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Native and Phosphorylated Bovine Adrenal Tyrosine 3-Monooxygenase. Interactions with Tetrahydropterins and Substrate and Stability of the Formed 4a-Hydroxy-Tetrahydrobiopterin

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Summary

The catalytic activity of tyrosine 3-monoxygenase (tyrosine hydroxylase) is dependent on a tetrahydropterin cofactor and enzyme-bound iron. The oxidation of tetrahydrobiopterin by bovine adrenal tyrosine hydroxylase was studied by high performance liquid chromatography (HPLC). The first pterin product detected during catalytic turnover, 4a-hydroxy-tetrahydrobiopterin, was isolated by HPLC and the pseudo first-order rate constant of its dehydration to quinonoid dihydrobiopterin was estimated. The $t_{1/2}$ was found to be 45 and 72 s in 100 and 5 mmol/L Tris-HCl, respectively, at pH 7.5 and 23 °C.

The rate of the 4a-hydroxy-tetrahydropterin formation was found to increase on phosphorylation of the enzyme by cyclic AMP-dependent protein kinase. The phosphorylation did not significantly change the electron paramagnetic resonance (EPR) spectrum of tyrosine hydroxylase. However, in the presence of substrate or cofactor and dithiothreitol, the EPR spectral properties were strikingly different from those observed prior to phosphorylation. Our findings are in accordance with a mechanism in which the covalently bound phosphate activates the enzyme by facilitating changes in the redox-state of the enzyme-bound iron.

Introduction

The most well-defined biological role of tetrahydropterins in mammals is their function as electron donors in the monooxygenase reactions catalyzed by the three aromatic amino acid hydroxylases, i. e. phenylalanine, tyrosine and tryptophan hydroxylase (for reviews, see refs. 1–3). These enzymes show a high degree of

sequence homology and are postulated to have similar reaction mechanisms (1–4). During catalytic turnover, tetrahydropterins are oxidized to dihydropterins in the tyrosine hydroxylase reaction (1–3, 5), e. g.

The formed dihydrobiopterins (qBH₂ and 7,8-BH₂) can in turn be regenerated to tetrahydrobiopterin (BH₄) by dihydropteridine reductase and dihydrofolate reductase, respectively (2). Although the overall stoichiometry of the hydroxylation reaction (equation 1) is known, the precise function of the tetrahydro-

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Enzymes: Tyrosine 3-monoxygenase, tyrosine hydroxylase (EC 1.14.16.2); Phenylalanine 4-monoxygenase, phenylalanine hydroxylase (EC 1.14.16.1).

Abbreviations: BH₄, 6(R)-tetrahydrobiopterin; 4a-OH-BH₄, 4a-hydroxy-tetrahydrobiopterin; qBH₂, quinonoid dihydrobiopterin; 7,8-BH₂, 7,8-dihydrobiopterin; 6-MPH₄, 6-methyltetrahydropterin; q6-MPH₂, quinonoid 6-methyldihydropterin; DOPA, 3,4-dihydroxy-phenylalanine; DTT, dithiothreitol; EPR, electron paramagnetic resonance; HPLC, high-performance liquid chromatography.

terins in the catalytic cycle is still unclear. In addition to their function as electron donors in the reduction of dioxygen, tetrahydropterins may also activate the enzyme, as observed for phenylalanine hydroxylase, in which the catalytically inactive Fe(III) bound to the enzyme appears to be reduced by the cofactor 2, 5, 6).

Like phenylalanine hydroxylase, bovine adrenal tyrosine hydroxylase also contains substantial amounts of high-spin Fe(III) (7, 8). However, in contrast to phenylalanine hydroxylase, recent studies on this enzyme (8) have revealed that tetrahydropterins reduce only a minor fraction of the Fe(III) present in the enzyme as isolated. Since a phosphorylated form of tyrosine hydroxylase has a more favorable K_m -value for its cofactor, and possibly also an increased V_{max} -value (1, 9), we have now further examined the reactions of the phosphorylated enzyme with the cofactor and the substrate.

