHODase insert (1.1 kb) was then subcloned into the *Nde*I and *Bam*HI restriction sites in pET11a.

Each of the eight histidines in the 39 kDa DHODase were changed individually to alanine; histidine 71 was also replaced by an asparagine. The overlapping primer pairs used to construct the histidine mutations are shown in Table 1. In the first round of PCR, the new DHODase plasmid was used as a template for two separate reactions in which (a) the forward histidine primer was combined with a reverse primer containing the nearest unique restriction site, and (b) the reverse histidine primer was combined with a forward primer containing the nearest restriction site (see Fig. 2 and Table 1). Following purification, the two PCR products were combined in the second round of PCR and amplified with the outside primers containing the nearest restriction sites. This fragment was then digested by the nearest unique restriction enzymes and subcloned into DHODase plasmid from which the corresponding wild-type fragment had been removed.

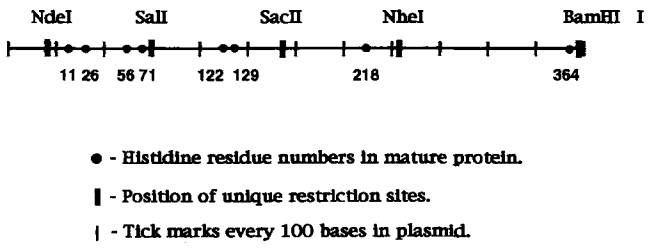

FIG. 2. Location of unique restriction sites and histidine residues in pET11a-DHODase. Mutants of histidines 11, 26, 56, and 71 were constructed by PCR amplification of the region encompassing the *Nde*I and *Sal*I restriction sites, followed by subcloning of the mutagenic fragment into pET11a-DHODase, which had been cut with the same enzymes. Histidine 122 and 129 mutagenic fragments were subcloned into the *Sal*I/*Sac*II position; histidine 218 mutagenic fragment was subcloned into the *Sac*II/*Nhe*I position, and histidine 364 was subcloned into the *Nhe*I/*Bam*HI position.

PCRs were performed in a Perkin–Elmer Gene Amp PCR System 9600. For the first round of PCR, each 100-μL reaction contained 0.1 μg plasmid template, 0.2 mM dNTPs, 100 pmol each of forward and reverse primer, 1.25 mM $MgCl_2$, 10 μL of 10× Ultma reaction buffer (Perkin–Elmer) and 3 U of Ultma DNA polymerase. A 5-min initial denaturation at 94° was followed by 25 cycles of the following: denaturation at 94° for 30 sec, annealing at 60° for 30 sec, polymerization at 72° for 30 sec. This was followed by a final 10-min polymerization step at 72°. Samples were maintained at 4° until used. Since each reaction yielded a single product (as determined by visualization of a single band on an agarose gel), PCR products were used directly in the second round of PCR following removal of primers, nucleotides, and polymerase using the Qiaquick-spin PCR purification columns (Qiagen). In the second round of PCR, equimolar amounts of overlapping fragments were combined with 100 pmol each of the

outside primers; all other conditions were identical to the first round of PCR. The resulting products were then digested with restriction enzymes corresponding to the closest unique restriction sites (Fig. 2) and subcloned into the DHODase plasmid cut with the same enzymes. Incorporation of the desired mutation and absence of nonspecific mutations were confirmed by fluorescent cycle sequencing of each plasmid.

Protein Expression

For protein expression, the pET11a-DHODase mutant plasmids were transformed into a pyrimidine auxotrophic strain of *E. coli*, TAP330, which lacks the endogenous gene for the bacterial DHODase [10]. Cells were grown overnight in Luria Bertani broth containing 100 μg/mL ampicillin and 0.00244% uridine at 37°. A 1:50 dilution of the overnight culture was then grown in the same medium to an optical density of 0.4 to 0.7 at 600 nm, after which protein expression was induced by the addition of IPTG to a final concentration of 100 μM. After 3 hr of further growth, the cells were harvested by centrifugation. After washing the cells with 0.85% NaCl to remove excess medium, the cell paste was frozen at −20° until used. For analysis, the cells were resuspended in lysis buffer (50 mM Tris, pH 8.0, 100 mM NaCl), lysed by sonication, and cell debris was removed by centrifugation. Expression levels were assessed by SDS–PAGE using equal amounts of total protein.

