Lys-D48 Is Required for Charge Stabilization, Rapid Flavin Reduction, and Internal Electron Transfer in the Catalytic Cycle of Dihydroorotate Dehydrogenase B of Lactococcus lactis

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Dihydroorotate dehydrogenase B (DHODB) catalyzes the oxidation of dihydroorotate (DHO) to orotate and is found in the pyrimidine biosynthetic pathway. The Lactococcus lactis lactate enzyme is a dimer of heterodimers containing FMN, FAD, and a 2Fe-2S center. Lys-D48 is found in the catalytic subunit and its side-chain adopts different positions, influenced by ligand binding. Based on crystal structures of DHODB in the presence and absence of orotate, we hypothesized that Lys-D48 has a role in facilitating electron transfer in DHODB, specifically in stabilizing negative charge in the reduced FMN isoalloxazine ring. We show that mutagenesis of Lys-D48 to an alanine, arginine, glutamine, or glutamate residue (mutants K38A, K48R, K48Q, and K48E) impairs catalytic turnover substantially (∼50–500-fold reduction in turnover number). Stopped-flow studies demonstrate that loss of catalytic activity is attributed to poor rates of FMN reduction by substrate. Mutation also impairs electron transfer from the 2Fe-2S center to FMN. Addition of methyamine leads to partial rescue of flavin reduction activity. Nicotinamide coenzyme oxidation and reduction at the distal FAD site is unaffected by the mutations. Formation of the spin-interacting state between the FMN semiquinone-reduced 2Fe-2S centers observed in wild-type enzyme is retained in the mutant enzymes, consistent with there being little perturbation of the supernexchange change paths that contribute to the efficiency of electron transfer between these cofactors. Our data suggest a key charge-stabilizing role for Lys-D48 during reduction of FMN by dihydroorotate, or by electron transfer from the 2Fe-2S center, and establish a common mechanism of FMN reduction in the single FMN-containing A-type and the complex multicenter B-type DHOD enzymes.

The dihydroorotate dehydrogenases (DHOD) are flavoproteins that participate in the de novo biosynthesis of pyrimidines. They are a heterogeneous family of enzymes that catalyze the conversion of dihydroorotate to orotate (Scheme 1) and the transfer of electrons to various redox acceptors (1). Class 1A DHODs (2, 3) are soluble dimeric enzymes, contain a FMN prosthetic group and are able to use fumarate as an electron acceptor. They are found in anaerobic yeasts, milk-fermenting bacteria, and some protozoa. The soluble class 1B enzymes, which are found in Gram-positive bacteria, contain FMN, FAD, and a 2Fe-2S center and use NAD+ as electron acceptor (4, 5). Lactococcus lactis and related milk-fermenting bacteria like Enterococcus faecalis are unusual in possessing two DHOD enzymes (i.e. DHODA (class 1A) and DHODB (class 1B)). The class 2 enzymes, which are found in eukaryotes and Gram-negative bacteria, are membrane-bound, contain FMN and are oxidized by ubiquinone (6–8).

The multicenter DHODB (EC 1.3.3.1) enzymes have been purified from a number of organisms including Lactococcus lactis, Bacillus subtilis, E. faecalis, and Clostridium oroticum (4, 9–11). A crystallographic structure for L. lactis DHODB is available (5). The enzyme is a dimer of heterodimers, with a catalytic subunit (termed subunit D) responsible for the oxidation of dihydroorotate to orotate and reduction of the FMN cofactor. Electron transfer subunits (termed K subunits) are involved in electron transfer and the binding/reduction of the electron acceptor NAD+ (5). The K subunits bind a 2Fe-2S center and FAD, and the electron transfer pathway is from FMN (subunit D) to 2Fe-2S center (subunit K) to FAD (subunit K) and NAD+. Substrate binding in the active site leads to conformational changes that occur in specific regions of the catalytic D-subunit (5). Notable among these are the relatively large movements for the side chains of Lys-D48 and Tyr-K232.

Kinetic isotope effect studies and analysis of the pH dependence of the reaction catalyzed by E. faecalis DHODB indicate a reversible reaction. Rate-limiting macroscopic isozonations have been identified in the reductive and oxidative half-reactions (11). Studies of double isotope effects on V and V(KDHO) obtained for deuteration at C6-proS and C5 of dihydroorotate have been used to propose a mechanism in which C5-proS proton transfer and C6-hydride transfer are concerted. This mode of oxidation of dihydroorotate prevents formation of an unstable carbamion intermediate (pKs ~ 20–21, Ref. 10). Active site cysteine and lysine residues have been proposed to act as a general base and electrostatic catalyst in C. oroticum DHODB (10).

