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Tetrahydrobiopterin protects phenylalanine hydroxylase activity in vivo: Implications for tetrahydrobiopterin-responsive hyperphenylalaninemia

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Abstract The natural cofactor of phenylalanine hydroxylase (PAH), tetrahydrobiopterin (BH_4), regulates the enzyme activity as well as being essential in catalysis. BH_4-responsive PAH deficiency is a variant of hyperphenylalaninemia or phenylketonuria (PKU) caused by mutations in the human PAH gene that respond to oral BH_4 loading by stimulating enzyme activity and therefore lowering serum phenylalanine. Here, we showed in a coupled transcription–translation in vitro assay that upon expression in the presence of BH_4, wild-type PAH enzyme activity was enhanced. We then investigated the effect of BH_4 on PAH activity in transgenic mice that had a complete or partial deficiency in the endogenous cofactor biosynthesis. The rate of hepatic PAH enzyme activity increased significantly with BH_4 content without affecting gene expression or *Pah*-mRNA stability. These results indicate that BH_4 has a chaperon-like effect on PAH synthesis and/or is a protecting cofactor against enzyme auto-inactivation and degradation also in vivo. Our findings thus contribute to the understanding of the regulation of PAH by its cofactor BH_4 on an additional level and provide a molecular explanation for cofactor-responsive PKU.
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Keywords: Tetrahydrobiopterin; Hyperphenylalaninemia; Phenylketonuria; Hepatic phenylalanine hydroxylase; Chaperon; Gene expression

1. Introduction

As an essential oxygen-activating cofactor for nitric oxide synthase (NOS) and aromatic amino acid hydroxylases, tetrahydrobiopterin (BH_4) is necessary for NO and monoamine neurotransmitter production, as well as for phenylalanine catabolism. Other functions associated with BH_4, some of which are less defined on a molecular level, include regulation of cell proliferation, self-protecting activity for NO toxicity, and regulation of apoptosis [1,2]. Most of these effects are secondary and can be attributed to either the intracellular availability of BH_4 for NO production or superoxide/peroxide formation from auto-oxidation of non-protein-bound BH_4 in the presence of molecular oxygen [3]. Although the enzymes involved in the synthesis of BH_4 are constitutively expressed in liver (but not necessarily in other organs), where BH_4 acts as cofactor of the enzyme phenylalanine hydroxylase (PAH), the regulation of the BH_4 biosynthesis is complex. BH_4 negatively regulates its synthesis by product feedback inhibition of the first enzyme in the synthesis pathway, the GTP cyclohydrolase I, while L-Phe is an activator [1]. BH_4 also exerts direct negative regulatory effects on the activity of the hepatic PAH, namely as an inhibitor of both the activation of the enzyme by preincubation with L-Phe [4] and of the rate of phosphorylation of the enzyme [5]. Moreover, in vitro, BH_4 inhibits the proteolytic degradation of PAH [6], but little is known about the in vivo significance of these findings or the regulation of PAH by BH_4 at the transcriptional and translational level.

Synthetic BH_4 is routinely used for replacement therapy to treat patients with one of the rare genetic defects of cofactor biosynthesis or regeneration [7]. Recently, a potential new application for BH_4 was put forward. This concerns the BH_4-responsive PAH deficiency [8,9], which is a subtype of classical phenylketonuria (PKU; for a recent review see also [10]). PKU is an autosomal recessive disorder and over 400 mutations have been reported causing various degrees of deficiency in hepatic PAH, and thus to abnormal and toxic accumulation of serum L-Phe [11]. PKU patients that respond to high doses of oral synthetic BH_4 carry specific mutations in the human PAH gene leading to either single amino acid alterations or small in-frame insertions or deletions. All these responsive mutant alleles exhibit potential residual PAH activity [12]. Based on the known 3D-structure for the tetrameric PAH and on the location or vicinity of mutations to the catalytic domain including the BH_4-binding site, or the regulatory or oligomerization domains, it has been proposed that the responsiveness to BH_4 is related to K_m variants with lowered affinity for BH_4 binding [13–16]. However, for BH_4-responsiveness of PAH-mutations found outside these domains, alternative explanations were discussed, including upregulation of gene expression, PAH-mRNA stabilization, and protection of PAH from misfolding and/or degradation [17–20]. Recent experimental analyses of wild-type or mutant PAH indicated that the response of PKU mutations may have a multifactorial basis [21].

