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Structural and mechanistic characteristics of dihydropteridine reductase: A member of the Tyr-(Xaa)$_3$-Lys-containing family of reductases and dehydrogenases

(short-chain dehydrogenases/conserved residues)

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ABSTRACT **Dihydropteridine reductase (EC 1.6.99.7) is a member of the recently identified family of proteins known as short-chain dehydrogenases. When the x-ray structure of dihydropteridine reductase is correlated with conserved amino acid sequences characteristic of this enzyme class, two important common structural regions can be identified. One is close to the protein N terminus and serves as the cofactor binding site, while a second conserved feature makes up the inner surface of an α-helix in which a tyrosine side chain is positioned in close proximity to a lysine residue four residues downstream in the sequence. The main function of this Tyr-Lys couple may be to facilitate tyrosine hydroxyl group participation in proton transfer. Thus, it appears that there is a distinctive common mechanism for this group of short-chain or pyridine dinucleotide-dependent oxidoreductases that is different from their higher molecular weight counterparts.**

Dihydropteridine reductase (DHPR; EC 1.6.99.7) is an enzyme whose ubiquitous distribution in mammalian tissues has always presented something of an enigma. It is well known that it is the source of the tetrahydrobiopterin cofactor used in the aromatic amino acid hydroxylation reactions, particularly in liver, adrenal, and nerve tissue (1); however, its function in other tissues is yet to be clearly resolved. DHPR is a dimeric protein of $M_r \approx 51,000$, and the recent crystal structure of the rat liver enzyme (2) shows that the dimer is formed by two identical monomers whose intimate interaction stems from the hydrophobic interplay of a quartet of helices: two from each subunit. The two active sites in the holoenzyme are structurally identical and are located some 30 Å apart, adjacent to the distal edges of opposing helices (αF in each monomer), which form part of the dimer interface. Their function is to catalyze the NADH-mediated reduction of quinonoid dihydrobiopterin to afford tetrahydrobiopterin (Fig. 1), which functions as an essential cofactor in the biosynthetic reactions that convert phenylalanine to tyrosine, tyrosine to dihydroxyphenylalanine, and tryptophan to dihydroxytryptophan. The reactions are essential to the generation of the catecholamines, and genetic defects in any of the reactions required to ensure tyrosine biosynthesis give rise to serious clinical malfunctions known collectively as phenylketonuria (3–5). For this reason, DHPR has received intense scrutiny by many laboratories over the past two or three decades (6). Moreover, this enzymatic reaction bears a superficial resemblance to the action of dihydrofolate reductase (7), insofar as each enzyme uses a reduced dinucleotide to catalyze the conversion of a substituted dihydropteridine to its tetrahydro analog, and thus interest has been further stimulated because of potential overlap with the field of folate metabolism. In spite of the superficial similarity, the active sites as well as the mechanisms of the two enzymes are quite different. Several observations relating to its structure and sequence have suggested that DHPR, but not dihydrofolate reductase, is a member of a larger class of dinucleotide binding proteins whose general purpose is to act as reductant or dehydrogenase, respectively, of polarized olefinic bonds or their concomitant reduced forms (8). Primary amino acid sequence alignments of these so called short-chain dehydrogenases indicate the presence of a strictly conserved Tyr-(Xaa)$_3$-Lys sequence, and it has been suggested that these residues may be part of the enzyme active site (9). The following report discusses these relationships in greater detail in view of the recently determined three-dimensional structure for rat liver DHPR (2).