We have reported reductive titrations of DHODB from L. lactis and demonstrated a five electron capacity (12). The mid-point reduction
potential of the 2Fe-2S center (−212 mV ± 3 mV), the oxidized/semiquinone ($E_1$) and semiquinone/hydroquinone ($E_2$) couples for the FMN ($E_1 = −301 ± 2 mV; E_2 = −252 ± 8 mV$) and FAD ($E_1 = −312 ± 6 mV; E_2 = −297 ± 5 mV$) are known from UV-visible spectrophotometric titration and EPR studies. A spin-interacting state between the FMN semiquinone species and the reduced 2Fe-2S center forms during reductive titration with dithionite. This spin-interacting state is characterized by an unusual EPR signal with very small rhombic anisotropy and g values 2.02, 1.99, and 1.96 (12). To begin to address the mechanism of flavin reduction and internal electron transfer in DHODB we have performed a series of stopped-flow studies of the wild-type L. lactis enzyme and mutants altered in the region of Lys-D48, which is located in the FMN-binding region. Herein, we report the first detailed kinetic characterization of DHODB using stopped-flow methods and demonstrate a key role for Lys-D48 in FMN reduction and internal electron transfer from the 2Fe-2S center to FMN. Our studies are consistent with a charge-stabilization role for Lys-D48 during electron transfer rather than the result of major perturbation of the reduction potential of the redox cofactors, or disruption of superexchange pathways that contribute to the efficiency of electron transfer between redox cofactors.

**EXPERIMENTAL PROCEDURES**

**Materials, Protein Expression, and Purification—**L-Cysteine, NAD$^+$, DHO, orotate, NADH, and D-hydroorotic acid sodium salt were purchased from Sigma. Wild-type DHODB was purified from *Escherichia coli* strain SØ6645 essentially as described previously (4, 12). Mutagenesis of the *L. lactis* DHODB catalytic subunit (Lys-D48) was carried out using the QuikChange mutagenesis procedure (13) as marketed in kit form (Stratagene) according to the manufacturer’s instructions. *E. coli* expression plasmid pFN3 (4), which encodes both D and K subunits of redox cofactors, or disruption of superexchange pathways that contribute to the efficiency of electron transfer between redox cofactors.

![Scheme 1](image)

**Steady-state Kinetic Assays—**DHOD activity was monitored by measuring changes in absorbance at 290 nm using an extinction coefficient of 6220 M$^{-1}$ cm$^{-1}$. Initial rates were calculated from the absorbance increase at 290 nm ($ε_{290} = 5960$ M$^{-1}$ cm$^{-1}$). One unit of activity is defined as the amount of enzyme that catalyzes the production of 1 μmol of orotate per second. Apparent $K_m$ and $k_{cat}$ values for L-dihydroorotate were determined at a fixed and saturating concentration of NAD$^+$ (900 μM). The data were fitted to the Michaelis-Menten equation using the Grafit software package (14).

**Stopped-flow Kinetic Measurements—**Unless stated otherwise, all kinetic studies were carried out under strict anaerobic conditions (<5 ppm O$_2$) within a glove box environment (Belle Technology). Reaction mixtures were prepared using an Applied Photophysics SX.18MV-R stopped-flow spectrophotometer contained within the glove box. Stopped-flow, multiple-wavelength absorption studies were carried out using a photodiode array detector and X-SCAN software (Applied Photophysics Ltd). Spectral deconvolution was performed by global analysis and numerical integration methods using PROKIN software (Applied Photophysics). For single-wavelength studies, absorption and fluorescence data collected were analyzed using nonlinear least squares regression analysis on an Acorn Risc PC microcomputer using Spectrakinetics software (Applied Photophysics). In the reductive half-reaction, experiments were performed by mixing enzyme in the appropriate buffer with an equal volume of reducing substrate/cofactor in the same buffer at the desired concentration. The concentration of sub-

8452A single beam diode array spectrometer with a 1-cm light path. Reactions were performed at 25 °C in 50 mM potassium phosphate/50 mM bis-Tris propane buffer pH 8.0, containing 1 mM EDTA, 2 mM pyruvate, 0.02 units/ml rabbit muscle L-lactate dehydrogenase (LDH), 900 μM NAD$^+$ and a fixed concentration of DHOD. The reaction was initiated by the addition of L-dihydroorotate and initial rates were calculated from the absorbance increase at 290 nm ($ε_{290} = 5960$ M$^{-1}$ cm$^{-1}$). One unit of activity is defined as the amount of enzyme that catalyzes the production of 1 μmol of orotate per second. Apparent $K_m$ and $k_{cat}$ values for L-dihydroorotate were determined at a fixed and saturating concentration of NAD$^+$ (900 μM). The data were fitted to the Michaelis-Menten equation using the Grafit software package (14).

**Preparation of Anaerobic Samples—**Buffer (50 mM potassium phosphate/50 mM bis-Tris propane, pH 8.0) was made anaerobic by bubbling argon gas through it for >2 h. Solutions were then placed in an anaerobic glove box (Belle Technology) overnight to remove any residual traces of oxygen. Protein samples were made anaerobic by passing through a small gel filtration (Bio-Rad 10 DG) column (housed in the glove box), which had been pre-equilibrated with anaerobic buffer. Substrate and coenzyme solutions were made by adding the appropriate solid to anaerobic buffer.