Activity Assays

Activity assays were performed in 96-well microtiter plates at ambient temperature (*ca.* 23°). Two different colorimetric assays were used to quantify DHODase activity: the NBT assay and the DCIP assay [10]. Both assays are coupled assays, based on the ability of active enzyme to transfer electrons through ubiquinone to an acceptor (NBT or DCIP) in the presence of dihydroorotate. In the case of the NBT assay, reduction of NBT is accompanied by an increase in absorbance at 610 nm. This assay is linear over a wider enzyme concentration range compared to the DCIP assay and was convenient for conditions employing either long incubation times (to test for residual activity) or high enzyme concentrations, as in the inhibitor titration studies (*vide infra*). For determination of Michaelis constants, the DCIP assay was used. Reduction of DCIP is stoichiometrically equivalent to oxidation of dihydroorotate [13] and is accompanied by a loss of absorbance at 610 nm ($\varepsilon = 21{,}500\ M^{-1}\ cm^{-1}$). To determine catalytic turnover constants, k_{cat} values, for crude enzyme preparations, an active site titration procedure was used [14–16]. In this case, the DHODase-specific, tight-binding inhibitor BQR was used to titrate the amount of active DHODase present in the cell lysate. For H26A-DHODase, which was refractory to BQR inhibition, an estimate of the relative activity compared with wild-type DHODase was made by dividing the V_{max} values for the two enzymes (NBT assay) by the respective total amount of protein in the whole cell lysates. Total protein was quantified using the method of Bradford [17] with BSA as the protein standard.

NBT ASSAY. A total reaction volume of 200 μL contained 200 μM ubiquinone in assay buffer (100 mM Tris, pH 8.0, 0.1% Triton X-100), 250 μM dihydroorotate, and 100 μM NBT. The reaction was initiated by the addition of dihydroorotate and NBT, and the increase in absorbance at 610 nm as a function of time was monitored. In cases where an inhibitor was present, the inhibitor (10 μL) was incubated for 5 min with the enzyme/ubiquinone solution (135 μL total volume) prior to addition of dihydroorotate and NBT.

DETERMINATION OF ACTIVE ENZYME CONCENTRATION USING INHIBITOR TITRATION. To determine the amount of active enzyme present in the cell lysate, the sample was titrated with the DHODase-specific tight-binding inhibitor BQR. The NBT assay was used to follow enzyme activity, as described above. Prior to substrate addition, enzyme was incubated with inhibitor for 5 min. The following final concentrations of BQR were used in the titration: 0, 2.5, 5, 7.5, 10, 15, 25, 50, 75, and 100 nM. The fractional velocity as a function of inhibitor concentration was determined, and the total enzyme concentration was calculated by direct fit of the data to the Morrison equation [18].

DCIP ASSAY AND DETERMINATION OF SUBSTRATE K_m. In general, a total reaction volume of 200 μL contained 200 μM ubiquinone in assay buffer (*vide supra*), 250 μM dihydroorotate, and 100 μM DCIP. The reaction was initiated by the addition of dihydroorotate and DCIP; the decrease of 610 nm absorbance was followed as a function of time. For the determination of dihydroorotate K_m, the ubiquinone concentration was held constant at 200 μM and the following dihydroorotate concentrations were tested: 0, 5, 10, 20, 25, 50, 75, and 100 μM. This provided substrate concentrations from 0.1 to 3.5 K_m (of wild-type enzyme). For the determination of ubiquinone K_m, the dihydroorotate concentration was held constant at 250 μM and the following ubiquinone concentrations were used: 0, 7.5, 15, 25, 40, 50, 75, and 100 μM. This provided substrate concentrations from 0.3 to 4 K_m (of wild-type enzyme). The data from each experiment were fit to the Henri–Michaelis–Menten equation using a nonlinear least squares method to determine values of K_m and V_{max} [16]. The kinetic constants used in Table 2 are the average values of two or three determinations for each mutant.

RESULTS AND DISCUSSION

The complete amino acid sequence of recombinant human DHODase is displayed in Fig. 1, where the 8 histidine residues of the protein are highlighted. Of these, 5 residues are conserved in the closely related (87% amino acid

TABLE 2. Enzyme kinetic parameters for wild-type (WT) and mutant recombinant human DHODase

Mutant	V_{max}/V_{max}^{WT}	K_m/K_m^{WT} DHO (μM)	K_m/K_m^{WT} Q_6 (μM)	k_{cat}/k_{cat}^{WT}
WT*	1.00	1.0	1.0	1.00
H11A	1.16	1.2	0.8	1.02
H26A	0.10	0.5	1.6	ND†
H56A	1.21	1.8	1.0	1.25
H71A	0	ND	ND	0
H71N	1.59	1.7	1.2	1.33
H122A	0.53	0.6	0.8	1.23
H129A	0	ND	ND	0
H218A	1.04	1.1	1.0	1.07
H364A	0	ND	ND	0

* The K_m value for DHO and the V_{max} for the wild-type enzyme were, within experimental error, the same as previously reported [10]. The average K_m value for Q_6 for the wild-type enzyme was 28 μM.