DHPR Is a Short-Chain Dehydrogenase

The newly recognized family of so-called short-chain dehydrogenases has recently been reviewed (9), not including DHPR. An alignment of primary amino acid sequences for 20 enzymes in this family has been compiled from which certain common features can be discerned. Of particular interest is the conclusion that only 6 of the 250-odd residues in a canonical short-chain dehydrogenase are strictly conserved among the sequences examined (9). A key finding in the current study is that DHPR is a member of this family (Fig. 2). One other member of this family of short-chain dehydrogenases, 20β-hydroxysteroid dehydrogenase (20βDH), has been structurally characterized (10), but not at high resolution. The overall three-dimensional structure of the two enzymes is clearly related as evidenced by a root-mean-square deviation of 2 Å for 160 C$^\alpha$ carbon atoms of DHPR superposed onto corresponding atoms of 20βDH. Hence, it is now possible to interpret general characteristics of these short-chain dehydrogenases in terms of the high-resolution x-ray structure (M. M. Skinner, N.H.X., J.M.W., and K.I.V., unpublished results) of one specific member of the class—namely, DHPR.

Consistent with the role of sequentially homologous residues in medium-chain dehydrogenases such as lactate dehydrogenase and liver alcohol dehydrogenase, Gly-13 and Gly-19 in DHPR, and presumably other short-chain dehydrogenases as well, occur in sharp turns associated with proper folding of the adenine binding domain. A third conserved residue, Gly-126 in DHPR, is situated near the C-terminal end of the dinucleotide fold, where it provides a short, tight linkage between helix αE and strand βE, which positions several side chains to interact directly with the nicotinamide mononucleotide portion of the cofactor (Fig. 3).

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Abbreviations: DHPR, dihydropteridine reductase; 20βDH, 20β-hydroxysteroid dehydrogenase.
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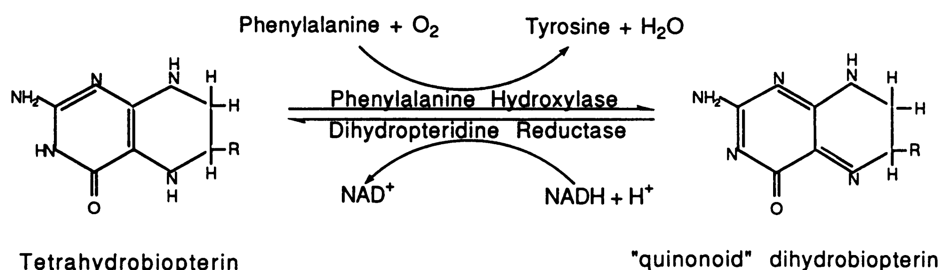

FIG. 1. Phenylalanine hydroxylase catalyzes the conversion of phenylalanine to tyrosine using tetrahydrobiopterin as a cofactor. During this conversion, the tetrahydropterin is converted to quinonoid dihydrobiopterin. DHPR then catalyzes the reduction of quinonoid dihydrobiopterin to tetrahydrobiopterin.

Sequence alignments by Persson *et al.* (9) point to a fourth residue, an aspartic acid (Asp-61 in DHPR), that they argue is strictly conserved in the short-chain dehydrogenase family and may be involved in hydrogen bonding to the coenzyme. In fact, Asp-61 in DHPR is located two turns from the N terminus of αD with its side chain projecting out into solution near one edge of the dimer interface. According to the alignment of Persson *et al.* (9), Asp-60 in 20βDH should be geometrically equivalent to Asp-61 in DHPR. Even though the three-dimensional structures of DHPR and 20βDH are very similar, as mentioned earlier, these two aspartic acid residues are 19 Å away from each other due to a difference in local structure caused by the absence of αC in DHPR (Fig. 4). The crystal structure indicates that Asp-60 in 20βDH is located at the C-terminal end of βC, which precedes αD in these two structurally related proteins. Connecting segments between βB and βC and between βC and αD appears to be quite variable in this family of enzymes, both with respect to the number of residues involved and the amino acid composition. In the absence of three-dimensional structural information, alignment schemes based on maximizing sequence homologies can prove misleading. In this case, the combined crystallographic evidence clearly indicates that the aspartic acid residue in question is not conserved in the short-chain dehydrogenase family.