Materials and Methods

Tyrosine hydroxylase was purified from the cytosolic fraction of bovine adrenal medulla homogenates, using a new large-scale procedure (8). Analyses of the phosphate content of various enzyme samples, prepared by this procedure, have shown the presence of somewhat varying amounts of phosphate, i.e. from below 0.2 to approx. 0.6 mol phosphate per mol monomer (8). The enzyme preparations used in the present study had specific activities of about 400 nmol/(min · mg) when assayed at pH 6.0, using 1 mmol/L 6-methyltetrahydropterin (6-MPH₄) as the cofactor and 0.3 mmol/L L-tyrosine as the substrate (10).

In the experiments using phosphorylated hydroxylase, the enzyme (1.5 g/L) was incubated with 0.1 g/L of the catalytic subunit of bovine heart cyclic AMP-dependent protein kinase (11), 4 mmol/L MgCl₂, 1 mmol/L [³²P]ATP, 20 μmol/L dithiothreitol (DTT), 100 mmol/L KCl and 4% (w/v) sucrose in 75 mmol/L K-phosphate, pH 7.0. After a reaction time of 15 min at 30 °C, the enzyme was subjected to gel filtration on a Sephadex G-25 column equilibrated with 200 mmol/L KCl in 100 mmol/L K-phosphate. Under these conditions, 0.6–0.8 mol phosphate per mol enzyme monomer was found to be incorporated into tyrosine hydroxylase (12). Thus, after phosphorylation, the enzyme contained at least 0.8 mol phosphate per mol monomer. In control experiments, the enzyme was incubated (without added ATP) and gel filtered under identical conditions.

Phenylalanine hydroxylase was purified from bovine liver by a slight modification (13) of the procedure of Shiman *et al.* (14).

6(*R*)-BH₄ and 6(*RS*)-methyltetrahydropterin (6-MPH₄) were from Dr. B. Schircks Laboratories (Jona, Switzerland). 4a-hydroxy-tetrahydrobiopterin (4a-OH-BH₄) was prepared by incubation of 50 μmol/L 6(*R*)-BH₄ with 20 μmol/L phenylalanine hydroxylase, 1 mmol/L dithiothreitol, 1 mmol/L phenylalanine, 0.2 g/L catalase and 100 mmol/L Tris-HCl, pH 8.4 (15). The enzyme was activated by incubation with 2 mmol/L L-phenylalanine at 25 °C for 2 min before the reaction was started by addition of cofactor. After a reaction time of 2 min at 25 °C, a 20 μL aliquot of the mixture was injected into the liquid chromatograph and the peak corresponding to the 4a-OH-BH₄ was collected at –80 °C (Fig. 1). Due to the rapid

Figure 1. Generation and isolation of tetrahydropterin oxidation products. For further details, see the main text.

decay of the 4a-hydroxy-tetrahydropterins, carefully controlled incubation time and temperature was necessary in order to obtain reproducible results.

HPLC analysis of pterins was performed on a Hewlett-Packard 60 × 4.6 mm i. d. (3 μm particle size) C₁₈ reversed phase column, equipped with a 30 × 4.6 mm i. d. C₁₈ precolumn (5 μm particle size) (15). The degassed mobile phase (ammonium acetate, ammonium formate, potassium phosphate or Tris-HCl) was pumped at 2.0 mL/min. The eluant was monitored with a Hewlett-Packard model 1040A diode array HPLC detector (Fig. 1).

X-band EPR spectra at 9.24 GHz microwave frequency were obtained with a Bruker ESP 300 EPR system equipped with an Oxford Instruments helium flow cryostat (ESR-9). The spectra of tyrosine hydroxylase (2 g/L in 150 μL) were obtained with 1 mW microwave power at 3.6 K, 10 Gauss field modulation and 11 min sweep time. The samples (in 200 mmol/L KCl and 100 mmol/L K-phosphate, pH 7.0) were kept in EPR tubes (i. d. = 4 mm); incubation of the enzyme with substrate or 6-MPH₄ and DTT was performed for 1 min at 25 °C, and the samples were subsequently frozen in ethanol kept at −80 °C and stored in liquid nitrogen.

