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Regulatory properties of tetrahydrobiopterin cofactor bound at the active site of phenylalanine hydroxylase

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Introduction

Phenylalanine hydroxylase (PAH) requires the reduced pterin cofactor (6*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin (6*R*-BH_4) to reduce the iron [(Fe(III)--->Fe(II)] at the active site, to activate dioxygen and hydroxylate the aromatic ring of the amino acid substrate L-phenylalanine (L-Phe). In addition, it also functions as a potent negative effector of the activation of the enzyme by L-Phe and the phosphorylation of Ser16 by cyclic AMP-dependent protein kinase (PKA)[1,2]. The rate of PKA-dependent phosphorylation is inhibited only by pterin cofactors [(6*R*-BH_4), (6*S*-BH_4) og 7,8-dihydro-L-biopterin (BH_2)] that are able to decrease the potency and efficiency of L-Phe as an activator of the hydroxylase [2]. These findings have been interpreted in terms of an interdomain conformational change induced by the pterin binding at the active site [2]. The recent X-ray crystal structures of three different truncated forms of the enzyme have yielded valuable new information on its structure and function [3], including a structural explanation for the inhibitory effects of the pterin cofactors as affered in the present study.

Materials and Methods

Expression in E. coli and purification of recombinant hPAH forms

Wild-type and mutant forms of the double truncated dimeric form deltaN102/deltaC24-hPAH were expressed as fusion proteins in *E. coli*, using the maltose-binding protein (MBP) as fusion partner, purified by affinity chromatography (as fusion protein) and size exclusion chromatography (after cleavage with the restriction protease factor Xa)[4]. The PAH activity was determined as described [5].

Site-specific mutagenesis

Mutations were introduced into the double truncated form of the wt-hPAH cDNA (deltaN102/deltaC24-hPAH) by PCR-based site-specific mutagenesis as described [4,6].

Crystallization, data collection, model building and refinement

The wild-type form of the double truncated form of hPAH (deltaN102/deltaC24-hPAH) was co-crystallized with the oxidized form of the natural cofactor 7,8-dihydro-L-biopterin (BH_2) [6] with a similar size and morphology as the original native crystals [7]. Data collection, model building and refinement were carried out as described [6,7].

Results and Discussion

Pterin binding at the active site of hPAH

The crystal structure (at 2.0 Å resolution) of the binary complex of dimeric catalytic domain (residues 118-424) of human PAH (hPAH) [6] could be superimposed on the crystal structure of the corresponding ligand-free native form [7] with a root-mean-square displacement of 0.26 Å. Electron density difference maps showed density that could be interpreted as the pterin ring, and allowed for the positioning of the ring as well as the O4 oxygen atom and the dihydroxypropyl side-chain [6]. The pterin binds in the second coordination sphere of the catalytic iron (the C4a atom is 6.1 Å away), and

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interacts through several hydrogen bonds to two water molecules coordinated to the iron, as well as to the main chain carbonyl oxygens of A322, G247 and L249 and the main chain amide of L249 (see Table 1 for atomic distances). The pterin ring forms an aromatic pi-stacking interaction with F254, and Y325 also contributes to the positioning of the pterin ring and its dihydroxypropyl side-chain by hydrophobic interactions. E286 hydrogen bonds to one of the water molecules coordinated to the iron as well as to a water molecule which hydrogen bonds to N3 of the pterin ring. Site-specific mutations of E286 (E286A/Q), F254 (F254A/L) and Y325 (Y325F) confirmed the important contribution of E286 and F254 to the normal positioning of the pterin cofactor and catalytic activity of hPAH. The most dramatic effect was observed on substitution of E286 (E286A) which resulted in a 38000-fold decrease in the k'_{cat}/K'_{m}-value (6*R*-BH_4 as the variable substrate).

	Distance (Å)
Fe-O4	3.8
Fe-C4a	6.1
Fe-N5	6.1
Fe-Wat3	2.3
Fe-Wat1	2.6
Wat1-O4	2.5
Wat3-O4	2.8

Table 1. Atomic distances between the active site iron, its ligated water molecules and 7,8-dihydro-L-biopterin.