The generation of 6-pyruvoyltetrahydropterin synthase (*Pts*) knockout mice, which are unable to biosynthesize BH_4, has recently been described [22,23]. Interestingly, the $Pts^{-/-}$ homozygotes showed normal tyrosine hydroxylase (TH) gene expression, as shown by Northern blot analysis of brain tissue, but a depletion of protein and activity with respect to the

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wild-type animals, while tryptophan hydroxylase (TPH) was not affected [22]. Furthermore, blood phenylalanine content was increased in $Pts^{-/-}$ mice [23]. However, the consequence of disruption of the *Pts* gene on PAH protein and activity has not been investigated in detail. Here, we have analyzed the effect of BH_4 on wild-type PAH expression and activity in untreated newborn mice in the *Pts* k.o., heterozygous, and wild-type background, and found that the natural cofactor for PAH does not affect gene expression but rather stimulates PAH enzyme activity and stability in a concentration-dependent manner, thus providing a rationale for BH_4-responsive hyperphenylalaninemia in humans.

2. Materials and methods

2.1. Coupled in vitro transcription–translation system

Coupled in vitro transcription–translation (TnT) of wild-type human PAH was carried out by using the TnT-T7 reticulocyte lysate system from Promega and the pcDNA3-hPAH expression vector as described [24], with various concentrations of added BH_4 up to 500 μM, kept reduced with 5 mM dithiothreitol, which was also present in the synthesis assays carried out in the absence of BH_4. After 30 min, the extracts were filtrated with MicroSpin™ G-25 columns (Amersham Biosciences) to eliminate free amino acids, BH_4 and other low molecular weight compounds prior to measurements of PAH activity at standard conditions using 15 μl of the filtrated extract.

2.2. Transgenic Pts mice

Hybrid mice from 129/Sv-C57BL/6 back-crossings with different genotypes regarding the *Pts* allele (wild-type, heterozygous and homozygous; 3–8 individuals from each group) were sacrificed at day one after birth and their livers were frozen immediately in liquid nitrogen. Liver homogenate was prepared at 4 °C in the presence of dithiothreitol and the protease inhibitors phenylmethylsulfonyl fluoride, leupeptin, and pepstatin as described [23].

2.3. Assay of PAH activity

The PAH activity assay was adapted from Ledleay et al. [25]. For every assay, liver homogenate containing 50–100 μg of total protein was used [23]. The appropriate amount of homogenate was adjusted to a final volume of 78 μl with water, followed by adding 22 μl of a master mix containing 0.6 mM phenylalanine, 3.6 U of catalase (Sigma), and 0.15 M KCl in a 0.2 M potassium phosphate buffer, pH 6.8. After preincubation at room temperature for 5 min, the reaction was started by adding 2 μl of 0.1 M dithiothreitol and 2 μl of 4.5 mM 6-methyltetrahydropterin (Schircks Laboratories) to the samples and incubated for 60 min at 25 °C. The reaction was stopped by incubating for 5 min a 96 °C heating block. For the blank, the appropriate amount of liver extract was adjusted to a final volume of 104 μl with water and incubated for 5 min in a 96 °C heating block. Samples were centrifuged for 5 min at 13 000 rpm, and the supernatant was filtered in an Ultrafree-MC filter device and centrifuged again at $5000 \times g$ for 15 min. Phenylalanine and tyrosine were quantified with a standard amino acid analyzer (Biochrom 20 Plus, Amersham Bioscienes). Alternatively, for the measurement of PAH in the TnT expression assays (see above), we measured activity as described [26], using standard concentrations of L-Phe (1 mM) and BH_4 (75 μM; (6*R*)-5,6,7,8-tetrahydro-L-biopterin, $BH_4 \cdot 2HCl$ from Schircks Laboratories, Jona, Switzerland) at pH 7.0 and 25 °C, with pre-activation of the enzyme by 5 min pre-incubation with L-Phe.