Results

Kinetics of conversion of 4a-hydroxy-tetrahydropterins to dihydropterins

The 4a-hydroxy-tetrahydropterin has recently been isolated and established as an immediate reaction product of both the phenylalanine and tyrosine hydroxylase reactions with both the naturally occurring and synthetic tetrahydropterins (2, 5, 15, 16). The formation of the 4a-hydroxy-tetrahydropterins by tyrosine hydroxylase (or phenylalanine hydroxylase) can be selectively measured using high-speed HPLC with UV-detection (15). The 4a-hydroxy-tetrahydropterins are, however, rapidly dehydrated to the quinonoid dihydropterins in a spontaneous (2, 16, 17) or enzyme catalyzed (2, 17) reaction (equation 2).

Using preparative high-speed HPLC, the enzymatically generated 4a-OH-BH₄ was isolated and stored at −80 °C (15). Its spontaneous decay to qBH₂ (equation 2) and the subsequent tautomerization to 7,8-BH₂ (equation 3) was followed selectively (Fig. 2A). The pseudo first-order rate constant (k_2 in equation 2) of the non-enzymatic dehydration of the 4a-OH-BH₄ in Tris-HCl, pH 7.5, was found to be slightly dependent on the ionic strength. Thus, the observed $t_{1/2}$ at 23 °C was approx. 1.2 min in 5 mmol/L Tris-

Figure 2. Nonenzymatic conversion of 4a-OH-BH₄ to qBH₂ and 7,8-BH₂ in Tris-HCl, pH 7.50, at 23 °C. (A) 4a-OH-BH₄, prepared as described in the Methods section, was incubated for 81 s–459 s in 100 mmol/L Tris-HCl before injection into the liquid chromatograph. During incubation and chromatography, the 4a-OH-BH₄ was gradually converted into qBH₂ and 7,8-BH₂. The traces represent the absorbance at 246 nm. The solvent front appears at 22 s (vertical arrow). The total incubation time (including retention time) is also indicated. (B) Pseudo first-order reaction kinetics for the conversion of 4a-OH-BH₄ to the qBH₂ on incubation at 23 °C in 5 mmol/L (●), 20 mmol/L (▲) or 100 mmol/L (■) Tris-HCl, pH 7.50.

HCl, 1.1 min in 20 mmol/L Tris-HCl and 45 s in 100 mmol/L Tris (Fig. 2B). For comparison, the observed $t_{1/2}$ was approx. 40 s in both 20 mmol/L ammonium formate, pH 7.5, or 20 mmol/L K-phosphate, pH 7.5.

The effect of cyclic AMP-dependent phosphorylation of tyrosine hydroxylase on the formation of 4a-OH-BH₄

The phosphorylation of tyrosine hydroxylase by cyclic AMP-dependent and other protein kinases is known to stimulate its catalytic activity (for a review, see ref. 1). These studies have all been based on the assay of DOPA as the product of the tyrosine hydroxylase reaction. Using assay conditions which favour the accumulation of 4a-hydroxy-tetrahydropterins (15–18), a close correlation has been observed between the amount of 4a-hydroxy-tetrahydropterins recovered by HPLC and the amount of tyrosine formed (15). From Figure 3 it is seen that the 4a-OH-BH₄ formation by tyrosine hydroxylase phosphorylated by the purified catalytic subunit of bovine cyclic AMP-dependent protein kinase, is increased about five-fold compared to the non-phosphorylated control enzyme. Under these conditions a similar increased formation of tyrosine from phenylalanine was also observed (data not shown).