Conformational changes in the protein and the cofactor on ligand binding

Some important conformational changes are seen in the active site upon pterin binding, including movements of the 245-250 loop and the 380's loop in the direction of the iron and thus allows for several important H-bonds to the pterin to be formed [6]. These are rather flexible loops as indicated by a high B-factor. Thus, the C_{alpha} atom of G247 moves about 1.3 Å towards the pterin ring, when compared to the native ligand-free structure. L248 side-chain changes its conformation as compared to the non-liganded structure, and now faces the active site. L255 also shifts its conformation in order to accomodate the dihydroxypropyl side-chain of the pterin. Mutations in G247 (G247V), L249 (L249F/H) and L255 (L255V/S) have been reported in patients with phenylketonuria (PKU database located at http://www.mcgill.ca/pahdb). Considering the important hydrogen bonds between the cofactor and these active site residues as well as the movements G247 undergo upon pterin binding, the dysfunction of the mutant enzyme forms can be easily understood. Recent NMR studies have shown a change in the conformation of the side-chain of BH_2 from a *trans* in solution to an almost *cis* in the bound state [8], which was also observed in the crystal structure with a torsional angle between the OH groups of about 53° [6]. The pterin cofactor is in an ideal orientation and conformation for dioxygen to bind in a bridging position between iron and the pterin.

In 1984 we proposed [1] a working model with four alternative conformations of rat PAH: the resting or ground state (G-state), the L-Phe activated state (P-state), the 6*R*-BH_4 inhibited state (B-state) and a state of catalytic turnover (BP-state). We have now structural evidence for conformational changes at the active site on pterin binding (see above). That additional conformational changes do occur on pterin binding to the full-length tetrameric enzyme is suggested by the inhibitory effect of 6*R*-BH_4 on both limited proteolysis, activation by L-Phe and phosphorylation of Ser16 in rat PAH by PKA (apparent K_i = 1.9 μM)[1,2]. The same effects have been observed for hPAH, with a similar inhibitor constant for 6*R*-BH_4 as in rat PAH [Solstad T and Flatmark T, unpublished data]. Furthermore, superposition of the binary hPAH-BH_2 complex onto the crystal structure of the ligand-free dimeric rat PAH [9], which contains both the regulatory and catalytic domains, reveals that the C2'-OH group of BH_2 is sufficiently close (2.7 Å) to form hydrogen bonds to Ser23 (Figure 1) through either the side-chain or the carbonyl oxygen of the main chain.

Figure 1. Putative interaction of the N-terminal autoregulatory sequence of rat PAH (in yellow) with 7,8-dihydro-L-biopterin (in green). The hPAH-BH_2 structure (PDB code 1DMW) was superimposed onto the corresponding region (residues 118-424) of the ligand-free rat PAH (PDB code 2PHM) containing the regulatory and catalytic domains. The C_{alpha}-ribbon of the protein backbone of the catalytic domain is shown in gray and the iron in red. The side-chains are not shown for clarity.

Thus, upon 6*R*-BH_4/BH_2 binding to the full-length PAH, a conformational change of the protein including a movement of the mobile N-terminal autoregulatory sequence [9] is likely to occur, forming the proper length hydrogen bonds to the pterin. These interactions gives a plausible structural explanation for the specific regulatory properties of the dihydroxypropyl side-chain of BH_4 (negative effector) in the full-length enzyme in terms of phosphorylation of Ser16 by PKA and activation by L-Phe [1,2]. Similar interactions with the N-terminal autoregulatory sequence was observed for iron-coordinated catecholamine also explaining the inhibitory effect of dopamine binding on the rate of phosphorylation of Ser16 in full-length hPAH by PKA [Solstad T and Flatmark T, unpublished data]. Thus, in order to explain the regulatory properties of 6*R*-BH_4 in the full-length form of PAH it is not necessary to have an additional binding site of the cofactor in the regulatory domain as recently postulated [9].

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