In NAD(H)-specific short-chain dehydrogenases there is an additional conserved aspartic acid residue, which is also conserved in DHPR (Asp-37), and it is hydrogen bonded to the adenyl ribose of the cofactor. The remaining two conserved residues, Tyr-146 and Lys-150 in DHPR, almost certainly have important catalytic functions for proteins in this family of short-chain dehydrogenases. Tyr-146 and Lys-150 are located near the N terminus of αF on the interior surface of the helix, where their respective side chains stack on top of one another and project into the substrate binding cavity.

There are additional features of helix αF that merit comment. Except for proline, glycine has the lowest propensity to exist in an α-helical conformation of all the naturally occurring amino acids. Blaber *et al.* (11) have argued that this low helix propensity for glycine is a result of (*i*) especially unfavorable entropic costs associated with folding the most conformationally flexible amino acid into a tightly constrained element of a secondary structure, and (*ii*) its lack of hydrophobic stabilization. It is striking that three of the first eight residues (145, 147, and 151) in the αF helix of DHPR are glycine even though it is very unusual to have such enrichment of glycine residues in an α-helix. Hence it looks highly probable that these residues have functional roles. It is interesting to note that both residues adjacent to Tyr-146 are glycine residues, and Lys-150 has a neighbor that is a glycine. Glycine residues give added flexibility to the chain, which might be required for events occurring during catalysis. One of the naturally occurring mutants¶ causing phenylketonuria has Gly-147 mutated to a serine, and we speculate that one of the causes for the loss of activity is the loss of flexibility. It is of further interest to note that in most of the other members of the short-chain dehydrogenase family, the conserved tyrosine and lysine have one or more glycine residues either adjacent or one removed from them. The C terminus of αF has two leucine residues one turn apart on the inner surface of the helix where they pack against two similarly positioned leucine side chains from αE forming part of a leucine-rich hydrophobic core. Finally, judging from their unusually low temperature factors, residues 149–156 in αF represent by far the most rigid portion of the entire DHPR structure. The average isotropic temperature factor for side chain atoms of Lys-150 is just over 2 Å^2.

```
                     130        135        140        145      150
DHPR    125   G [G] L L T L A G A K A A L D G T P G H I G [Y] G H A [K]--
PGDH    130   G [G] I I I N H S S L A G L H P V A Q Q P V [Y] C A S [K]--
17βDH   134   S [G] R V L V T G S V G G L H G L P F N D V [Y] C A S [K]--
DADH    131   G [G] I I C N I G S V T G F N A I Y Q V P V [Y] S G T [K]--
20βDH   131   G [G] S I V N I S S A A G L H G L A L T S S [Y] G A S [K]--
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FIG. 2. Alignment of a common selected region of five short-chain dehydrogenases. Strictly conserved residues are boxed. Residue numbers at the start of each line refer to each sequence, and those above refer to the rat liver DHPR. DHPR, rat liver DHPR; PGDH, human 15-hydroxyprostaglandin dehydrogenase; 17βDH, human 17β-hydroxysteroid dehydrogenase; DADH, *Drosophila melanogaster* alcohol dehydrogenase; 20βDH, *Streptomyces hydrogenans* 20βDH.

Substrate Binding and the Mechanistic Role of the Tyr-(Xaa)$_3$-Lys Couple