† ND = not determined.

sequence identity) rat enzyme—H26, H56, H122, H129, and H364—and may thus have some importance in protein structure or catalysis. Each of these residues was mutated individually to an alanine, and H71 was mutated additionally to an asparagine. The wild-type and mutant enzymes were expressed under the control of the inducible T7 promoter in the previously described TAP330 strain of *E. coli* [10]. This bacterial strain is auxotrophic for pyrimidine due to the lack of the endogenous gene for DHODase, providing a convenient expression system with no background enzymatic activity. Figure 3 illustrates the expression levels obtained in whole cell lysates for the wild-type and mutant DHODases, as determined by SDS–PAGE with Coomassie brilliant blue staining. It is clear from this figure that comparable expression levels were obtained for all of the recombinant enzymes examined here.

To assess the enzymatic activity of the mutant enzymes, cell lysates were clarified by centrifugation, and the supernatants were quantified for total protein and enzymatic activity as described under Materials and Methods. SDS–PAGE analysis of the clarified supernatants (data not shown) suggested similar levels of soluble enzyme for all of the mutants studied here, but in all cases the majority of the expressed enzyme was present as insoluble inclusion bodies that were devoid of enzymatic activity. The limiting quantities of soluble enzyme precluded efforts to purify each of the individual enzymes; the amount of soluble enzyme in these supernatants was sufficient, however, for enzyme activity measurements. The relative activity of each mutant was normalized by dividing its V_{max} value (normalized for total protein concentration) by that of the wild-type enzyme. These data are summarized in Table 2. As seen in Table 2, most of the histidine to alanine replacements had a minimal effect on the relative activity of the enzyme. Three mutants, however, led to a complete loss of enzymatic activity: H71A, H129A, and H364A. For these three mutants, attempts were made to determine enzymatic activity with long incubation times (up to overnight incubation with substrate) and with increased substrate and enzyme concentrations. Even under these extreme conditions, no enzymatic activity was detected.

The lack of activity for the H71A mutant was particularly surprising since this residue is not conserved in the closely related rat enzyme (Accession Number X80778). In the rat enzyme, H71 is replaced by an asparagine residue. We therefore evaluated a human DHODase mutant in which H71 was replaced by asparagine. As seen in Table 2, the H71N mutant had relative activity comparable to that of the wild-type human enzyme. The combined data for the H71A and H71N mutants suggest that a histidine residue is not required at position 71, but that this site is less permissive than most of the other histidine locations within the enzyme. That asparagine can replace histidine at this site suggests either a requirement for a nitrogen-containing side chain capable of H-bonding interactions, or a requirement for a certain degree of steric bulk at this location; neither of these potential requirements would be fulfilled by an alanine residue at this site.

The other two enzymatically inactive mutants both involve histidine residues that are conserved between the human and rat enzymes. The inactivity of the H364A mutant was also somewhat surprising in that this residue is only two amino acids away from the carboxyl terminus of the enzyme. We note, however, that there is a very high

