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The effect of tetrahydrobiopterin on the thermostability of human phenylalanine hydroxylase as studied by circular dichroism spectroscopy

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Introduction

Mammalian phenylalanine hydroxylase (phenylalanine 4-monooxygenase, PAH, EC 1.14.16.1) catalyzes the hydroxylation of L-phenylalanine (L-Phe) to L-tyrosine (L-Tyr), the rate limiting step in the only catabolic pathway of phenylalanine. PAH is a non-heme iron-containing enzyme which depends on tetrahydrobiopterin (BH₄) to catalyze the incorporation of molecular oxygen into the ring of L-Phe (1). A dysfunction of this enzyme caused by mutations in the PAH gene results in a serious disease in humans, phenylketonuria. It has been proposed that *in vivo* regulation of PAH is dependent primarily upon the actions of L-Phe and BH₄ (2,3). Thus, L-Phe is considered to be an activator, while BH₄ blocks this substrate activation by forming an inactive BH₄-enzyme complex. This complex formation seems to control the metabolic availability of BH₄ and is important in protecting the cofactor from degradation when L-Phe levels are low in the cell (2). Moreover, it has also been shown that BH₄ protects rat PAH from proteolytic degradation of the N-terminal region (4). In this work we have studied the stability changes induced on human PAH (hPAH) upon binding of BH₄ and its oxidized analogue dihydrobiopterin (BH₂), using circular dichroism.

Materials and Methods

Purification of hPAH. Expression of recombinant wild-type hPAH in *E. coli* (TB1) as fusion protein with maltose-binding protein, purification of the fusion protein by affinity chromatography on amylose resin, cleavage by factor Xa and further purification of the tetrameric form by size exclusion chromatography was performed as described (5,6). The concentration

of purified hPAH was estimated by the absorbance at 280 nm, using the absorption coefficient A_{280} (1 mg ml⁻¹cm⁻¹) = 1.0 (5).

Circular dichroism (CD) detected thermal denaturation. CD temperature scan curves of hPAH were recorded on a Jasco J-715 spectropolarimeter (Jasco, Corp.) equipped with PTC-348WI Peltier element (Jasco, Corp.). Stock solutions (50 mM) of BH₄ (Schircks Laboratories) were prepared in 10 mM HCl. Samples containing hPAH (15 μM subunit) in the absence and presence of either 0.5 mM BH₄ or 0.5 mM BH₂ (Schircks Laboratories) were freshly prepared in 20 mM Na-phosphate buffer, 0.2 M NaCl, pH 7.0, using quartz cuvettes with a path length of 1 mm for the measurements. The thermal denaturation of hPAH was monitored at 222 nm at a scan rate of 0.7 °C/min. Analysis of the data and the determination of the midpoint melting temperature (T_m) values were performed by noise reduction and differentiation of the scans using the Standard Analysis program provided with the CD instrument.

Results and discussion

We have recently studied the thermostability of hPAH and the effects of truncations and L-Phe binding by infrared spectroscopy, differential scanning calorimetry and CD (7 and unpublished results). These studies show that the denaturation of the enzyme results in two unfolding transitions with T_m -values at 45 and 54 °C, corresponding to the N-terminal regulatory and C-terminal catalytic domains of hPAH, respectively (Fig. 1). Moreover, the addition of L-Phe stabilized both domains and increased domain-domain interactions (data not shown). We have now studied by CD the effects

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Figure 1. Heat induced unfolding of hPAH. (A) Change in the CD signal at 222 nm for hPAH, in the absence of ligand (—); in the presence of 0.5 mM BH₄ (----); and in the presence of 0.5 mM BH₂ (---). (B) Differentiation of the data in A.

of BH₄ and BH₂ on the thermal induced denaturation of hPAH. Although none of these pterins induced significant changes in the CD spectrum (from 185 to 310 nm) of hPAH at 25 °C (data not shown) they seem to affect the stability and the domain interactions in the enzyme. Thus, in the presence of BH₄ the T_m of the CD-detected thermal transition corresponding to the catalytic domain increased to 58 °C (Fig. 1 and Table 1). BH₄ did not affect the T_m for the unfolding of the regulatory domain, although the transition becomes sharper and more separated from the second transition (Fig. 1). Although the subunit is more compact and stable in the presence of BH₄ (4), the less concerted unfolding of the domains may indicate a weakening of the interactions between the regulatory and catalytic domains in BH₄ complexed hPAH. Thus, the negative regulator BH₄ has an opposite effect in the net interactions between the major domains than the activator L-Phe. In the presence of the cofactor analogue BH₂ the overall thermostability of hPAH is increased (Fig. 1 and Table 1), which is a characteristic effect of the binding of a ligand to one of the domains of a protein with interacting domains.

Table 1. T_m values obtained for hPAH in the CD detected thermal denaturation

No ligand added	0.5 mM BH ₄	0.5 mM BH ₂
45	46	50
54	58	58

The recent structural study on the conformation of BH₂ bound at the active site of hPAH by NMR and docking (8) has shown that the dihydroxypropyl side-chain of BH₂ interacts with Ser251 and Ala322 in the enzyme. These residues in turn have polar and non polar interactions with the inhibitory autoregulatory N-terminal sequence (9). We have assumed that these interactions are the basis for the regulatory conformational changes induced by BH₄ (8). However, our present results indicate the existence of subtle differences in the binding of the natural and the oxidized cofactor analogue and the conformational changes that they induce in hPAH. Moreover, the results obtained in this work with hPAH differ from those obtained with the homologous enzyme tyrosine hydroxylase, which, as seen by infrared-detected denaturation, seems to be significantly destabilized by the binding of both BH₂ and BH₄ (10). This different effect of the pterins could be related to the specific regulatory properties elicited by the natural cofactor on the two enzymes (1).

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