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Charge Stabilization in Dihydroorotate Dehydrogenase B

Lys-D48, Conformational Mobility, and Interactions with the FMN Isoalloxazine and Tyr-K232/2Fe-2S Center in Native and Orotate-bound Enzyme—Crystal structures of native and orotate-bound L. lactis DHODB have indicated differences between the two structures that potentially have major implications for the mechanism of flavin reduction and internal electron transfer (5). These structural differences involve (i) a flexible loop that contains the catalytic cysteine residue that is proposed to abstract a proton from the C5 atom of dihydroorotate, (ii) a re-orientation of Lys-D170 so that it hydrogen bonds to the FMN in the orotate-bound structure, and (iii) a re-orientation of Met-D247 so that the thioether group interacts with the FMN ribityl O3 and Lys-D170 (Fig. 1). Residue Lys-D48, however, undergoes the largest structural change. In the structure of the native enzyme, the Lys-D48 side chain is positioned away from the FMN. In this conformation, the Ne atom of Lys-D48 is located close to the Cε1 and Cβ1 atoms of Tyr-K232. Additionally, the Ne atoms of Lys-K247 and Lys-D48 resided closer together than in the orotate-bound structure.
D170 are also positioned close to Tyr-K232 (Fig. 1B). The binding of orotate to the active site of DHODB brings about a re-positioning of Lys-D48 such that the side chain now makes hydrogen bonding interactions with the N5 and O4 atoms of the FMN isalloxazine ring. Moreover, the carboxyl group of orotate forms a hydrogen bond with the side chain of Lys-D48, suggesting this interaction is important in binding of orotate to the enzyme. In this conformation, Lys-D48 occupies a position similar to that of the equivalent lysine residue found in L. lactis DHODA (3, 17). We hypothesized that in the orotate-bound form of DHODB, the hydrogen bonds formed between the Ne group of Lys-D48 and the FMN N5 and O4 atoms might modulate the reductive half-reaction chemistry through a variety of effects. These might include (i) alteration of the electronic properties of the FMN, (ii) an influence on the geometry of substrate binding, (iii) charge stabilization of the FMN (at the O4 position) during FMN reduction, and (iv) possible disruption of the degree of electronic coupling between the FMN and 2Fe-2S center which might affect the rate of electron transfer from FMN to the 2Fe-2S center. The implications of this conformational mobility for mechanism are unclear, and for this reason we have investigated the importance of this Lys-D48 in the catalytic cycle by directed mutagenesis and analysis of the electron transfer properties of four mutant forms of the enzyme (K48A, K48Q, K48R, and K48E) designed to alter the charge and size of the side chain at position D48. Each mutant enzyme was purified as described previously for the wild-type enzyme and was assembled stoichiometrically with FAD, FMN, and the 2Fe-2S center. The spectral properties of the purified mutant proteins were essentially identical to those of the wild-type enzyme (12). The electronic absorption spectra of the oxidized forms of wild-type and all K48 mutant DHODB enzymes had absorption maxima at 453 nm and 376 nm. The absorption shoulder at 540 nm from the iron-sulfur cluster is also observed in all oxidized forms (12). Initially, we studied in detail, by stopped-flow and steady-state methods, the kinetics of flavin reduction and electron transfer in wild-type DHODB. These studies are presented below and form a framework for comparison with the mutant enzymes (also reported below).

Reduction of Wild-type DHODB with NADH—Rapid mixing studies of the reduction of DHODB by a stoichiometric amount of NADH (the non-physiological direction of electron transfer) indicated that enzyme reduction proceeded in two observable kinetic phases (Fig. 2, A and B). In the first kinetic phase (A → B, 51 s⁻¹), there is evidence of partial reduction of the flavin cofactors and the development of long wavelength absorption at around 550–650 nm, consistent with the development of some blue semiquinone. Further reduction of the flavin cofactors occurs in a subsequent kinetic phase (B → C, 13 s⁻¹) to yield partially (∼50%) reduced DHODB. With an excess (10-fold) of NADH over enzyme, the two kinetic phases are maintained: however, the first kinetic phase is substantially faster (270 s⁻¹) indicating that the observed rate constant for this phase is dependent on NADH concentration. Following completion of the second kinetic phase, the reduction level of the enzyme is greater following mixing with 10-fold excess NADH compared with the stoichiometric mix (Fig. 2C). The first spectrum obtained in the diode array spectral series is very similar to that of the purified oxidized enzyme, suggesting that the binding of NADH does not substantially perturb the electronic properties of the enzyme-bound FAD.

To obtain values of rate constants for each kinetic phase as a function of NADH concentration, we performed stopped-flow studies with detection at a single wavelength or by fluorescence detection. Absorption changes monitored at 452 nm following rapid mixing of DHODB with NADH were biphasic, consistent with the photodiode array data (Fig. 3A). The concentration dependence of both kinetic phases was investigated under pseudo first-order conditions at this wavelength (Fig. 3B). The first kinetic phase showed a linear dependence on NADH concentration with rate constant 6.9 ± 0.1 × 10⁶ M⁻¹ s⁻¹, which represents reduction of the FAD cofactor of DHODB by NADH. The second kinetic phase was essentially independent of NADH concentration over the range 10–35 μM, and probably reflects internal electron transfer to the 2Fe-2S center and FMN and/or loss of NAD⁺. Above 40 μM the apparent independence was lost (Fig. 3B), but the mechanistic origin for this is uncertain. Also, the relatively small amplitude changes associated with the slow phase (Fig. 2C) complicates any attempt to rigorously analyze the concentration dependence of this phase. Rapid mixing studies of DHODB with NADH using fluorescence detection (excitation 340 nm, emission 450 nm) to monitor coenzyme oxidation produced monophasic reaction transients (Fig. 3C). Observed rate constants calculated from these fluorescence transients were essentially the...
same as those measured in the fast phase of the absorption transients (Fig. 3A). These data confirm that the first kinetic phase observed in the absorption transients is attributable to coenzyme oxidation.