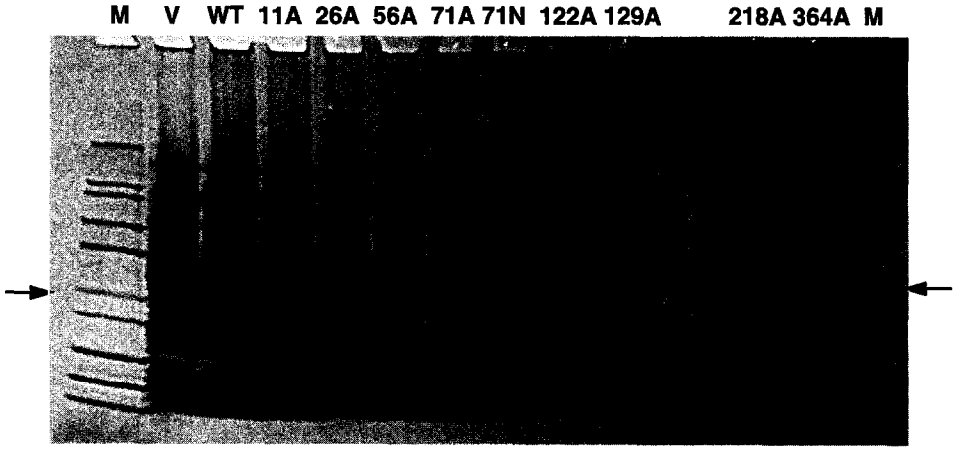

FIG. 3. SDS–PAGE of whole cell lysates from recombinant *E. coli* containing the genes for the wild-type (WT) and mutant forms of human DHODase. The labels above the lanes of the gels refer to the histidine residue replacement within DHODase, with the following exceptions: M = molecular weight markers; V = vector alone. The arrows at the left and right margins point to the protein band representing the expressed DHODase. See Materials and Methods for further details.

level of amino acid conservation in the carboxyl terminus of the mammalian enzymes, perhaps suggesting an important structural or functional role for this portion of the protein. It is clear from the present results that both H129 and H364 are required for enzymatic activity. The specific role that each of these residues play in human DHODase, however, cannot be determined from the present data. These residues may each play a direct role in catalysis, may act as ligands for the putative metal cofactors of the enzyme, or may be required to maintain the structural integrity of the native enzyme. Further studies with the purified mutant enzymes will be required to address this issue more definitively.

For the mutants that displayed enzymatic activity we wished to assess more quantitatively the effects of the mutations on enzyme kinetics. We therefore determined the K_m values for DHO and Q_6 and the k_{cat} value for the overall enzymatic reaction from Henri–Michaelis–Menten kinetic studies. The values of these parameters, relative to the wild-type enzyme, are summarized in Table 2. Among the active mutants we see at most a 2-fold difference in the K_m value for either substrate. Likewise, the k_{cat} values of the active mutants were all comparable to the wild-type enzyme, with the exception of the H26A mutant. This mutant displayed about a 10-fold decrease in V_{max}, but comparable DHO and Q_6 K_m values relative to the wild-type enzyme. Hence, the effect of this mutation must be to perturb catalytic conversion of substrate to product (k_{cat} effect), without a significant effect on substrate binding (K_m effect).

To quantify k_{cat} for the enzymatic reaction, we required an accurate determination of the concentration of active enzyme in the cell extracts used here. To obtain estimates of the concentrations of the enzymes, we performed active site titrations with the tight-binding inhibitor BQR (see Fig. 4A for the structure of this inhibitor), as previously described [14–16, 18]. In the course of these measurements, we made the surprising discovery that one of the mutants, H26A, was insensitive to inhibition by BQR. Figure 4B illustrates the concentration-dependent inhibition of the recombinant enzymes by BQR. For the wild-type and active mutants other than H26A, the IC_{50} value (i.e. the inhibitor concentration that effects half-maximal inhibition) for BQR fell between 6 and 10 nM. In the case of the H26A mutant, however, we observed less than 25% inhibition of activity at any concentration tested. The lines drawn through the data points in Fig. 4B represent the nonlinear least squares best fit to equation 1:

$$\frac{v_i}{v_0} = \frac{1}{1 + \left(\frac{[I]}{IC_{50}}\right)^n} \qquad (1)$$

where v_i/v_0 is the fractional velocity of the enzyme in the presence of inhibitor at concentration $[I]$, and n is a term that relates to the degree of inhibitor binding cooperativity. For the H26A mutant, the data are not sufficient to provide