Solution of the rat DHPR–NADH binary complex at a resolution of 2.3 Å reported recently (2) has led to a proposed model for substrate binding at the enzyme active site. However, the natural instability of the quinonoid dihydro substrate, the absence of known specific competitive inhibitors for the pteridine binding site, and the poor affinity of NAD$^+$ for the protein ($K_d \approx 0.1$ mM) have created problems in obtaining direct crystallographic evidence to support or contradict specific features of the hypothetical model. Therefore, graphic simulation has been employed (2) to create an active-site model (Fig. 5). Analysis of this model suggests that certain amino acids have sufficiently close proximity to the substrates to participate in the reductive reaction. The pteridine appears to be sandwiched between the nicotinamide ring of NADH and Trp-86, with the phenolic side chain of Tyr-146 and the ε-amino group of Lys-150 being within 3–4 Å of the pteridine 4-keto group and nicotinamide ribose 2′ and 3′

¶Smooker, P. M., Howell, D. W., Dianzani, I. & Cotton, R. G. H. (1992) Sixth International Conference on Pteridines and Related Biogenic Amines and Folates, June 7–10, 1992, Seoul, Korea.

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                                *         * *                 *         *       * *
              5     |<--  βA  -->|     |<---        αB    --->|<---      βB    --->|
DHPR  :       E A R R V L V Y G G R - G A L G S R C V Q A F R A R N W W V A S I D V 38
20βDH : M N D L S G K T V I I T G G A R G L G A L A A R Q A V A A G A R V V L A D V 38
        1           |<---βA  -->|     |<---        αB    --->|<---      βB    --->|

                                                    *
                                                |<---   βC  --->|   |<--- αD --->
DHPR  : - - V E N E E A S - - - - - - - - - - - A S V I V K M T D S F T E Q A D 61
20βDH : L D E E G A A T A R E L G D A A R Y Q H L D V T I - - - - - - - - - - - 63
        |<---      αC     --->|           |<--- βC --->|

                    *                     * *           * *       *
        <---      αD    --->|             |<---       βD      --->|
DHPR  : Q - V T A E V G K L L G - D Q K - V D A I L C V A G G W A G G N 90
20βDH : E E C W Q R V V A Y A R E E F G S V D G L V N N A G I S T G M F 95
          |<---        αD       --->|     |<---       βD      --->|

                                                    *
                  |<---                   αE                      --->|
DHPR  : A K S K S L F K N C D L M W K Q S I W T S T I S S H L A T K H L K E - - G 125
20βDH : L E T E - S V E R F R K V V D I N L T G V F I G M K T V I P A M K D A G G 131
                  |<---                   αE                      --->|

        *                 *   *   *             * *     *     *     *         *
        |<--- βE --->|                      |<---             αF            --->|
DHPR  : G L L T L A G A K A A L D G T P G M I G Y G M A K G A V H Q L C Q S L A G 162
20βDH : G S I V N I S S A A G L M G L A L T S S Y G A S K W G V R G L S K L A A V 168
        |<--- βE --->|                      |<---             αF            --->|

                                  *   *
                      |<---     βF    --->|
DHPR  : K N S G M P S G A A A I A V L P V T L D T P M N R K S M P E A D F S S W 198
20βDH : E L G T D - - R I R V N S V H P G M T Y T P M T A E T G I R Q D E G N Y 202
                      |<---     βF    --->|

                                                                      *         *
                              |<---       αG      --->|             |<--- βG ---->
DHPR  : - - - - - - - - - T P L E F L V E T F H D W I T G N K R P N S G S L I Q V V 227
20βDH : P N T P M G R V G N E P G E I A G A V V K L A S D T S S Y V T G A E L A V D 240
                              |<---       αG      --->|             |<--- βG --->|

        <---βG--->|<---   βH  --->|
DHPR  : - - T T D G K T E L T P A Y F  240
20βDH : G G W T T G P T V K Y V M G Q  255
```