Reduction of NADH by Reduced DHODB—We also investigated electron transfer from DHODB to NADH (the physiological direction of electron transfer) using stopped-flow methods. DHODB was titrated with dithionite under anaerobic conditions to introduce 5 electrons into the redox cofactors. The 5 electron reduced enzyme was then mixed with NADH and electron transfer monitored at 452 nm. Reaction transients were biphasic (Fig. 3D), and the rate constant for the fast kinetic phase exhibited a linear dependence on NADH concentration (rate constant $7.80 \pm 0.01 \times 10^3 \text{ M}^{-1} \text{s}^{-1}$; Fig. 3E).

**FIGURE 3.** Dependence of the observed rates of flavin reduction/oxidation on NADH/NADH concentration for wild-type DHODB. Reaction conditions: 50 mM bis-Tris propane/50 mM phosphate buffer, pH 8.0; 4 °C. A, absorption transient at 452 nm observed on mixing wild-type DHODB (2.3 $\mu$M) with NADH (30 $\mu$M). Transient is fitted to a standard biphasic expression (rate constants, 196 s$^{-1}$ and 8.7 s$^{-1}$). Inset, plot of residuals from the fit to a biphasic rate expression. B, plot illustrating the NADH concentration dependence of the rate for the fast (filled circles) and slow (open circles) phases of the absorption change observed at 452 nm and for the fluorescence change (monitored at 450 nm; open squares) with wild-type DHODB. C, fluorescence transient (excitation 340 nm, emission 450 nm) observed on mixing wild-type DHODB (5 $\mu$M) with NADH (30 $\mu$M). Transient is analyzed using a standard single exponential expression (rate constant, 172 s$^{-1}$). Inset, plot of residuals from the fit to a monophasic rate expression. D, absorption transient at 452 nm observed on mixing wild-type 5 electron-reduced DHODB (2.3 $\mu$M) with NADH (400 $\mu$M). Transient is fitted to a standard biphasic expression (rate constants, 30.6 s$^{-1}$ and 4.8 s$^{-1}$). Inset, plot of residuals from the fit to a biphasic rate expression. E, plot illustrating the NADH concentration dependence of the rate for the fast (filled circles) and slow (open circles) phases of the absorption change observed at 452 nm on mixing 5 electron-reduced DHODB with NADH.
kinetic phase was independent of NAD\(^+\) concentration in the range 0 to 300 \(\mu\)M NAD\(^+\); Fig. 3E), above this NAD\(^+\) concentration the rate constant increases about 8-fold (range 300–500 nM NAD\(^+\)). The mechanistic basis for this elevation in rate constant at high NAD\(^+\) concentration is uncertain. The slow kinetic phase made only a small contribution (~15%) to the overall absorption change observed at 452 nm. We infer that the first kinetic phase represents electron transfer from FADH\(_2\) to NAD\(^+\); the second rate constant we attribute also to reduction of NAD\(^+\), but this kinetic phase also incorporates internal electron transfer from the reduced FMN and 2Fe-2S centers to FAD prior to hydride transfer to NAD\(^+\). The lower value of the rate constant for NAD\(^+\) reduction (7.80 ± 0.01 × 10\(^{4}\) m\(^{-1}\) s\(^{-1}\)) compared with the rate constant for FAD reduction by NADH is consistent with the known mid-point reduction potentials for NADH/NAD\(^+\) and the FADH\(_2\)/FAD couples.

Reduction of Wild-type DHODB by Dihydroorotate—Rapid mixing studies of the reduction of DHODB with a stoichiometric concentration of dihydroorotate produced two observable kinetic phases (Fig. 4, A and B). The first kinetic phase (A → B, 11 s\(^{-1}\)) is accompanied by a bleaching of the flavin absorption spectrum and some development of long wavelength signature attributed to flavin neutral semiquinone at 550–650 nm. The development of a small amount of neutral semiquinone signature is attributed to electron transfer from the FMN to the 2Fe-2S center as the equilibrium position is attained for this kinetic phase. Further reduction of the enzyme cofactors occurs in the second kinetic phase (B → C, 4.7 s\(^{-1}\)), which is attributed to further electron transfer giving rise to an equilibrium distribution of enzyme species in which all three cofactors are to some extent reduced. Such an equilibrium distribution is consistent with the similarity in the values for the mid-point reduction potentials for the three cofactors that we measured previously with wild-type enzyme (12). Studies with excess (10-fold over enzyme concentration) dihydroorotate produces a more complex series of absorption changes giving rise to four observable spectral species of DHODB and reduction of the enzyme beyond the 2 electron level (Fig. 4, C and D). With the membrane-bound dihydroorotate dehydrogenase of *E. coli*, binding of DHO in the dead time of the stopped-flow instrument gives rise to a ~20 nm red-shift in the spectrum of the enzyme-bound FMN (18). With DHODB, no such spectral shift was observed and the first spectrum recorded in the diode array dataset was essentially identical to that of the oxidized pure enzyme. This highlights a key difference in DHO binding between the different types of DHODB enzyme. Binding in DHODB apparently does not lead to electronic perturbation of the enzyme-bound FMN.

