The Comparative Interaction of Quinonoid (6*R*)-Dihydrobiopterin and an Alternative Dihydropterin Substrate with Wild-Type and Mutant Rat Dihydropteridine Reductases†

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ABSTRACT: Kinetic parameters and primary deuterium isotope effects have been determined for wild-type dihydropteridine reductase (EC 1.6.99.7) and the Ala133Ser, Lys150Gln, Tyr146His, Tyr146Phe single, and Tyr146Phe/Ala133Ser and Tyr146Phe/Lys150Gln double mutant enzyme forms using the natural substrate, quinonoid (6*R*)-L-*erythro*-dihydrobiopterin (qBH_2) and an alternate substrate, quinonoid 6,7-dimethyldihydropteridine (q-6,7-diMePtH_2). Mutation at either Tyr146 or Lys150 resulted in pronounced changes in kinetic parameters and isotope effects for both pterin substrates, confirming a critical role for these residues in enzyme-mediated hydride transfer. By contrast, the Ala133Ser mutant was practically indistinguishable from wild-type enzyme. The changes observed, however, were quite different for the two pterin substrates. Thus, k_{cat} for q-6,7-diMePtH_2 decreased across the series of mutants from a value of 150 s^{-1} for wild-type enzyme to essentially zero activity for the Tyr146Phe/Lys150Gln double mutant. Conversely, k_{cat} for qBH_2 increased 3−11-fold across the same series of mutants from the wild-type value of 23 s^{-1}. For both pterin substrates, the K_m (K_{Pt}) increased several orders of magnitude upon mutation of Tyr146 or Lys150, with the greater relative increase using qBH_2. Significant primary deuterium isotope effects on k_{cat} ($^Dk_{cat}$) and k_{cat}/K_{Pt} ($^D(k_{cat}/K_{Pt})$) observed for the Tyr146 and Lys150 mutants varied depending on the pterin substrate used and ranged up to a maximum value of 5.5−6. For qBH_2, where $^Dk_{cat} < {}^Dk_{cat}/K_{Pt}$ was consistently observed, the rate determining step is ascribed to release of the tetrahydropterin product. For q-6,7-diMePtH_2, where in all cases $^Dk_{cat} = {}^Dk_{cat}/K_{Pt}$, catalysis is probably limited by an isomerization step occurring prior to hydride transfer. Modeling studies in which qBH_2 was docked into the binary E:NADH complex provide a structural rationale for the observed differences between the two pterin substrates. The natural substrate, qBH_2, displays a higher affinity for the enzyme active site, presumably due to interaction of the dihydroxypropyl side chain of the substrate with a polar loop of residues containing Asn186, Ser189, and Met190. The location of this loop within the three-dimensional structure is consistent with putative substrate binding loops for other members of the short chain dehydrogenase/reductase (SDR) family, which includes dihydropteridine reductase.

Dihydropteridine reductase (DHPR) (EC 1.6.99.7) catalyzes the NADH-mediated reduction of quinonoid dihydrobiopterin (qBH_2) to (6*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin (BH_4). In liver, adrenal, and nerve tissues, BH_4 is the cofactor for the enzymatic hydroxylation of the aromatic amino acids: phenylalanine, tyrosine, and tryptophan (Kuhn & Lowenberg, 1985; Kaufman & Kaufman, 1985; Shiman, 1985). Figure 1 illustrates the reductive pathway to tetrahydrobiopterin and depicts the link between the hydroxylases and DHPR. Genetic defects in the hydroxylation process give rise to clinical disorders known as phenylketonuria (PKU) (Kaufman et al., 1975; Armarego et al., 1984; Whiteley et al., 1993), which includes defects in the structure of DHPR, resulting in a diminished efficiency of qBH_2 reduction.

DHPR has an ordered kinetic mechanism in which NADH binds first and NAD^+ is released last; a typical bi-bi sequential reaction initially shown by inhibition studies (Askenes & Ljones, 1980) and later corroborated by the binary E:NADH crystal structure (Varughese et al., 1992). Previous experiments to ascertain the rate-determining step for the reaction catalyzed by wild-type DHPR used isotope and stopped-flow rapid reaction techniques with quinonoid 6,7-dimethyl dihydropteridine (q-6,7-diMePtH_2) as the substrate (Poddar & Henkin, 1984). Presteady state rates were observed to be comparable to steady state rates, and no burst of NADH utilization was detected, indicating that product off was not the rate-limiting step. Also, no difference in reaction rate was observed using the deuterated and non-

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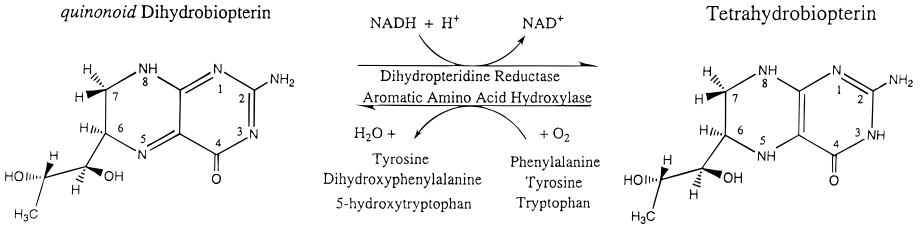

FIGURE 1: Enzymatic cycling of tetrahydrobiopterin between DHPR and the aromatic amino acid hydroxylases. The alternative substrate is the racemic quinonoid 6,7-dimethylpteridine in which the 6-dihydroxypropyl and a 7-hydrogen substituent are replaced by methyl groups. The mechanism described for the reduction pathway indicates protonation occurs at the pteridine 0−4 position; however, the conventional 3,4-amido structure is used for the tetrahydropteridine in this figure as rapid equilibration would occur after product release (Blakley, 1969).

deuterated nicotinamide cofactors in the steady state. Together, these observations suggested that a slow isomerization step, possibly a conformational change, occurs at the ternary E:NADH/q-6,7-diMePtH_2 complex stage of the reaction prior to hydride transfer. Whether the same holds true, however, for the natural substrate, qBH_2, has not been determined.