Figure 3. The effect of phosphorylation of tyrosine hydroxylase on the formation of 4a-OH-BH₄ and qBH₂. Unphosphorylated (trace a) or phosphorylated (trace b, upper trace) tyrosine hydroxylase (2 g/L) was incubated with 0.5 mmol/L BH₄ in the presence of 1 mmol/L DTT, 2 mmol/L L-phenylalanine, 0.2 g/L catalase and 100 mmol/L Tris-HCl, pH 8.4. After incubation for 2 min at 25 °C, a 20 µl aliquot was injected into the liquid chromatograph, and the absorbance was measured at 300 nm. The vertical arrow indicates components of the incubation mixture (Tris and ATP) eluting at or shortly after the solvent front.

Phosphorylation of tyrosine hydroxylase facilitates changes in the EPR spectrum of the enzyme-bound Fe(III)

Tyrosine hydroxylase as isolated contains 0.6–0.9 mol Fe per mol subunit enzyme. Its EPR spectrum shows that the enzyme-bound Fe(III) has *g*-values around 7.0, 5.2 and 1.9. This set of *g*-values is typical of high-spin Fe(III) species in an environment of slightly distorted axial symmetry (*E/D* ~ 0.04 ± 0.01). On addition of L-tyrosine this EPR signal is virtually unchanged (8). Under aerobic conditions the presence of an excess of 6-MPH₄ (0.2 mmol/L) plus DTT (2 mmol/L) induces moderate changes of the EPR spectra, even in the presence of L-tyrosine, while the addition of a strong reducing agent such as dithionite (10 mmol/L), makes the iron EPR silent, indicating that the iron is reduced to Fe(II) (8).

Figure 4 shows the effect of phosphorylation on the EPR spectrum of tyrosine hydroxylase, in the presence of either substrate or cofactor and DTT. While phosphorylation itself has small effects on the EPR

Figure 4. EPR spectra of phosphorylated tyrosine hydroxylase. (a) After incubation with 1.5 mmol/L L-tyrosine, (b) only standard incubation medium (control), (c) after incubation with 1.7 mmol/L D-tyrosine, and (d) after incubation with 1 mmol/L 6-MPH₄, 1.7 mmol/L D-tyrosine and 5 mmol/L DTT. All incubations were performed for 1 min at 25 °C. The spectra are not corrected for the small dilution upon adding substrate or cofactor (7% dilution in Figs. 4a and 4c, 9% dilution in Fig. 4d); pH was kept between 7.0 and 7.2.

spectrum of the resting enzyme (Fig. 4b), the spectrum in the presence of either substrate or cofactor and DTT is markedly changed by phosphorylation. Thus, after incubation with 1.5 mmol/L L-tyrosine (Fig. 4a), the signal intensity of the species with $g_{\max}$ around 7.0 was significantly reduced. A new high-spin Fe(III) species with a $g_{\max}$ -value of 9.2 appeared, and the contribution around $g = 4.3$ was considerably increased. In comparison, incubation of the phosphorylated enzyme with 1.7 mmol/L D-tyrosine resulted in an EPR spectrum (Fig. 4c) which was similar to that of the phosphorylated enzyme control (Fig. 4b). Aerobic incubation with 2 mmol/L 6-MPH₄ and 10 mmol/L DTT makes the previously EPR detectable Fe(III) with $g_{\max} \sim 7.0$ of the phosphorylated enzyme EPR silent, presumably due to reduction of its ferric iron to Fe(II) (12). This reduction is slightly influenced by the presence of D-tyrosine (Fig. 4d). In comparison, if the non-phosphorylated enzyme is incubated with 2 mmol/L 6-MPH₄ and 10 mmol/L DTT, more than 75% of the $g_{\max} \sim 7.0$ species is still present (data not shown).