The concentration dependence of the three kinetic phases was investigated in single wavelength stopped-flow studies at 452 nm. The first kinetic phase shows a hyperbolic dependence on dihydroorotate concentration (Fig. 5A). Fitting to plots of observed rate constant versus DHO concentration using a hyperbolic expression produced a dissociation constant for the complex of oxidized enzyme and dihydroorotate of 244 ± 9 \(\mu\)M and a limiting rate constant for flavin reduction of 522 ± 8 s\(^{-1}\). The second (B → C, ~22 s\(^{-1}\)) and third (C → D, ~1.6 s\(^{-1}\)) kinetic phases are essentially independent of substrate concentration in the pseudo first-order regime and are attributed to internal electron transfer and further reduction of the enzyme by DHO (Fig. 5B). The lack of a dependence of the rate constants for these two phases on DHO concentration is consistent with the intrinsic rate of FMN reduction by substrate being faster than the rate of internal electron transfer within DHODB.\(^3\)

\(^3\) Alternatively, some step in the course of flavin reduction subsequent to formation of the Michaelis complex (e.g. product release) could also rate-limit electron transfer and account for the lack of dependence of these rate constants on DHO concentration.
Steady-state analyses were extended to include studies with NADH as reductant and orotate or molecular oxygen as electron acceptor (Table 2). Unlike orotate, molecular oxygen is capable of accepting electrons from the FAD and FMN cofactors. With orotate and NADH, mutation of Lys-D48 has little effect on the Michaelis constant for orotate, but leads to a significant reduction in the turnover number with this substrate (more than a 1000-fold decrease in $k_{\text{cat}}$ for K48Q DHODB compared with wild-type DHODB; Table 2). In reactions with NADH and molecular oxygen, there is at most only a 5-fold decrease in the rate of steady-state turnover for the K48Q mutant when compared with the wild-type enzyme. These data are consistent with the effects of mutation being restricted to the FMN-containing active site in the D-subunit.

**Stopped-flow Studies of the Reduction of Mutant DHODB Enzymes with Dihydroorotate**—Our steady-state studies have indicated that mutation of Lys-D48 leads to impaired steady-state turnover values for the mutant enzymes (Table 1). To assess the effects of these mutations on the kinetics of flavin reduction and subsequent internal electron transfer in DHODB we performed a series of stopped-flow studies with the substrate dihydroorotate (data summarized in Table 3). Global analysis of time-dependent absorption changes indicated that reduction of the K48R enzyme with excess substrate occurs in three kinetic phases (Fig. 6, A and B). In the first kinetic phase ($A \rightarrow B$), the flavin absorption is partially decreased. The degree of flavin reduction in the first kinetic phase is slightly less than that seen for comparable studies with the wild-type enzyme, perhaps reflecting a perturbed distribution of enzyme species at the end of this kinetic phase (Fig. 4). Some long wavelength changes develop which we attribute to the development of neutral blue semiquinone as the 2-electron reduced form of the enzyme adjusts to an equilibrium distribution at the end of the first kinetic phase. In the second kinetic phase ($B \rightarrow C$), minor absorption changes occur at long wavelength, but there is no overall change in the reduction level of the flavins. The mechanistic origins of these minor spectral changes are unclear, but they might be related to relatively slow

TABLE 1

| Enzyme   | Apparent $K_m$ DHO (μM) | Apparent $k_{\text{cat}}$ (s$^{-1}$) |
|----------|-------------------------|--------------------------------------|
| Wild-type  | $90 \pm 6$                | $49.3 \pm 0.8$                        |
| K48E      | $367 \pm 16$             | $0.73 \pm 0.01$                       |
| K48R      | $44 \pm 5$               | $0.42 \pm 0.01$                       |
| K48Q      | $131 \pm 11$             | $0.11 \pm 0.01$                       |
| K48A      | $195 \pm 12$             | $0.12 \pm 0.01$                       |

* For the wild-type enzyme, at a fixed concentration of L-DHO (2 mM), $K_m$ (NAD$^+$) = 62 ± 4 μM and $k_{\text{cat}} = 55 \pm 1$ s$^{-1}$.

Steady-state parameters of wild-type and mutant DHODB enzymes using DHO as electron donor and NAD$^+$ as electron acceptor.

TABLE 2

| Kinetic parameter | Wild-type DHODB | K48Q DHODB |
|------------------|-----------------|------------|
| $K_m$ DHO (μM)   | $90 \pm 6$      | $131 \pm 11$ |
| $K_m$ NAD$^+$ (μM) | $62 \pm 4$    | ND         |
| $k_{\text{cat}}$ (DHO, s$^{-1}$) | $49.3 \pm 0.8$ | $0.11 \pm 0.01$ |
| $k_{\text{cat}}$ (orotate, s$^{-1}$) | $21.3 \pm 2.2$ | $14.4 \pm 0.9$ |
| $k_{\text{cat}}$ (NADH, μM)$^b$ | $5.6 \pm 0.9$  | $1.2 \pm 0.5$  |
| $k_{\text{cat}}$ (Orate, s$^{-1}$)$^b$ | $2.2 \pm 0.1$  | $3.7 \pm 0.3$  |
| $k_{\text{cat}}$ (O$_2$, s$^{-1}$)$^b$ | $11.7 \pm 0.3$ | $11.7 \pm 0.3$ |

* ND, not determined.