This report describes the determination of the kinetic parameters and kinetic isotope effects for the NADH/NADD-mediated reduction reaction catalyzed by wild-type and Ala133Ser, Lys150Gln, Tyr146His, Tyr146Phe, Tyr146Phe/Ala133Ser, and Tyr146Phe/Lys150Gln mutants of DHPR using the natural substrate, qBH_2, and the alternative substrate, q-6,7-diMePtH_2. Evidence has been obtained for a change in the relative contribution of various steps in the mechanism to the overall rate limitation of the DHPR-catalyzed reaction. Analysis of isotope effects obtained for wild-type and mutant enzymes has allowed assignment of rate-limiting steps in the reaction sequence. In addition, the ability to estimate hydride transfer rates in several instances provides insight into the roles that Tyr146, Lys150, and a Ser mutation introduced at position 133 play in the enzymatic activity of DHPR and provides a foundation for the participation of the Tyr-$(Xaa)_3$-Lys motif and serine in a general mechanism for all members of the SDR family.

EXPERIMENTAL PROCEDURES

Mutants of DHPR were generated by overexpression of the pKQV4 plasmid in the DH5α strain of *Escherichia coli* as described previously (Kiefer et al., 1996). The only novel mutant prepared for this report, namely the Ala133Ser/Tyr146Phe double mutant, was assembled using a composite of the previously reported individual mutation sequences. Protein purification, elimination of trace impurities and isolation of apoenzyme by HPLC, and SDS−PAGE analysis were performed as in previous DHPR studies (Matthews et al., 1991; Kiefer et al., 1996). 2-Amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropterin was obtained from CalBiochem (La Jolla, CA) and (6*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin from Dr. B. Schircks Laboratories (Jona, Switzerland).

Kinetic Measurements. DHPR initial velocities were determined using a Cary 219 spectrophotometer by following the oxidation of NADH at 340 nm according to the procedure of Webber and Whiteley (Webber et al., 1978). Kinetic constants were obtained from duplicate or triplicate measurements of initial rates under conditions that varied pterin and NADH(D) concentrations. Reactions were carried out in 0.04 M Tris pH 6.8 at 25 °C. The substrates q-6,7-diMePtH_2 and qBH_2 were generated *in situ* from the corresponding tetrahydropterin using 0.5 mM $K_3Fe(CN)_6$. Including a strong oxidant such as $K_3Fe(CN)_6$ in the reaction mixture is necessary to generate the dihydro substrate *in situ*, because quinonoid dihydropteridines are unstable, making any attempt at purification of the pterin substrate prior to use in enzymatic assays futile (Armarego et al. 1984). Duplicate blank rates measured in the absence of enzyme were thus used to correct the observed rates for nonenzymatic breakdown of the quinonoid dihydropteridine substrate and oxidation of NADH (Armarego et al., 1983). In order to confirm the reproducibility of the enzymatic rates, an alternate assay procedure employing hydrogen peroxide and peroxidase as oxidant (Armarego et al., 1983) was also used in selected experiments. In every case the rates observed were consistent with those observed with $K_3Fe(CN)_6$. All results were analyzed using the Fortran fitting programs of Cleland (Cleland, 1979), with all concentrations of substrates and enzyme measured spectrophotometrically.

Kinetic Isotope Effects. Stereospecifically deuterium labeled (4*S*)-[4-D]NADD and unlabeled NADH were prepared in identical manner adding glucose-*1-d* (or 1-H) and NAD^+ in a 1:1 ratio to a mixture containing glucose-6-phosphate dehydrogenase, 40% DMSO, and 60 mM phosphate buffer, pH 8, as described previously (Viola et al., 1979). High-quality DMSO (Pierce, Rockford, IL) was necessary for >80% conversion of NAD^+ to NADH. NAD^+ was obtained from Sigma (St. Louis, MO), glucose-6-phosphate dehydrogenase from CalBiochem (La Jolla, CA), and glucose-*1-d* from Isotec, Inc. (Miamisburg, OH). After incubation for 24 h at 25 °C the NADD(H) was separated from the reaction mixture by chromatography on a Q-sepharose column using a published protocol (Orr & Blanchard, 1984). Fractions with $A_{260}/A_{340} < 2.5$ were pooled and lyophilized for later use. To ensure true comparison of the kinetic results obtained, both the deuterated and non-deuterated coenzymes used in the initial velocity experiments were prepared and purified using the same procedures.

Initial velocity experiments to look for solvent isotope effects were carried out in the presence of D_2O. All deuterated buffers were made by lyophilizing the corresponding H_2O buffer at the required pH and then resuspending the residue in D_2O. D_2O (>98% D content) was obtained from Isotec, Inc.

Model for the Ternary Complex. qBH_2 was positioned in the wild-type active site according to a previously described procedure by restricting N5 of the pteridine substrate molecule to a distance of ∼3.3 Å from the C4 position of the nicotinamide ring of bound NADH (Kiefer et al., 1996; Varughese et al., 1992). The best geometrical fit that avoids acute steric interactions has the *re*face of the pteridine ring rotated above the nicotinamide with the 6-dihydroxypropyl

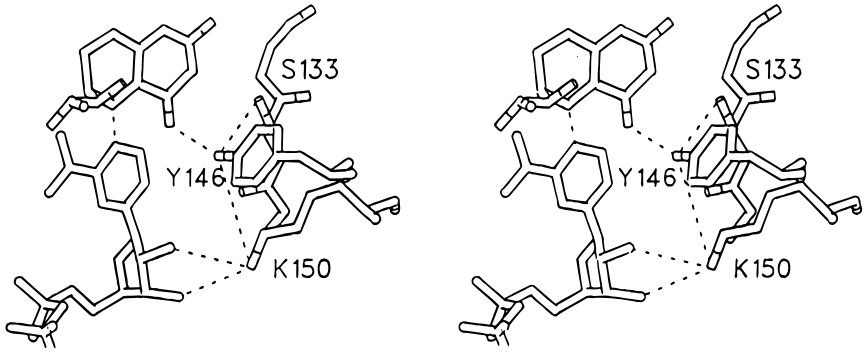

FIGURE 2: Stereoview of the ternary complex model of the A133S mutant of DHPR with NADH and qBH_2 highlighting the interaction (dashed lines) between Tyr146, Lys150, and Ser133 and the pterin substrate.