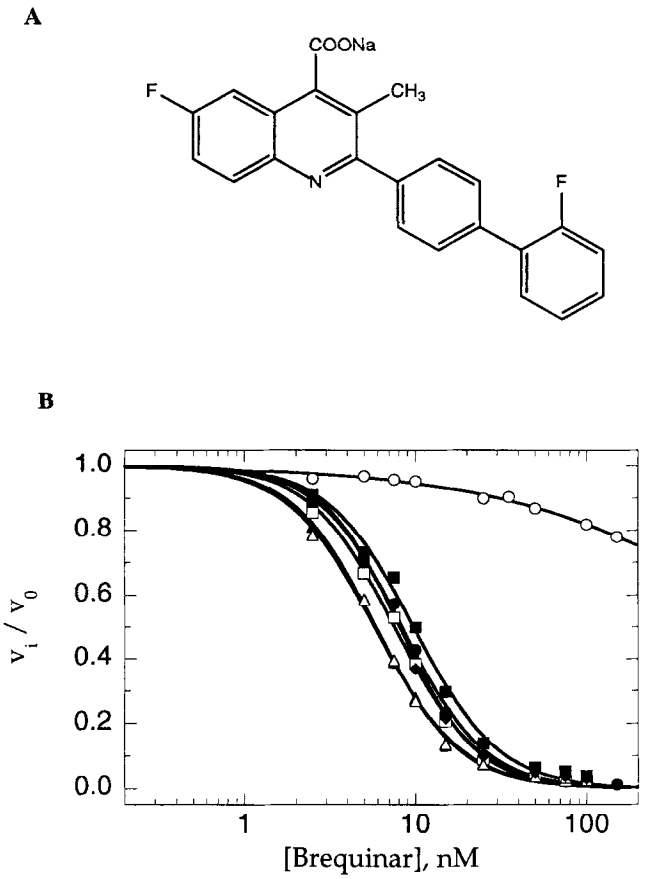

FIG. 4. (A) Chemical structure of BQR. (B) Concentration–response plot for inhibition of wild-type and mutant human DHODase by BQR. The symbols represent data points for the enzymes as follows: wild type (●); H11A (■); H26A (○); H56A (▲); H71N (□); H122A (△); and H218A (◆). The lines drawn through the data points represent the nonlinear least squares best fits to equation 1.

a good estimate of the IC_{50} from this type of curve fitting. However, the extrapolated value derived from curve fitting of the data for the H26A mutant was 1337 nM; this should be considered to be an estimated lower limit on the true IC_{50} for this mutant. Nevertheless, it is clear from the data in Fig. 4B that the H26A mutation decreases the sensitivity of the enzyme to BQR by at least 167-fold relative to the wild-type enzyme. The most straightforward interpretation of this result is that H26 is located within the BQR binding pocket of the enzyme, although an indirect effect of the H26A substitution on BQR binding cannot be ruled out completely. An alternative explanation for the above result is that mutation of H26 to alanine perturbs the overall conformation or stability of the enzyme, hence affecting its interactions with the inhibitor. We note that the H26A mutant displayed a 10-fold reduction in specific activity relative to the wild-type enzyme, which might be interpreted also as an indication of a global effect of the mutation on enzyme structure. This reduction in activity is small compared with the large perturbation in BQR binding, and must be viewed with some caution, as it is based on

normalization of the respective V_{max} values for total protein concentration. While the SDS–PAGE analysis illustrated in Fig. 3 indicates similar expression levels for the wild-type and H26A mutant, small differences in expression level could exaggerate the activity difference between these enzymes. Furthermore, the lack of significant perturbation of the Michaelis constants for either DHO or Q_6 in the H26A mutant argues against a global structural perturbation for this enzyme.

The data presented here suggest that H26 plays an important role in the binding of BQR to human DHODase. One can envision a number of modes of molecular interaction between a histidine residue and BQR, including hydrogen bonding, van der Waals interactions, and ring stacking. A detailed description of these interactions and the identification of other residues important for BQR binding will require further mutagenesis studies of human DHODase. Nevertheless, the present data provide the first structural insights into the interactions of BQR with the human enzyme. BQR and its structural analogs [19, 20] are the most potent inhibitors of human DHODase to be reported. Their good oral bioavailability and pharmacokinetic profiles, together with their demonstrated efficacy for blocking organ rejection in animal allograft and xenograft transplantation, portend the successful use of this inhibitor class as immunoregulatory agents. Understanding the interactions of the enzyme with this inhibitor is an important step towards the development of future antiproliferative compounds that target DHODase, perhaps leading ultimately to new drugs for use in immunoregulatory therapies. Continued mutagenesis and biophysical studies of DHODase may help to better refine the structural determinants of inhibitor binding to this enzyme.

We thank William Pitts, Bruce Jaffee, and Ron Magolda for their continued interest in this work and for helpful discussions. J.P.D. acknowledges the support of the DuPont Merck Pharmaceutical Co. postdoctoral program.