2.4. Western blot analysis

Quantification of hepatic PAH by Western blot analysis was performed as described [26], using affinity-purified polyclonal rabbit anti-rat PAH (1.6 μg/ml) as the primary antibody, and 3–100 ng human PAH protein, in which range the method was quantitatively linear.

2.5. RNA isolation and quantitative PCR

20–30 mg of mouse liver was used for RNA isolation according to the manufacturer's protocol (QIAmp RNA Blood Mini Kit from Qiagen). Random primed cDNA was prepared from 1 μg total RNA using the Reverse Transcription System from Promega. Quantitative PCR was performed using Taqman technology and an AbiPrism 7700 sequence detector and the TaqMan Universal PCR Master Mix (Applied Biosystems). Probe and primer sequences were as follows: MPAH3-TM-Probe: 5′-CTT TTG CTG CCA CAA TCC CCC G-3′ (5′-FAM and 3′-TAMRA-labeled); MPAH1-TM-FOR: 5′-CCG AGA GTT TCA ATG ATG CCA-3′; MPAH2-TM-REV: 5′-TCA TAG CGA ACG GAG AAG GG-3′. For controls, standard probes for GAPDH or 18S ribosomal RNA were used. All probes and sequences were synthesized by Microsynth (Balgach, Switzerland).

3. Results and discussion

3.1. BH_4 enhances PAH activity under in vitro conditions

Recently, pulse-chase analyses in a cell-free coupled TnT eukaryote system have been applied to investigate the effect of BH_4 on the activity and stability of PAH expressed in the absence and the presence of BH_4 [21]. As seen by size-exclusion chromatography, the in vitro synthesized protein has a similar oligomeric distribution into tetrameric and dimeric forms as the enzyme from liver or obtained by expression in *Escherichia coli* [27] both in the absence and the presence of BH_4 during synthesis [21]. Nevertheless, the presence of the cofactor results in a moderate stimulation of the activity of the newly synthesized PAH, and also appears to protect the enzyme against the rapid inactivation observed in the TnT system, possibly by shielding the active site from reactive oxygen species [21]. These experiments, however, were performed at very high concentration of BH_4 (500 μM), far above the physiological intracellular concentration of BH_4 in mouse and human liver, which have been reported to be 5 and 10 μM, respectively [21,28,29]. We here studied the synthesis of $[^{35}S]$Met-labeled PAH in the TnT system for 30 min at 30 °C in the presence of various concentrations of BH_4 up to 500 μM. The in vitro synthesized enzyme showed the characteristic double band corresponding to the phosphorylated (~51 kDa) and unphosphorylated (~50 kDa) enzyme (Fig. 1A). The amount of protein was found to increase slightly with BH_4 concentration up to 100 μM BH_4 (maximal $22 \pm 3\%$ increase in total protein content), and no change was observed in the similar distribution of phosphorylated and unphosphorylated PAH (Fig. 1A). The specific activity of the synthesized PAH measured at standard conditions (1 mM L-Phe and 75 μM BH_4) increased more significantly than the protein amount when expression was carried out in the presence of BH_4, with an optimum range for BH_4 at 25–100 μM under the conditions used (Fig. 1B). The stimulation by BH_4 of PAH activity expressed in vitro is most probably related to a direct effect on the enzyme, since activation of transcription or mRNA stabilization can be ruled out in this in vitro protein expression system. Moreover, according to some recent results based on TnT assays combined with pulse-chase methodology [21], we anticipate increased amount of protein, and consequently of activity, for a number of PAH mutant proteins in PKU patients, such as A309V, V388M, and Y414C, for which it was observed in vitro that BH_4 prevents protein degradation (see also the discussion below on sub-saturating levels of BH_4 with respect to PAH).