Table 1: Kinetic Parameters for q-6,7-diMePtH_2 with Wild-Type and Mutant Rat DHPR

enzyme	k_{cat}[a] (s^{-1})	K_{NH}[b] (μM)	K_{Pt}[c] (μM)	k_{cat}/K_{NH} ($\mu M^{-1}\,s^{-1}$)	k_{cat}/K_{Pt} ($\mu M^{-1}\,s^{-1}$)	$^{D}k_{cat}$[d]	$^{D}(k_{cat}/K_{NH})$[d]	$^{D}(k_{cat}/K_{Pt})$[d]
wild-type	153[e]	13[e]	27[e]	11.9	5.7	0.9 ± 0.2	1.0 ± 0.1	0.9 ± 0.1
Ala133Ser	152	22	8	6.9	19.0	1.1 ± 0.1	0.8 ± 0.2	0.9 ± 0.1
Lys150Gln	52	13	290	4.0	0.2	2.9 ± 0.7	1.4 ± 0.6	3.0 ± 1.2
Tyr146His	36	8	170	4.5	0.2	4.5 ± 0.9	1.3 ± 0.8	4.6 ± 1.5
Tyr146Phe	17	3	460	5.7	0.04	~8		
Tyr146Phe/Ala133Ser	2	2	100	1.0	0.02	7.0 ± 2.0	1.0 ± 0.7	5.5 ± 2.1
Tyr146Phe/Lys150Gln								

[a] The k_{cat} values are based on the dihydropteridine reductase monomer molecular weight of 25 420. [b] K_{NH} is the K_m for the reduced nicotinamide cofactor, NADH. [c] K_{Pt} is the K_m for the quinonoid dihydropterin substrate. [d] $^{D}k_{cat} = (k_{cat,H}/k_{cat,D})$ and similarly, $^{D}(k_{cat}/K_{NH}) = (k_{cat}/K_{NH})_H/(k_{cat}/K_{NH})_D$; $^{D}(k_{cat}/K_{Pt}) = (k_{cat}/K_{Pt})_H/(k_{cat}/K_{Pt})_D$. [e] Taken from Kiefer et al., 1996.

Table 2: Kinetic Parameters for qBH_2 with Wild-Type and Mutant Rat DHPR

enzyme	k_{cat}[a] (s^{-1})	K_{NH}[b] (mM)	K_{Pt}[c] (mM)	k_{cat}/K_{NH} ($mM^{-1}\,s^{-1}$)	k_{cat}/K_{Pt} ($mM^{-1}\,s^{-1}$)	$^{D}k_{cat}$[d]	$^{D}(k_{cat}/K_{NH})$[d]	$^{D}(k_{cat}/K_{Pt})$[d]
wild-type	23	5	0.4	4.6	57.5	1.0 ± 0.1	0.9 ± 0.3	1.3 ± 0.4
Ala133Ser	20	4	0.4	5.0	50.0	1.0 ± 0.1	1.0 ± 0.3	1.4 ± 0.4
Lys150Gln	269	79	28	3.4	9.6	2.1 ± 0.3	0.9 ± 0.3	5.2 ± 1.8
Tyr146His	139	24	7	5.8	19.9	2.4 ± 0.2	1.2 ± 0.2	5.9 ± 0.9
Tyr146Phe	138	51	308	2.7	0.5	7.0 ± 1.5	0.7 ± 0.3	5.8 ± 1.9
Tyr146Phe/Ala133Ser	74	8	24	9.3	3.1	2.2 ± 0.2	0.8 ± 0.3	8.0 ± 2.0
Tyr146Phe/Lys150Gln	~200	~20	>1000	~10	<0.2	~5		

[a] The k_{cat} values are based on the dihydropteridine reductase monomer molecular weight of 25 420. [b] K_{NH} is the K_m for the reduced nicotinamide cofactor, NADH. [c] K_{Pt} is the K_m for the quinonoid dihydropterin substrate. [d] $^{D}k_{cat} = (k_{cat,H}/k_{cat,D})$ and similarly, $^{D}(k_{cat}/K_{NH}) = (k_{cat}/K_{NH})_H/(k_{cat}/K_{NH})_D$; $^{D}(k_{cat}/K_{Pt}) = (k_{cat}/K_{Pt})_H/(k_{cat}/K_{Pt})_D$.

substituent pointing away from the nicotinamide ring (see Figure 2). This model forms the basis for the structural and mechanistic explanations described in this report.

RESULTS

The kinetic parameters and primary deuterium isotope effects measured using NADH versus NADD are significantly different for the two dihydropterin substrates. Tables 1 and 2 contain the essential kinetic parameters as well as the $^{D}k_{cat}$ and $^{D}k_{cat}/K_m$ values for the reaction catalyzed by wild-type and mutant DHPR enzymes using q-6,7-diMePtH_2 and qBH_2, respectively. The large uncertainties for the isotope effects shown in Tables 1 and 2 are due to the inherent limitations of the enzyme assay, which are unavoidable as discussed in the Methods section. Thus, high concentrations of pterin substrate required for analysis of several DHPR mutants resulted in high blank rates due to breakdown of the quinonoid dihydropteridine substrate and nonenzymic oxidation of NADH. To minimize the blank rate contribution, the maximum pterin concentration was limited to ≤ 200 μM, which created somewhat greater uncertainty in the kinetic parameters of mutants with large pterin K_m values (e.g., Tyr146Phe in Table 1 and Tyr146Phe/Lys150Gln in Table 2). In addition, the rapid enzyme-mediated oxidation of NADH adds uncertainty when obtaining kinetic parameters for mutants with a small NADH K_m value (e.g., Tyr146Phe and Ala133Ser/Tyr146Phe in Table 1). Nevertheless, the results are consistent and trends are clearly discernable.

Alternate Substrate, q-6,7-diMePtH_2. As shown in Table 1, k_{cat} for enzyme-catalyzed reduction of q-6,7-diMePtH_2 is highest for wild-type DHPR (153 s^{-1}) and then decreases upon mutation of either Tyr146 or Lys150, from 3-fold for the Lys150Gln mutant to an undetectable level of activity for the Tyr146Phe/Lys150Gln double mutant. The decrease in measured K_m for NADH (K_{NH}) parallels this change, and k_{cat}/K_{NH} remains essentially constant over the series of mutant DHPR enzymes. The somewhat lower figure for the double mutant reflects the above-mentioned problems associated with using very low concentrations of NADH in the assay. The results for k_{cat}/K_{NH} are consistent with the proposed ordered bi-bi kinetic mechanism, since in that case k_{cat}/K_{NH} represents the diffusion-limited on-rate for NADH binding to the free enzyme. The average measured $k_{cat}/K_{NH} \sim 6 \times 10^6$ M^{-1} s^{-1} is in the range expected for NADH binding to an enzyme (Fersht, 1985).