* Measured at atmospheric concentrations of O$_2$.

* Measured at saturating concentrations of NADH.

Comparison of observed rate constants obtained for each kinetic phase in the reduction of DHODB and mutants by DHO

All parameters have associated errors of <5%. All measurements carried out at 25 °C except for reduction of wild-type DHODB with DHO, which was measured at 4 °C. Reduction of wild-type DHODB by DHO is too fast at 25 °C to capture the first kinetic phase. Values of rate constants shown for mutant enzymes were determined at a DHO concentration of 1 mM. Values of rate constants were independent of DHO concentration at values >1 mM DHO.

TABLE 3

| Enzyme   | $k_1$ | $k_2$ | $k_3$ |
|----------|-------|-------|-------|
| Wild-type | 522   | 22    | 1.6   |
| K48R     | 1.24  | 0.18  | 0.014 |
| K48E     | 2.8   | 0.04  | 0.002 |
| K48Q     | 0.43  | 0.1   | 0.01  |
| K48A     | 0.3   | 0.03  | 0.002 |

* The value of $k_i$ for wild-type enzyme is a limiting rate constant at saturating substrate concentration calculated from a plot of the observed rate constant versus DHO concentration.

FIGURE 5. Concentration dependence of observed rate constants for each reaction phase in the reduction of DHODB with DHO. Main panel, plot illustrating the DHO concentration dependence of the rate ($k_{\text{cat}}$) of the fast phase observed at 452 nm for wild-type DHODB. The limiting rate for flavin reduction for the fast phase (estimated using the Strickland equation) was calculated to be 522 ± 8 s$^{-1}$, and the dissociation constant for the oxidized enzyme-dihydroorotate complex is 244 ± 9 μM. Inset, concentration dependence of the rate for the intermediate (open circles) and slow (closed circles) phases of the triphasic transitions observed at 452 nm. Reaction conditions: 50 mM bis-Tris propane/50 mM phosphate buffer pH 8.0; 4 °C. Enzyme concentration, 2.3 μM.
product release. In the third kinetic phase (C → D), there is further substantial bleaching of the flavin absorption at 452 nm as the enzyme is reduced further by substrate.

The rate constants for each kinetic phase are substantially slower than those associated with the absorption changes for the wild-type enzyme (Table 3). Comparable studies with the K48E, K48A, and K48Q mutant enzymes also indicated that flavin reduction is substantially compromised compared with the wild-type enzyme (Table 3) and absorption changes associated with enzyme reduction were similar to those observed for the K48R DHODB.4

We have shown that addition of methyamine to stopped-flow reactions of the mutant DHODB enzymes results in partial rescue of the FMN reduction rate in a concentration-dependent manner (supplementary material, Fig. S3). Methyamine has no affect on the flavin reduction kinetics for wild-type DHODB. That the FMN reduction activity of the mutant enzymes is partially restored on addition of methyamine suggests a role for Lys-D48 in electrostatic stabilization of developing negative charge on the O4 atom of the FMN during reduction of the enzyme by dihydroorotate.

**Flavin Reduction Levels during Steady-state Turnover of Wild-type and Mutant Enzymes Revealed by Enzyme-monitored Turnover**—We also probed steady-state turnover in the wild-type and K48Q enzymes using the enzyme monitored turnover method to assess the effects of mutating Lys-D48 on the reduction level of the enzyme. Profiles from enzyme monitored turnover studies using (i) NADH and oxygen and (ii) NADH and orotate as substrates with wild-type and K48Q enzyme are shown in Fig. 7. With wild-type enzyme, the steady-state spectrum (spectrum 2) indicates that the enzyme is essentially fully reduced during turnover with oxygen (Fig. 7A) or orotate (Fig. 7C) as electron acceptors, indicating that enzyme reduction and internal electron transfer within wild-type enzyme are fast relative to reoxidation of the cofactors by orotate or oxygen. Comparable studies with the K48Q enzyme indicate that the flavins are approximately only half reduced during steady-

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4 Given the slow rate constant for the fast phase in the mutant enzymes, it was not possible to measure enzyme-substrate dissociation constants using stopped-flow methods because the first and second kinetic phases could not be resolved at lower substrate concentrations.
state turnover (Fig. 7, B and D), suggesting more of a balance between the rates of reductive and oxidative processes. The FAD reduction kinetics of the K48Q mutant are essentially unaffected by mutation as demonstrated by stopped-flow mixing studies of FAD reduction by NADH (Fig. 8). That the flavin reduction level during steady-state turnover is less in the mutant enzyme compared with wild-type suggests a bottleneck on the rate of electron transfer from the 2Fe-2S center to FMN. The steady-state spectrum, therefore, predominantly represents reduced FAD and oxidized FMN.