Discussion

High-speed liquid chromatography as an analytical tool in the analysis of unstable tetrahydropterins

During catalytic turnover, both phenylalanine and tyrosine hydroxylase oxidize the tetrahydropterin cofactors, like tetrahydrobiopterin, tetrahydroneopterin and 6-methyl-tetrahydropterin, to their respective 4a-hydroxy-tetrahydropterins (equation 1) (15). These compounds have previously been observed spectroscopically as transient intermediates (2, 15–18) or analyzed at subzero temperatures (2, 19). Thus, stopped-flow spectroscopy has provided valuable information about the kinetics and spectral properties of such intermediates formed in the phenylalanine and tyrosine hydroxylase reactions (15, 18). In order to obtain additional spectral and kinetic information about the oxidation of the cofactor in these reactions, we have developed a HPLC method for the spectroscopic characterization, quantitation and isolation of the unstable pterins formed in the enzymatic reactions (15). Thus, the HPLC analysis of pteridines (20, 21) has been extended to include also short-lived pterins. In the present study, this technique has been used to examine the spontaneous dehydration of 4a-OH-BH₄. The rate of dehydration of 4a-hydroxy-tetrahydropterins (k_2 in equation 2) has been estimated spectroscopically for the synthetic BH₄ analogue 6-MPH₄ in a complex reaction mixture (2, 17). For the biopterin derivative, however, the rate of tautomerization of the quinonoid intermediate dihydropterin (k_3 in equation

3) is about 10 times faster, and this reaction therefore interferes in the estimation of the rate constant for the dehydration of 4a-OH-BH₄ (k_2 in equation 2) under similar experimental conditions (16, 17). Using the rapid HPLC method, which separates the different pterins, the two sequential reactions can be studied separately (Fig. 2). The rate of disappearance of 4a-OH-BH₄ (k_2 in equation 2) was found to be only moderately influenced by the nature and concentration of the buffers employed. This is in contrast to the observed effects of buffer ions on the rate of tautomerization of the quinonoid dihydropterins (k_3 in equation 3) (22).

The role of iron in the tyrosine hydroxylase reaction

Although iron seems to be essential for the catalytic activity of tyrosine hydroxylase, the mechanistic role of the enzyme-bound iron is not understood. The Fe(II) state seems to be catalytically important, since the enzyme is inhibited by low concentrations of Fe(II) chelators (8). This is analogous to phenylalanine hydroxylase and rat pheochromocytoma tyrosine hydroxylase, in which Fe(II) is postulated to be the only active iron species (2, 15, 16). However, a considerable amount of the iron in tyrosine hydroxylase exists as Fe(III) (8), which has been studied by various spectroscopic techniques (7, 8, 23). Thus, tyrosine hydroxylase as isolated has a blue-green colour (7, 8), and its resonance Raman spectra have been assigned to a coordination of the feedback inhibitors adrenaline and noradrenaline to the enzyme-bound high-spin Fe(III) (23, 24). In analogy with studies on model complexes, evidence has been obtained in support of a catecholamine coordination which may lower the redox potential of the iron and thus stabilize the Fe(III) state (12, 23, 24).

After phosphorylation, the Fe(III) species with $g_{\max} \sim 7.0$ is made EPR-silent by incubation with 6-MPH₄ and DTT (in the absence of added substrate), indicating that this Fe(III) species is reduced to Fe(II). The phosphorylation of tyrosine hydroxylase does not seem to change the reaction stoichiometry (equation 1), since a comparably increased formation of both 4a-OH-BH₄ and tyrosine was observed and no new reaction products were detected. Similarly, the phosphorylated enzyme reacts in a strictly stereospecific manner with tyrosine, i. e. only the L-enantiomer (Fig. 4a), as previously observed for the catalytic reaction (1, 15).

While tyrosine hydroxylase as isolated has many spectroscopic features which are distinct from those of phenylalanine hydroxylase, the phosphorylated enzyme shows some similarities to the non-phosphoryl-

ated form of phenylalanine hydroxylase. For this enzyme, the direct formation of quinonoid dihydropterin is accompanied by the reduction of its Fe(III), in an activating reaction, which can be inhibited by catecholamines (2, 5, 6). The molecular events responsible for the activation of tyrosine hydroxylase by phosphorylation (25) is presently not known. Our working hypothesis is that it decreases the affinity of the feedback inhibitors to the Fe(III) (Andersson, K. K. unpublished observations), thus facilitating changes in redox-state of the enzyme-bound iron (12, 24).

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