The K_m value for q-6,7-diMePtH$_2$ (K_{Pt}) increases significantly with mutation of either Tyr146 or Lys150, such that k_{cat}/K_{Pt} values decrease from 30-fold for the Tyr146His and Lys150Gln mutants to more than 250-fold for the Tyr146Phe/Ala133Ser double mutant. k_{cat}/K_{Pt} is equal to the first-order rate constant for reaction at low q-6,7-diMePtH$_2$ substrate concentrations and is thus a measure of the catalytic efficiency for reduction by a particular DHPR enzyme. Interestingly, because K_{Pt} decreases 3.5-fold for the Ala133Ser mutant while k_{cat} remains constant, the Ala133Ser mutant enzyme is actually a more efficient catalyst of q-6,7-diMePtH$_2$ reduction than is the wild-type DHPR. Substituting serine for Ala133 has a similar effect of lowering the K_{Pt} value when combined with the Tyr146Phe mutation, as can be seen by comparing the K_{Pt} values for the Tyr146Phe and Tyr146Phe/Ala133Ser mutants in Table 1.

Isotope effect measurements reveal further details of the kinetic mechanism for DHPR-mediated reduction of q-6,7-diMePtH$_2$. For all of the enzymes tested, $^D(k_{cat}/K_{NH})$ does not differ significantly from a value of 1.0. This finding is again consistent with an ordered mechanism, since $^Dk_{cat}/K_m$ for the first substrate to bind must be equal to 1.0 (Cleland, 1979). For the wild-type DHPR and the Ala133Ser mutant enzyme, $^Dk_{cat}$ and $^Dk_{cat}/K_{Pt}$ values are also equal to 1.0, indicating complete masking of the intrinsic primary deuterium isotope effect on hydride transfer for these two enzymes. Mutation of either Tyr146 or Lys150 results in an increase in both $^Dk_{cat}$ and $^D(k_{cat}/K_{Pt})$ with both values increasing equally to a maximum of 5.5−6.0 for the Tyr146Phe and Tyr146Phe/Ala133Ser mutants. As will be shown below, this parallel increase in observed isotope effects for q-6,7-diMePtH$_2$ reduction with $^Dk_{cat} = {}^Dk_{cat}/K_{Pt}$ for each DHPR enzyme tested is quite different from the results obtained using the natural substrate, qBH$_2$.

Natural Substrate, qBH$_2$. As shown in Table 2, k_{cat} for qBH$_2$ reduction by wild-type DHPR (23 s^{-1}) is decreased 6-fold compared with k_{cat} for q-6,7-diMePtH$_2$ (150 s^{-1}), but because the K_{Pt} value is decreased 60-fold, the net result is a 10-fold *increase* in k_{cat}/K_{Pt} or catalytic efficiency for reduction of the natural substrate. The Ala133Ser mutant DHPR is kinetically indistinguishable from wild-type enzyme with respect to qBH$_2$ catalysis. Upon mutation of Tyr146 or Lys150, k_{cat} increases from 3- to 11-fold, with the maximum value measured for the Lys150Gln mutant (269 s^{-1}). However, because K_{Pt} values increase dramatically for these same mutants (up to >2500-fold for the Tyr146Phe/Lys150Gln double mutant), the net result is a 6-fold and 3-fold decrease in k_{cat}/K_{Pt} or catalytic efficiency for qBH$_2$ reduction catalyzed by the Lys150Gln and Tyr146His mutants, respectively, relative to wild-type DHPR. The most dramatic decrease in k_{cat}/K_{Pt} value is seen for the Tyr146Phe mutants; the Tyr146Phe single mutant shows a 115-fold decrease, while for the Tyr146Phe/Lys150Gln double mutant k_{cat}/K_{Pt} decreases more than 280-fold. Interestingly, conversion of Ala133 to serine in the Tyr146Phe/Ala133Ser double mutant results in a 6-fold recovery in catalytic efficiency relative to the single Tyr146Phe mutant, due primarily to a nearly 13-fold drop in K_{Pt} value. The Ala133Ser mutation thus appears to exert a similar effect on k_{cat}/K_{Pt} for reduction of either dihydropteridine substrate. Considering NADH, the calculated k_{cat}/K_{NH} values for qBH$_2$ reduction are again constant and equal to the values obtained using q-6,7-diMePtH$_2$, as they should be for an ordered mechanism in which NADH binding occurs prior to dihydropterin.

Comparing the isotope effect values shown in Table 2 reveals significant differences between the two dihydropterin substrates. As predicted, $^Dk_{cat}/K_{NH} = 1.0$ within experimental error for all the enzymes tested. For both wild-type and Ala133Ser mutant DHPR samples, the calculated $^Dk_{cat}$ and $^Dk_{cat}/K_{Pt}$ values are also not significantly different from 1.0. As observed for the alternative substrate, the $^Dk_{cat}$ and $^Dk_{cat}/K_{Pt}$ values for qBH$_2$ reduction both increased upon mutation of either Tyr146 or Lys150, up to a similar maximum value of 5.5−6.0. However, for qBH$_2$ catalysis the increase in $^Dk_{cat}$ value did not parallel the increase in $^Dk_{cat}/K_{Pt}$ value. Thus, for either the Lys150Gln, Tyr146His, or Tyr146Phe/Ala133Ser DHPR mutants, $^Dk_{cat}/K_{Pt}$ has already increased to the maximum value while $^Dk_{cat}$ remains in the range 2.2 ± 0.2. In addition, the increase in $^D(k_{cat}/K_{Pt})$ value from essentially 1.0 for wild-type enzyme to the maximum value of 5.5−6.0 for Lys150Gln and Tyr146His mutant DHPR occurs despite less than a 6-fold decrease in k_{cat}/K_{Pt} value. Two mutants, namely Tyr146Phe and Tyr146Phe/Lys150Gln, display maximal $^Dk_{cat}$ and $^D(k_{cat}/K_{Pt})$ isotope effects. Interestingly, in addition to the 6-fold recovery in catalytic efficiency noted previously, the conversion of Ala133 to serine in the Tyr146Phe/Ala133Ser double mutant also results in a masking of the observed $^Dk_{cat}$ value from the full effect observed for the Tyr146Phe single mutant DHPR. As will be described below, these subtle differences in the expression of isotope effects on k_{cat} and k_{cat}/K_{Pt} seen for the two dihydropterin substrates and for the various DHPR mutants reveal a great deal about the relative rates of steps within the overall kinetic mechanism.

Kinetic studies comparing reaction rates determined in H_2O versus D_2O were carried out using wild-type and mutant DHPR with the two different dihydropterin substrates. However, no significant solvent isotope effects were detected, indicating that proton transfer steps, as opposed to hydride transfer, do not contribute significantly to the overall rate limitation of the reaction for either dihydropterin substrate with either wild-type or the various DHPR mutant enzymes tested.