References

1. Jaffee BD, Jones EA, Loveless SE and Chen SF, The unique immunosuppressive activity of brequinar sodium. *Transplant Proc* **25:** 19–22, 1993.
2. Makowka L, Sher LS and Cramer DV, The development of brequinar as an immunosuppressive drug for transplantation. *Immunol Rev* **136:** 51–70, 1993.
3. Bartlett RA, Dinitrijevic M, Mattar T, Zielinski T, Germann T, Rude E, Thoenes GH, Küchle CCA, Schorlemmer H-U, Bremer E, Finnegan A and Schleyerbach R, Leflunomide (HWA 486), a novel immunomodulating compound for the treatment of autoimmune disorders and reactions leading to transplantation rejection. *Agents Actions* **32:** 10–21, 1991.
4. Williams JW, Xiao F, Foster P, Clardy C, McChesney L, Sankary H and Chong AS-F, Leflunomide in experimental transplantation. Control of rejection and alloantibody production, reversal of acute rejection, and interaction with cyclosporin. *Transplantation* **57:** 1223–1231, 1994.
5. Xiao F, Chong A, Foster P, Sankary H, McChesney L, Koukoulis G, Yang J, Frieders D and Williams JW, Leflunomide controls rejection in hamster to rat cardiac xenografts. *Transplantation* **58:** 828–834, 1994.
6. Chen SF, Perrella FW, Behrens DL and Papp LM, Inhibition of dihydroorotate dehydrogenase activity by brequinar sodium. *Cancer Res* **52:** 3521–3527, 1992.
7. Davis JP, Cain GA, Pitts WJ, Magolda RL and Copeland RA, The immunosuppressive metabolite of leflunomide is a potent inhibitor of human dihydroorotate dehydrogenase. *Biochemistry* **35:** 1270–1273, 1996.
8. Williamson RA, Yea CM, Robson PA, Curnock AP, Gadher S, Hambleton AB, Woodward K, Bruneau J-M, Hambleton P, Moss D, Thomson TA, Spinella-Jaegle S, Morand P, Courtin O, Sautés C, Westwood R, Hercend T, Kuo EA and Ruuth E, Dihydroorotate dehydrogenase is a high affinity binding protein for A771726 and mediator of a range of biological effects of the immunomodulatory compound. *J Biol Chem* **270:** 22467–22472, 1995.
9. Greene S, Watanabe K, Braatz-Trulson J and Lou L, Inhibition of dihydroorotate dehydrogenase by the immunosuppressive agent leflunomide. *Biochem Pharmacol* **50:** 861–867, 1995.
10. Copeland RA, Davis JP, Dowling RL, Lombardo D, Murphy KB and Patterson TA, Recombinant human dihydroorotate dehydrogenase: Expression, purification and characterization of a catalytically functional truncated enzyme. *Arch Biochem Biophys* **323:** 79–86, 1995.
11. Minet M, Dufour M-E and Lacroute F, Cloning and sequencing of a human cDNA coding for dihydroorotate dehydrogenase by complementation of the corresponding yeast mutant. *Gene* **121:** 393–396, 1992.
12. Innis MA, Gelfand DH, Sninsky JJ and White TJ, *PCR Protocols: A Guide to Methods and Applications*. Academic Press, San Diego, CA, 1990.
13. Miller RW, Dihydroorotate dehydrogenase (*Neurospora*). *Methods Enzymol* **51:** 63–69, 1978.
14. Bieth J, Some kinetic consequences of the tight binding of protein-proteinase-inhibitors to proteolytic enzymes and their application to the determination of dissociation constants. In: *Proteinase Inhibitors, Bayer-Symposium V*, pp. 463–469. Springer, New York, 1974.
15. Williams JW and Morrison JF, The kinetics of reversible tight-binding inhibition. *Methods Enzymol* **63:** 437–467, 1979.
16. Copeland RA, *Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis*. Wiley-VCH, New York, 1996.
17. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
18. Morrison JF, Kinetics of the reversible inhibition of enzyme-catalyzed reactions by tight-binding inhibitors. *Biochim Biophys Acta* **185:** 269–286, 1969.
19. Batt DG, Copeland RA, Dowling RL, Gardner TL, Jones EA, Orwat MJ, Pinto DJ, Pitts WJ, Magolda RL and Jaffee BD, Immunosuppressive structure–activity relationships of brequinar and related cinchoninic acid derivatives. *Bioorg Med Chem Lett* **5:** 1549–1554, 1995.
20. Chen SF, Papp LM, Ardecky RJ, Rao GV, Hesson DP, Forbes M and Dexter DL, Structure–activity relationship of quinoline carboxylic acids. *Biochem Pharmacol* **40:** 709–715, 1990.