We also performed enzyme monitored turnover studies with (i) dihydroorotate and oxygen as substrates and (ii) dihydroorotate and NAD\(^+\) using both wild-type and K48Q enzymes (Fig. 9). With the wild-type enzyme, the steady-state spectrum indicates that both flavin cofactors are essentially reduced during turnover with either electron acceptor (Fig. 9, A and C). In the K48Q enzyme, with dihydroorotate and NAD\(^+\) as substrates, both the FMN and FAD cofactors are essentially oxidized during steady-state turnover (Fig. 9D). This suggests a kinetic bottleneck in enzyme reduction (i.e. FMN reduction by DHO), which is consistent with our stopped-flow studies (Table 3). The situation with molecular oxygen as electron acceptor is more complex (Fig. 9B). In this case, the absorption profile at 452 nm for the K48Q enzyme with dihydroorotate and oxygen has
more phases than the corresponding profile obtained with dihydroorotate and NAD\(^+\) (Fig. 9, B and D, insets). The steady-state phase (spectrum 2, Fig. 9D), following depletion of oxygen, FMN is reduced (yielding spectrum 3), but there is a delay in obtaining further reduction of the enzyme (spectrum 4). The reasons for this are not clear, but we note that in stopped-flow studies of enzyme reduction with DHO alone (Fig. 6B) we obtained evidence for two intermediates with very similar absorption properties (species B and C in Fig. 6B). We speculated that conversion of species B to C might involve a slow product release step (i.e. slow product release from an enzyme species containing reduced FMN). Assuming this to be the case, the slow product release step would occur prior to conversion of spectrum 3 to spectrum 4 and this might account for the delayed further reduction of the enzyme (i.e. conversion of species 3 to 4). Despite difficulties in assigning observed spectral changes to mechanistic steps after the steady-state phase, it is clear with both NAD\(^+\) and dioxygen as electron acceptor that the flavins are essentially oxidized during steady-state turnover. This observation is consistent with impaired FMN reduction by substrate and a change in rate-determining step compared with wild-type enzyme.

Retention of the FMN/2Fe-2S Spin-interacting State on Mutating Lys-D48—EPR spectra of dithionite-reduced mutant forms of DHODB altered at Lys-D48 exhibit signals at 15K. An example is shown in Fig. 10A. We have previously established that these signals arise from the 2Fe-2S cluster and from a spin-spin interaction (spin-coupling) between this cluster and the flavosemiquinone form of the FMN cofactor (12). The rhombic 2Fe-2S cluster signal exhibits the same g values, 2.04, 1.95, 1.89, in all four mutants studied, K48R, K48Q, K48E, and K48A, and these values are the same as those determined from the EPR spectrum of the wild-type protein (12). Because the g values are dependent on the geometry of the cysteine residues ligating the cluster (19), the maintenance of these g values in the mutant enzymes suggests that the mutations do not affect the structure of the 2Fe-2S cluster. The spin-coupled signal is formed in all the mutant proteins, showing that spin-coupling is not specific for the presence of a lysine residue at position Lys-D48. This also suggests that the superexchange paths that contribute to the efficiency of electron transfer (20) between the FMN and 2Fe-2S cluster are essentially unperturbed. However, the formation of the spin-coupled signal in the mutants shows an altered dependence on the number of electron equivalents supplied when compared with wild type protein. Fig. 10B shows maximum formation of coupled signal at three electron equivalents for all four mutants, compared with just over two...
FIGURE 11. Molecular graphics representation of the change in side-chain location for Lys-D48 and interactions formed on binding dihydroorotate. The arrows indicate the flow of electrons following proton abstraction from the C5 atom of dihydroorotate by Cys-D135. In this scheme, proton transfer is shown from the e-amino group of Lys-D48 to the O4 atom of the FMN isoaflavin ring (i.e. the proposed charge neutralization scheme). A and B represent crystallographically defined water molecules. The figure was produced using PyMol software (23).

electron equivalents for wild-type enzyme (12). Given the origin of the spin-coupled signal and that the EPR signal of the 2Fe-2S cluster is unperturbed, this suggests a decrease in the mid-point potential of the oxidized-flavosemiquinone couple of the FMN. EPR experiments conducted at a higher temperature, 120 K, allow for measurement of the uncoupled flavosemiquinone spectrum in isolation (12). Quantitation of flavosemiquinone from these spectra (Fig. 10C) shows the same trend as the spin-coupled signal, but because only the flavosemiquinone signal appears in such spectra the quantitation is more accurate and allows for more subtle trends to be observed. The maxima in Fig. 10C may suggest that natural non-coupled flavosemiquinone is most difficult to form in K48R and easiest to form in the K48E mutant, although the data are not sufficient to allow for a determination of the potentials involved. Furthermore, the shapes of the curves beyond the maxima (i.e. when yet more electron equivalents have been added) suggest that reduction to the flavosemiquinone to the hydroquinone (i.e. the 2-electron reduced) state of the FMN cofactor is also more difficult in some mutants, particularly the K48E mutant. This suggests that the mid-point potential for the second electron reduction is also more negative in these mutants than in wild-type enzyme.

The qualitative observations concerning the stabilization of redox states of the FMN in the mutant enzymes arising from our analysis of EPR data were confirmed using potentiometric analysis, as described previously for wild-type enzyme (12). A detailed description of the methods used and the determined potentials for the mutant enzymes are found in supplemental data (Fig. S4). Overall the potentiometric data indicate that the flavin and 2Fe-2S couples are perturbed by only a small extent following mutation, and that the small perturbations observed are consistent with the need to reduce the mutant proteins further to maximize the signal for the spin-interacting state.