DISCUSSION

Previous studies have suggested that the overall slow step in the reaction catalyzed by DHPR occurs prior to hydride transfer. Thus, using wild-type enzyme and the alternate substrate q-6,7-diMePtH$_2$, no primary deuterium isotope effect was detected during steady state turnover, and no burst of NADH oxidation was observed in the presteady state (Poddar & Henkin, 1984). The results shown in Table 1 support this conclusion for wild-type enzyme and the alternate pterin substrate but reveal trends in both the kinetic parameters and isotope effects as mutations are introduced into the enzyme active site, which are quite different from those observed for reduction of the natural substrate, qBH$_2$. It is this substrate-dependent difference which defines the rate limitation of key steps within the kinetic mechanism, and thus could correlate with changes in substrate and enzyme active site structure.

In order to interpret our results, we begin by considering the ordered bi-bi kinetic mechanism shown as Scheme 1, in which NADH binds first to the free enzyme, followed by

Scheme 1: DHPR Mechanism

$$E + NADH \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_{NADH} + q\text{PtH}_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_{NADH}^{q\text{PtH}2} \xrightarrow{k_3}$$

$$E_{NAD^+}^{\text{PtH}4} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E_{NAD^+} + \text{PtH}_4 \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} E + NAD^+$$

Scheme 2: Expanded "k_3" Step Including a Prehydride Conformational Step

$$E_{NADH}^{q\text{PtH}2} \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} E_{NADH}^{*\,q\text{PtH}2} \xrightarrow{k_7} E_{NAD^+}^{\text{PtH}4}$$

dihydropterin substrate, and products are released in the order tetrahydropterin first, followed by NAD^+. The catalytic step (denoted by k_3) is considered to be irreversible since it has not been possible to demonstrate the reverse catalytic reaction (Kiefer, 1996; Poddar & Henkin, 1984). The following kinetic expressions can be readily derived from Scheme 1 for k_{cat} and k_{cat}/K_{Pt}

$$k_{cat} = (k_3k_4k_5)/(k_4k_5 + k_3k_4 + k_3k_5) = k_3/(1 + k_3/k_5 + k_3/k_4) \quad (1)$$

$$k_{cat}/K_{Pt} = k_2k_3/(k_3 + k_{-2}) = K_2k_3/(1 + k_3/k_{-2}) \quad (2)$$

and the corresponding isotope effects on k_{cat} and k_{cat}/K_{Pt}

$$^Dk_{cat} = (^Dk_3 + k_3/k_4 + k_3/k_5)/(1 + k_3/k_4 + k_3/k_5) \quad (3)$$

$$^D(k_{cat}/K_{Pt}) = (^Dk_3 + k_3/k_{-2})/(1 + k_3/k_{-2}) \quad (4)$$

where $K_2 = (k_2/k_{-2})$ is the equilibrium constant for the second step, k_3 is the catalytic step with NADH, and $^Dk_3 = (k_{3H}/k_{3D})$ is the ratio of rate constants for NADH versus NADD. These expressions for k_{cat} and k_{cat}/K_{Pt} have been cast in a form that emphasizes the important rate constant ratios which contribute to the observed rate.

Alternative Substrate, q-6,7-diMePtH$_2$. As outlined in Table 1, the results for the alternative substrate q-6,7-diMePtH$_2$ show the following trends. k_{cat} for wild-type enzyme starts high (153 s^{-1}) and then decreases with each mutation until essentially no activity is left in the Tyr146Phe/Lys150Gln double mutant. $^Dk_{cat}$ and $^D(k_{cat}/K_{Pt})$, which start at 1.0 for wild-type and the Ala133Ser mutant, increase in parallel fashion for all mutants with $^Dk_{cat} = ^D(k_{cat}/K_{Pt})$. The parallel increase in $^Dk_{cat} = ^D(k_{cat}/K_{Pt})$ requires that the same step in the reaction control the overall rate and expression of the isotope effect at both low and high pterin substrate concentrations. The absence of a presteady state burst for q-6,7-diMePtH$_2$ reduction by wild-type DHPR (Poddar & Henkin, 1984) requires that k_4 and k_5 be much faster than k_3 ($k_3/k_4 \ll 1$ and $k_3/k_5 \ll 1$ in eq 1), thus ruling out product release as the controlling step. In fact, the only step which satisfies these criteria and is consistent with all four expressions (eqs 1−4) is the catalytic step itself, k_3. However, because $^Dk_{cat} = ^D(k_{cat}/K_{Pt}) = 1.0$ for wild-type enzyme, k_3 cannot simply represent hydride transfer, but must include a preceding non-isotope-dependent step. As shown in Scheme 2, the catalytic step has therefore been separated into two steps: a reversible isomerization step (k_6) followed by an irreversible hydride transfer step (k_7). It is the former step, represented by k_6, which we ascribe to the slow step in the reduction of q-6,7-diMePtH$_2$ catalyzed by wild-type DHPR.

Using Scheme 2, we can rationalize in qualitative terms the results obtained with q-6,7-diMePtH$_2$, by replacing k_3 and $^Dk_3$ in eqs 1 and 3 with the following expressions

$$k_{cat} = \text{“}k_3\text{”} = k_6\,k_7/(k_{-6} + k_6 + k_7) = k_7f_{E^*}/[1 + (k_7/k_6)f_{E^*}] \quad (5)$$

$$^Dk_{cat} = \text{“}^Dk_3\text{”} = [^Dk_7 + (k_7/k_6)f_{E^*}]/[1 + (k_7/k_6)f_{E^*}] \quad (6)$$

where $K_6 = (k_6/k_{-6})$ is the equilibrium constant for the isomerization step, $f_{E^*} = K_6/(1 + K_6)$ represents the equilibrium fraction of ternary E:NADH/q-6,7-diMePtH$_2$ complex present in the active form, and $^Dk_7 = (k_{7H}/k_{7D})$ is the ratio of rate constants for hydride versus deuteride transfer. Analogous expressions can be derived for eqs 2 and 4 as follows:

$$k_{cat}/K_{Pt} = k_2k_6k_7/(k_{-2}\,k_{-6} + k_{-2}\,k_7 + k_6\,k_7) = K_2K_6k_7/[1 + (k_7/k_{-6})(1 + k_6/k_{-2})] \quad (7)$$

$$^D(k_{cat}/K_{Pt}) = [^Dk_7 + (k_7/k_{-6})(1 + k_6/k_{-2})]/[1 + (k_7/k_{-6})(1 + k_6/k_{-2})] \quad (8)$$