FIG. 3. Sequence alignment of DHPR and 20βDH based on the crystal structures. Structurally conserved residues are indicated by asterisks. Residues 39–44 of DHPR make up a loop in the same direction as αC of 20βDH. The αD helices of both enzymes are located near the adenine part of NADH, but they are displaced by two helical turns. The large loop regions before αG, 181–198 of DHPR and 185–211 of 20βDH, form parts of the active sites. Their differences can be attributed to different substrate specificity.

hydroxyl groups, respectively. It is interesting that the Tyr-$(Xaa)_3$-Lys sequence allows these two conserved amino acids to stack on top of each other within the α-helical structural motif such that both side chains point toward the substrate. This observation coupled with the conservation of this unique motif in the large family of proteins known as the short-chain dehydrogenases or aldo-keto reductases (Figs. 3 and 6) (9) suggests that there might be specific mechanistic consequences in this pairing. Clearly at the ambient cellular pH ≈ 7 the ε-amino group of Lys-150, which has a pK ≈ 10, will be protonated. Moreover, the phenolic group of Tyr-146, which also has a pK ≈ 10, will not have a great tendency to donate a proton. However, kinetic studies on the Tyr-146 → Phe mutant (J.M.W., unpublished results) show K_m values similar to the native enzyme and a k_{cat} decrease of two orders of magnitude. Therefore, it appears likely that Lys-150, by its proximity to the phenolic group, must influence the role of Tyr-146 in the reductive process. The Lys-150 ε-amino group

FIG. 4. Stereoview of the binary complex of DHPR with NADH. Gly-13, Gly-19, and Gly-126 have structural roles. Tyr-146 and Lys-150 have important catalytic roles.

FIG. 5. Stereoview of the active site of DHPR showing amino acids pertinent to binding and a possible binding mode for quinonoid dihydrobiopterin.

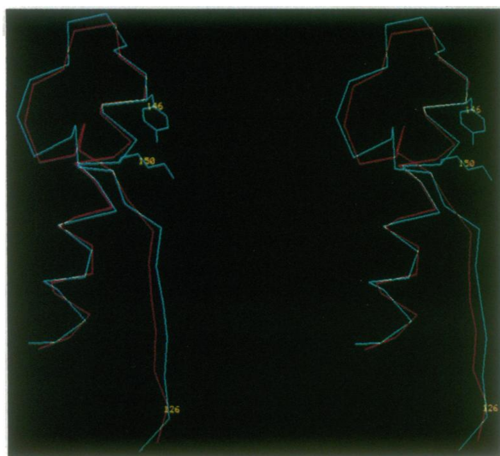
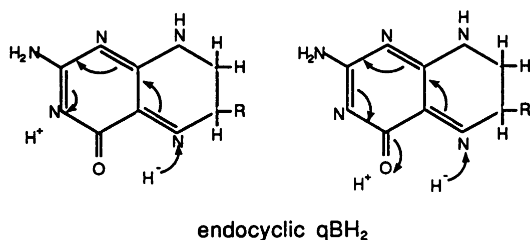


FIG. 6. Stereoview of the alignment of the Tyr-(Xaa)$_3$-Lys-containing region of rat DHPR (blue) with the corresponding region of 20βDH (red).

also plays an additional role, donating a hydrogen bond to the 2′-hydroxyl of the nicotinamide ribose of NADH. This interaction appears important in orienting the reduced nicotinamide such that the pro-S hydrogen is positioned for transfer to the pteridine N-5 position. Asn-186 also plays an important role here donating a strong hydrogen bond to the carboxamide substituent of the nicotinamide.

Kinetic evidence has shown that the hydrogen transfers in this reductive process go to centers prone to hydrogen exchange (12, 13), and ground-state electron density calculations have shown the pteridine 5-position could be receptive to hydride transfer (8). Therefore, the events outlined below, the projected reductive pathway for quinonoid dihydrobiopterin (qBH$_2$),



endocyclic qBH$_2$

are consistent with the molecular requirements of this reductive process. Two structures are shown above because at this stage it is unknown whether proton donation occurs directly to the 4-amido oxygen of the pteridine or indirectly to the N-3 position via a water molecule known to reside in this vicinity. It is of interest in the case of DHPR that the oxidized dinucleotide product of the reaction has little affinity for the enzyme ($K_d \geq 0.1$ mM) in contrast to that of NADH ($K_d \approx 0.02$ μM). It could be hypothesized that the Lys-150 ε-amino group has a further role to play—namely, that of contributing to removal of NAD$^+$ after reaction has occurred. That such a notion could have some foundation is supported by the observation that the uncharged adenine-uracil dinucleotide analog of NAD$^+$ has a $K_d \approx 0.35$ μM for DHPR in contrast to $K_d \approx 0.1$ mM for NAD$^+$ itself. Additional support for the important role suggested for the Tyr-(Xaa)$_3$-Lys motif comes from work with the DHPR mutants. The rat enzyme has been cloned and expressed in *Escherichia coli* (1), and Tyr-146 → Phe and Lys-150 → Gln mutants have been isolated and characterized (8). As described earlier, the former mutant has an altered k_{cat}, and this is reflected in the low specific activity (≈300 units/mg for wild-type down to 1 unit/mg) and in the