DISCUSSION

Our kinetic data demonstrate that FMN reduction by (i) dihydroorotate, or (ii) electron transfer from the 2Fe-2S, is impeded in the different Lys-D48 mutant enzymes. The change in the position of the Lys-D48 side-chain on binding orotate in the active site positions the e-amino group of the side chain within hydrogen bonding distance of the FMN O4 atom, the substrate carboxyl group and a water molecule (Fig. 11). Additionally, the e-amino group of Lys-D170 is in close proximity to the FMN N10 atom. The immediate environment of Lys-D170 suggests hydrogen bonds form to a ribityl alcohol group, the FMN O3 atom and a water molecule, which might have a role in stabilization of the reduced FMN, although the geometry is less favorable for this role compared with that observed for Lys-D48. The location of Lys-D48 in orotate-bound DHODB suggests that neutralization of the semi- or dihydroquinone FMN is possible through formal proton transfer to the FMN O4 atom by the e-amino group of Lys-D48 (Fig. 11). The compromised kinetics of FMN reduction by dihydroorotate (Table 3), and reverse electron transfer from the 2Fe-2S center (Fig. 7, B and D), are consistent with such a role for Lys-D48. Moreover, the partial rescue of FMN reduction activity observed on the addition of methylamine to the mutant DHODB enzymes (Fig. S3, supplemental data) is also consistent with this proposed role for Lys-D48.

We were surprised to find that our EPR (Fig. 10) and potentiometry studies (supplemental data Fig. S4) indicate mutation of Lys-D48 does not perturb substantially the mid-point reduction potentials of the flavin and 2Fe-2S cofactors. Given the proposed role of Lys-D48 in charge neutralization (through formal proton transfer to the FMN O4 atom) one might expect the mid-point potentials of the FMN to be considerably perturbed as a result of mutation. We emphasize, however, that both the potentiometry and EPR measurements were made with enzyme reduced by dithionite and not the substrate dihydroorotate. At this stage, it is not known if reduction of the cofactors by dithionite is sufficient to recruit the side chain of Lys-D48 to the position observed in the crystal structure of the DHODB-orotate complex, or whether additional interactions (e.g. the interaction of Lys-D48 with the carboxylate group of dihydroorotate) are also required to promote this conformational change. If reduction by dithionite is unable to recruit Lys-D48 to the position seen in the enzyme-orotate complex, then the environment of the FMN in both wild-type and mutant forms of DHODB will be similar in our EPR and potentiometric titrations. In this case, one might expect only minor perturbation of the mid-point potentials of the FMN and 2Fe-2S centers, consistent with our experimental observations. We suggest that if charge neutralization of the O4 atom requires proton transfer, and in a situation where reduction by dithionite does not recruit Lys-D48 to fulfill this role, it is likely this proton could be recruited from solvent under equilibrium titration conditions. In a stopped-flow turnover situation using dihydroorotate as substrate, we expect this proton transfer to be from Lys-D48 in wild-type enzyme. In

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5 We emphasize that EPR studies are performed at low temperature unlike kinetic and potentiometric measurements (using spectroelectrochemical methods). Also, in kinetic studies we cannot rule out the possibility that charge-transfer states might contribute to long wavelength absorption. For these reasons, a comparison and rationalization of the degree of semiquinone stabilization using these different methods is unreliable.
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the mutant proteins, however, this proton transfer would need to be from solvent and this could account for the slow FMN reduction rates observed with the mutant enzymes.

The retention of the spin-interacting state in the mutant DHODB enzymes indicates clearly that superexchange pathways between the FMN_{red} and reduced 2Fe-2S center are essentially unperturbed. We infer, therefore, that the slow rates of electron transfer from the 2Fe-2S center to FMN, as observed in our enzyme monitored turnover studies (Fig. 7, B and D), are not attributed to weaker electronic coupling between these two centers. This is consistent with the robust nature of electronic coupling in proteins over relatively short distances, which buffers against adverse affects on the coupling matrix element following mutation of an electron tunneling pathway (21).

Studies with the class 2, membrane-associated human DHOD have also implied a major role for an active site lysine residue in charge stabilization during reduction of the enzyme-bound FMN by dihydroorotate (22). In this case, the redox chemistry involves only a single FMN cofactor, as electron transfer is from substrate to FMN and then to ubiquinone. The crystal structure of the class 1A DHOD enzyme from L. lactis also reveals an active site lysine residue that is appropriately positioned to assist in charge stabilization/protonation in the reductive half-reaction. Our studies with the more complex class 1B enzyme have established similarities in the requirement for an active site lysine residue to stabilize or neutralize negative charge in the FMN isoalloxazine ring, and thus emphasize a common mechanism for facilitating rapid electron transfer from substrate to the FMN cofactor in both soluble and membrane-bound DHOD enzymes.

Concluding Remarks—Lys-D48 is required to maintain rapid rates of FMN reduction and internal electron transfer in DHODB through a mechanism that involves electrostatic stabilization of, or charge neutralization by proton transfer to, the O4 atom of the FMN isoalloxazine ring. Mutation of Lys-D48 does not affect electronic coupling between the 2Fe-2S and FMN centers as superexchange pathways are maintained in mutant enzymes, giving rise to the characteristic spin-interacting state seen in wild-type enzyme. Our study establishes a common mechanism for enhancing electron transfer in both soluble (class 1B) and membrane-bound DHOD (class 2) enzymes and emphasizes the need to stabilize the reduced form of the flavin isoalloxazine ring in flavoprotein catalysis.

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