In order to observe $^Dk_{cat} = ^D(k_{cat}/K_{Pt})$, eqs 6 and 8 must simplify such that the same partition ratio controls the expression of the intrinsic isotope effect on k_7. This will be true if two conditions are satisfied. First, the pterin substrate must not be "sticky" (Cleland, 1979) ($k_{-2} \gg k_6$), and hence $(k_6/k_{-2}) \approx 0$ for q-6,7-diMePtH$_2$ catalysis. Second, the isomerization step must be unfavorable ($k_{-6} \gg k_6$), such that $K_6 \approx 0$. Under these conditions, the partition ratio (k_7/k_{-6}) becomes the dominant factor controlling expression of the isotope effect in eqs 6 and 8. Thus, if $k_7 \gg k_{-6}$, a dominant forward propensity exists and the reaction is committed to proceed forward through catalysis once the isomerization has occurred. In that case, k_{cat} becomes equal to k_6, and because k_6 corresponds to a non-isotope-dependent step, we have $^Dk_{cat} = ^Dk_6 = 1.0$. Conversely, for $k_7 \ll k_{-6}$, the rate-limiting step becomes hydride transfer, with $k_{cat} = f_{E^*}k_7 \approx K_6k_7$ leading to full expression of the intrinsic isotope effect $^Dk_{cat} = ^Dk_7$.

The effect of mutations on the kinetic parameters and isotope effects observed for q-6,7-diMePtH$_2$ reduction can best be described by considering that the overall reaction rate is controlled by only two rate constants, namely k_6 and k_7. As mutations are introduced into the enzyme active site and the hydride transfer rate is slowed, the $k_7/(k_6 + k_{-6})$ ratio decreases resulting in an overall decrease in k_{cat}. Thus, for wild-type and Ala133Ser mutant DHPR where $^Dk_{cat} = 1.0$, the $k_7/(k_6 + k_{-6})$ ratio must be quite large in order to completely mask the intrinsic isotope effect. The fact that the Tyr146Phe and Tyr146Phe/Ala133Ser mutants exhibit a full isotope effect of ~6 implies that $k_7/(k_6 + k_{-6}) << 1$. For the Tyr146His and Lys150Gln enzyme forms, where $1 < ^Dk_{cat} < ^Dk_7 \approx 6$, eq 7 predicts an intermediate value for the $k_7/(k_6 + k_{-6})$ ratio.

Equations 5, 6, and 8 can be employed to yield quantitative estimates for several of the rate constants describing q-6,7-diMePtH$_2$ reduction. Thus, given the k_{cat} values of 52 and 36 s^{-1} and $^Dk_{cat} = ^D(k_{cat}/K_{Pt}) = 3.0$ and 4.5 for Lys150Gln and Tyr146His mutants, respectively, and assuming $^Dk_7 = 6.0$, an average value derived from combining the maximum

isotope effects observed in Tables 1 and 2, using either substrate and within the predicted range of 4−12 for a hydride transfer step (Klinman, 1978), one obtains $k_6 = 87$ and 120 s^{-1} and $k_7 \geq 1300$ and 515 s^{-1}. The estimates for k_7 represent minimum values, because $0 \leq f_{E^*} \leq 1$ and hence $k_7 f_{E^*} < k_7$. The rate of the isomerization step for Lys150Gln- and Tyr146His-mediated catalysis is thus approximately equal to the $k_{cat} = k_6$ value for wild-type and Ala133Ser enzyme (150 s^{-1}), suggesting that the isomerization step occurs at the same rate regardless of the nature of the active site mutation. This result is consistent with an isomerization step at the ternary complex stage of the reaction consisting of a structural reorganization of either the enzyme itself, the substrate(s), or perhaps both. Assuming $k_6 \approx 120$ s^{-1} for all enzyme forms, eq 5 provides estimates for the hydride transfer rate of $k_7 \geq 190$ and ≥ 20 s^{-1} for the Tyr146Phe and Tyr146Phe/Ala133Ser mutants, respectively.

The Tyr146 and Lys150 mutant results can be contrasted with those for wild-type enzyme (and Ala133Ser mutant) where we observe $k_{cat} = 150$ s^{-1} and $^{D}k_{cat} \sim 1.0$. Given $^{D}k_7 = 6.0$ and $k_6 \approx k_{cat}$, eq 6 requires that $k_7/(k_6 + k_{-6}) \geq 10$, and k_7 for hydride transfer must be ≥ 1500 s^{-1}. Thus, using the alternate substrate, q-6,7-diMePtH$_2$, mutation of either Lys150 or Tyr146 is seen to result in a dramatic decrease in the k_7 value for hydride transfer, but relatively little change in the k_6 value. The fact that product release does not contribute significantly to rate limitation of the reaction with q-6,7-diMePtH$_2$ further requires that k_4 and k_5 have values for wild type enzyme that are greater than $10k_6$ (k_4 and $k_5 >$ 1500 s^{-1}). As we will show in the next section, such is not the case for catalysis with the natural substrate, qBH$_2$.

Natural Substrate, qBH$_2$. The trends shown in Table 2 for the kinetic parameters and isotope effects determined using qBH$_2$ are quite different from those described in the preceding section for q-6,7-diMePtH$_2$ reduction. Thus, k_{cat} with wild-type enzyme starts out 6.5-fold slower than for q-6,7-diMePtH$_2$, but k_{cat}/K_{Pt} is 10-fold higher, due to the 60-fold decrease in K_{Pt} value. As mutations are introduced, k_{cat} increases, opposite to what is observed with q-6,7-diMePtH$_2$. However, the most important difference is revealed by the isotope effects, where $^{D}k_{cat} \leq {}^{D}(k_{cat}/K_{Pt})$ in each case where significant isotope effects are detected. These results have two consequences. First, for qBH$_2$ reduction, a step outside the region of the mechanism corresponding to k_{cat}/K_{Pt} (i.e. prior to the qBH$_2$ binding step or after the first irreversible step) must now contribute to the overall rate-limitation of the reaction in order to selectively reduce expression of the isotope effect on k_{cat}. Second, the fact that $^{D}(k_{cat}/K_{Pt})$ for the Tyr146His mutant has already reached the maximum value despite only a 3-fold decrease in k_{cat}/K_{Pt} indicates that the (k_7/k_{-6}) ratio must be quite small, and further that the forward isomerization rate, k_6, must be fast. Thus, with the natural substrate $k_3 = f_{E^*}k_7 \leq k_7$, with $k_3 = k_7$ if no isomerization step is present. As was found with q-6,7-diMePtH$_2$, hydride transfer with the natural substrate is completely rate limiting for the Tyr146Phe mutant DHPR and $^{D}k_{cat} = {}^{D}(k_{cat}/K_{Pt}) \approx 6.0$.