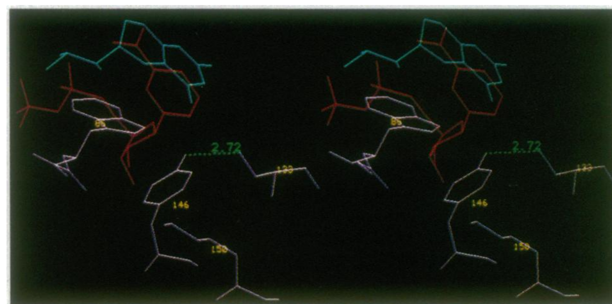


FIG. 7. Hypothetical arrangement of a Ser-133 replacement of Ala-133 in the rat DHPR structure.

latter case down to 50 units/mg. Clearly these two amino acids are critical for the reductive process.

As mentioned previously, only one other enzyme in this group of short-chain dehydrogenases has been crystallized and structurally characterized, 20βDH from *Streptomyces hydrogenans* (10). Superposition of the Gly → Tyr-(Xaa)$_3$-Lys region of the two enzymes (Fig. 6) shows a remarkable identity between the two three-dimensional structures, suggesting a potential similarity in mechanism for the two enzymes. However, there is a clear mechanistic distinction in that DHPR is a reductase with a strong forward impetus and a very low affinity for NAD$^+$, whereas for most members of the short-chain dehydrogenase family the equilibrium lies in favor of the oxidized species. Thirteen residues upstream from the conserved Tyr-146 in DHPR there is an alanine residue, which is replaced by serine in 19 of 20 short-chain dehydrogenase sequences aligned by Persson *et al.* (9). Model-building experiments with DHPR suggest that an Ala-133 → Ser replacement could position a Ser-133 side chain to hydrogen bond with the Tyr-146 hydroxyl (Fig. 7). The serine side chain, by virtue of its hydrogen-bonding ability, could provide a path for proton abstraction—a path that is absent in DHPR.

The fundamental distinction in reaction pathway between DHPR and the dehydrogenases, however, most probably relates both to the properties of substrate and products and to the structural and chemical characteristics of the active site. There is a strong energetically favorable impetus for the quinonoid dihydropteridine to be converted to the tetrahydro product. This is apparent from the former's ready reduction by thiols and also by its noncatalytic reduction with NADH. This is not the same for the keto-alcohol interconversion. The presence of another path, as is described above, could contribute to proton loss from the oxygen of the alcohol substrate, along with the hydride transfer from the adjacent carbon to NAD$^+$.

Conclusions

A large family of proteins exists in nature variously called short-chain dehydrogenases (reductases) that have two important common structural regions, one in the vicinity of the N terminus that binds a reduced or oxidized pyridine dinucleotide cofactor and the second on the interior surface of an α-helix that positions a tyrosine side chain in close spatial proximity to the lysine residue four residues removed in linear sequence. The prime function of this Tyr-Lys couple may be to facilitate tyrosine hydroxyl group participation in proton-transfer reactions, a feature that normally does not readily occur at the usual cellular pH. There is thus a clear distinctive common mechanism for this group of pyridine dinucleotide-containing oxidoreductases that is different from other reductases or dehydrogenases. For each member of the short-chain dehydrogenase family, the target or product of reaction is a polarized bond of the C=N or C=O

variety, and it is suspected that the recognition of this entity has been crucial to the evolutionary common features exhibited by the various proteins of this family.

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