The derived kinetic expressions again provide quantitative estimates for several rate constants describing qBH$_2$ reduction. The slow step outside the k_{cat}/K_{Pt} region for wild-type enzyme we assign to tetrahydropterin product release (or the isomerization step that allows release), described by k_4 in Scheme 1. Similar to the approach used for q-6,7-diMePtH$_2$, we then proceed to analyze the results using a simple model in which k_3 and k_4 predominantly control the overall turnover rate, as shown in eqs 9−11:

$$k_{cat} = 1/(1/k_3 + 1/k_4) = k_3/(1 + k_3/k_4) \quad (9)$$

$$^{D}k_{cat} = (^{D}k_3 + k_3/k_4)/(1 + k_3/k_4) \quad (10)$$

$$^{D}(k_{cat}/K_{Pt}) = (^{D}k_3 + k_3/k_{-2})/(1 + k_3/k_{-2}) \quad (11)$$

As with q-6,7-diMePtH$_2$, k_{-2} is faster than the slow step in k_3, giving $^{D}(k_{cat}/K_{Pt}) = {}^{D}k_3$. For the three mutants (Lys150Gln, Tyr146His, and Tyr146Phe/Ala133Ser) which display $^{D}k_{cat} \sim 2.2$, substitution into eqs 9 and 10 indicates that in each case hydride transfer contributes roughly 25% to overall rate limitation of the reaction, with k_4 contributing the remaining 75%. The estimated k_3 values are in the 310−1100 s^{-1} range, while k_4 is estimated to be in the 100−350 s^{-1} range.

The results for the DHPR mutants can again be compared with those for wild-type enzyme. Thus, for wild-type enzyme (and Ala133Ser mutant) where we observe $^{D}k_{cat} = 1.0$, $k_4 = 23$ s^{-1} must be the overall rate-limiting value for k_{cat}, resulting in complete masking of the isotope effect on hydride transfer. In order to not see any significant contribution from hydride transfer (i.e. an observed $^{D}k_{cat} \leq 1.1$ with $^{D}k_3 = 6.0$), $k_3/k_4 > 50$, and thus k_3 (which includes hydride transfer) must be >1150 s^{-1}.

Comparison of the Two Substrates. The two pterin substrates used in this study vary only in their substituents at the 6 and 7 positions. The natural substrate has a polar dihydroxypropyl group at the 6 position, and q-6,7-diMePtH$_2$ has a nonpolar methyl group at the 6 and 7 positions. Thus, the major significant difference between the two pterin substrates is the longer polar and hydrophilic side chain at the 6 position in qBH$_2$. The sample of q-6,7-diMePtH$_2$ used in this study contains a racemic mixture of 6,7-dimethyl isomers; however, it should be noted that the unnatural isomers are poor substrates for DHPR-mediated reduction (Armarego et al., 1983). The differential control of the reaction pathway between the two pteridine substrates clearly relates to this large polar substituent of qBH$_2$. With the natural substrate, conformational or isomeric changes associated with its initial accommodation are rapid and only with release of product do the associated changes become rate determining, whereas with the alternate pteridine substrate the product is rapidly lost. In this case the isomeric slow step precedes hydride transfer. It would appear that with qBH$_2$ the large polar substituent of the substrate precludes or accelerates the slow initial protein or substrate isomeric or conformational accommodation step.

The overall reaction rate for each substrate can be approximated to the sum of contributions from two steps, hydride transfer and a second slow step which differs for each substrate. With wild-type enzyme, hydride transfer is fast and the reaction rate is controlled by the pteridine-characterized slow step. As mutations are introduced, hydride transfer is slowed and becomes overall rate-limiting. This simple two-step mechanistic approximation has allowed estimation of the hydride transfer rates for each enzyme form. For the lysine and tyrosine mutants, the calculated range of hydride transfer rates of $\geq 20-1300$ and $\geq 138-1075$ s^{-1} for q-6,7-diMePtH$_2$ and qBH$_2$ reduction, respectively, is

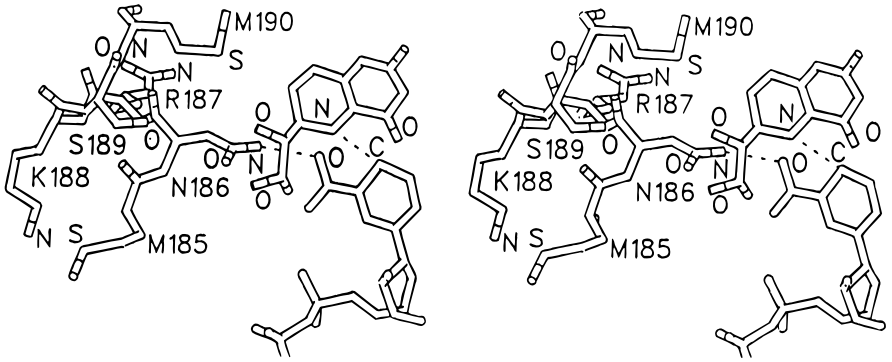

FIGURE 3: Stereoview of the ternary DHPR complex highlighting the environment of the dihydroxypropyl side chain of qBH_2 and the loop region of DHPR. The dashed lines indicate the hydrogen bonding of Asn186 with the NADH carboxamide group and the proposed hydride transfer between N5 of $qBH_2$and C4 of NADH.

comparable for the two substrates. However, the pterin off rate k_4 is clearly different for the two substrates, with $k_4 \geq 1500\ s^{-1}$ for q-6,7-diMePtH_2, but only $20-350\ s^{-1}$ for qBH_2. Thus, with the dihydroxypropyl group present, the off rate is at least 1 order of magnitude slower than with the methyl substituent. In structural terms, when the polar side chain is absent the only major point of interaction between enzyme and pterin is Tyr146. Consequently, with q-6,7-diMePtH_2, changes in the observed k_{cat} and calculated k_7 values clearly reveal the mutational effects on enzymatic reduction. Thus mutating Tyr146 or Lys150 decreases the rate and mutating both residues completely eliminates enzymatic reduction.

The Tyr-$(Xaa)_3$-Lys motif exhibits a strong influence on the ability of the reduced nucleotide to efficiently transfer hydride to the substrate (Kiefer et al., 1996). Catalysis is probably achieved via a hydrogen bond between the hydroxyl of Tyr146 and O4 of the pteridine (see Figure 2). Any mutation disrupting this interaction will decrease the hydride transfer rate. It is known from crystal structures of mutants that a water molecule present in the active site could compensate for the loss of the TyrOH−pterin interaction (Kiefer et al., 1996). Clearly, the trends of the data with qBH_2 suggests that only with the greater affinity of qBH_2 for the active site is the water in Tyr146Phe/Lys150Gln probably able to assist in the enzymatic reduction.

From modeling the natural pterin substrate with the dinucleotide bound E:NADH complex, the source of affinity of the dihydroxypropyl side chain for the enzyme appears to be a highly polar loop, residues 185−190 (185MNRKSM190). Figure 3 contains a close up view of the modeled ternary complex in the vicinity of the dihydroxypropyl side chain of the natural substrate. Included in this figure are residues Asn186, Ser189, and Met190. All of these polar groups are in the vicinity of the polar side chain. Additionally, Asn186 interacts with the carboxamide of the nicotinamide cofactor. Structurally common to the SDR family is an asparagine or a threonine interacting with the nicotinamide cofactor, as Asn186 does in DHPR. The asparagine and threonine have been shown to be sensitive to mutation, decreasing the stability of the binary dinucleotide enzyme complex (Kiefer et al., 1996; Jörnvall et al., 1995). Together, this loop positions both NADH and qBH_2 for hydride transfer.

Other members of the SDR family contain substrate specific loops structurally located in a similar position on the enzyme, between βF and αG opposite Tyr146 and Lys150 (Kiefer, 1996; Varughese et al., 1994). The loops vary in size according to the dimensions of the substrate. For example, the UDP-glucose epimerase requires a very large loop, creating an entirely separate domain to accommodate its substrate (Thoden et al., 1996c; Thoden et al., 1996c; Thoden et al., 1996c; Sakai et al., 1995c). A ternary versus binary X-ray crystallographic study of 7α-hydroxysteroid dehydrogenase (Tanaka et al., 1996), a short-chain dehydrogenase, revealed a significant reorganization of this substrate specific loop upon formation of the ternary complex and thus indicates a dynamic role associated with substrate affinity. An analogous loop region in *Drosophila* alcohol dehydrogenase (DADH) has been proposed to be dynamic based on a similarity between DADH and lactate dehydrogenase (Pouplana & Fothergill-Gilmore, 1994). The loop in lactate dehydrogenase folds over the active site in a comparable manner to that observed in the 7α-hydroxysteroid dehydrogenase.

Because the interaction of the equivalent loop is associated with both substrates in DHPR, it would appear that the loop is also important for pterin orientation and thus critical for q-6,7-diMePtH_2 alignment at the active site. The absence of the polar interaction that exists between the dihydroxypropyl side chain of qBH_2 and the loop may lead to the repositioning of the loop to allow steric interactions with the smaller side group of q-6,7-diMePtH_2. Thus, the isomerization step controlling the rate with the alternate substrate probably includes this folding of the loop toward q-6,7-diMePtH_2. The examples of loop closure by other SDR members suggests the closure of the polar loop is a general feature of the dehydrogenase reaction. The kinetic measurements presented in this report indicate the loop interaction could be rate limiting either by reorganization or affinity for the pterin.

Serine Mutants. The serine mutants of DHPR, Ala133Ser and Ala133Ser/Tyr146Phe were prepared to examine the role of the conserved active site serine common to other members of the SDR family. However, unlike the dramatic effects observed upon mutation of either Tyr146 or Lys150, the results of the Ala133Ser mutation were much more subtle. Thus, introduction of an Ala133Ser mutation into the Tyr146Phe mutant lowered the K_{Pt} value for both substrates, but only resulted in an increase in catalytic efficiency for qBH_2 reduction. Conversely, introduction of a single Ala133Ser mutation into wild-type DHPR leads to a 3-fold increase in catalytic efficiency for q-6,7-diMePtH_2, but no change in kinetic parameters for qBH_2. Thus, while the extra polar group provided by substitution of Ala133 by Ser-OH

does appear to facilitate interaction with the substrate at the active site, it does not appear to be important in the reduction reaction mediated by DHPR.

It should be noted that DHPR acts exclusively as a reductase in contrast to many dehydrogenases of the SDR family. In a reductase, the primary function of the enzyme active site is to transfer a hydride and proton to the substrate, while in a dehydrogenase the opposite is true. Consequently, the catalytic Tyr-OH must function primarily as a proton donor in the reductase as opposed to a proton acceptor in the dehydrogenase reaction. The role of the conserved active site serine residue may therefore be different for other members of the SDR family. For example, the serine hydroxyl may help stabilize the tyrosine anion, Tyr-O^-, which would facilitate the dehydrogenase reaction (Mckinley-Mckee et al., 1991; Varughese et al., 1994). A detailed kinetic comparison of the wild-type and serine mutant enzymes for one or more of the dehydrogenase members of the SDR family, similar to the studies described in this paper for DHPR, should provide further insight into the function, if any, of a serine at the active site.

CONCLUSION

Steady state kinetics and NADH(D) isotope effects presented for wild-type and mutant forms of DHPR suggest that the rate-determining step for reduction of either substrate is an isomerization step occurring either prior to hydride transfer in the case of the synthetic substrate q-6,7-diMePtH_2, or after catalysis is complete, with release of the reduced pterin product, in the case of the natural substrate, qBH_2. The difference between the two substrate activities is ascribed to a more favorable initial interaction between the enzyme active site and the dihydroxypropyl side chain of the natural substrate, resulting in a more rapid structural reorganization to yield the productive ternary complex prior to catalysis. With this extra interaction the reversal of the same structural reorganization after catalysis to allow product release is rate limiting with qBH_2, thus differing from q-6,7-diMePtH_2. It is postulated that a particular substrate specific polar loop of residues common to all members of the SDR family of enzymes may be important in this structural reorganization step. The Tyr-$(Xaa)_3$-Lys motif in DHPR and other members of the SDR family interacts with the substrate, probably in the form of a hydrogen bond, and any mutation of either tyrosine or lysine weakens this interaction. Furthermore, adding a serine to the active site of DHPR does not significantly help in pteridine reduction but could possibly function in the dehydrogenation reaction of other members of the SDR family.

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