3.2. Newborn mice defective for BH_4-cofactor biosynthesis exhibit normal PAH gene expression but reduced protein and enzyme activity

To our knowledge, no animal model with a BH_4-responsive mutant PAH is available to investigate the effect of BH_4 on

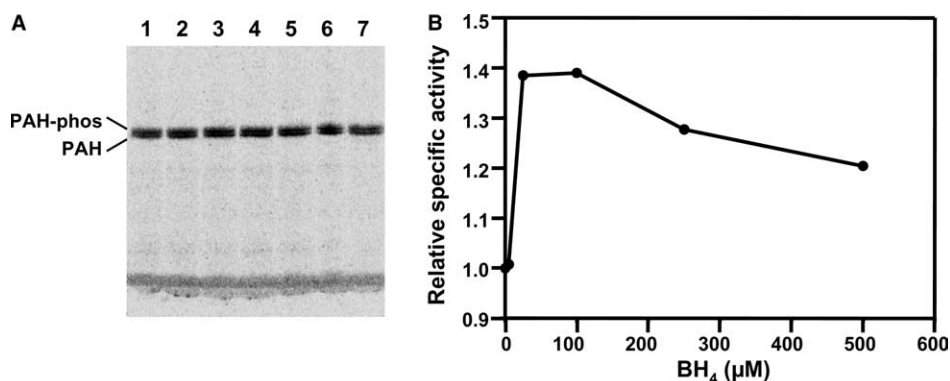

Fig. 1. Effect of BH_4 on the expression of wild-type human PAH in the in vitro TnT system. (A) Lanes 1–7: autoradiography of the expressed enzyme, phosphorylated (PAH-phos; 51 kDa) and unphosphorylated (PAH; 50 kDa) in the presence of 0, 5, 10, 25, 100, 250, and 500 μM BH_4 in the TnT assays. (B) PAH activity of enzyme expressed in the TnT system in the absence and presence of the indicated concentrations of BH_4. The values are given relative to the activity of the control PAH synthesized in the absence of BH_4 and are calculated in mU/cpm. Five mM dithiothreitol was present in the reactions, whereby the reducing agent did not affect the amount of PAH expressed or activity of the TnT mixtures, i.e., similar values in the absence of BH_4 and in the presence or absence of 5 mM dithiothreitol.

hepatic PAH in vivo. We thus analyzed untreated mice that were compromised for cofactor biosynthesis by harboring a homozygous or heterozygous deletion allele for *Pts*. The *Pts* gene codes for the second BH_4-biosynthetic enzyme, the 6-pyruvoyl-tetrahydropterin synthase [1]. Homozygous $Pts^{-/-}$ mice generated by targeted deletion were born with no visible developmental abnormalities, including normal liver, but died soon after birth due to neurotransmitter deficiency [23]. As previously reported, liver extracts of newborn wild-type mice contain 21.06 ± 0.43 pmol BH_4 per mg of protein, whereas heterozygous *Pts*-animals exhibit reduced BH_4 levels (12.53 ± 0.60 pmol/mg) and *Pts* knockout mice have only traces of liver BH_4 (0.87 ± 0.22 pmol/mg) [23]. We now compared BH_4 content to PAH gene expression and activity in the liver of newborn mice (Fig. 2). The PAH activity of wild-type animals was 1.05 ± 0.14 mU/mg, and it was reduced to 0.62 ± 0.04 mU/mg in heterozygous and to 34% that of the wild-type mice in homozygous *Pts* knockout (0.36 ± 0.07 mU/mg; Fig. 2B). In contrast, mRNA levels for *Pah*, quantified using real-time PCR technology, showed basically no difference between liver samples of wild-type, heterozygous, and knockout animals (see Fig. 2C; the relative values with respect to GAPDH controls were: wild-type 2.49 ± 1.49, range 1.18–4.39; heterozygous 2.91 ± 1.70, range 1.09–6.84, and knockout 2.30 ± 1.03, range 1.0–3.62). Gene expression and/or mRNA stability for the *Pah* gene was thus not responsible for the reduction of PAH activity.

We then determined the amount of PAH protein per total protein in liver extracts by quantitative Western blot analysis (Fig. 3). The wild-type mice had 1.8 ± 0.63 μg PAH protein/mg, while the heterozygous animals had 0.81 ± 0.10 μg/mg, and the knockout mice had 0.34 ± 0.10 μg/mg. Thus, the PAH protein represents 0.18% of the total liver protein in newborn wild-type mice and this value was reduced to 0.034% for the knockout animals. The PAH content in wild-type mice corresponds to about 40 pmol of PAH subunit/mg total protein, and is similar to the values reported in rat hepatocytes (i.e., 41.7 pmol PAH subunit/mg protein, corresponding to 9.1 μM intracellular PAH subunit [30] as well as to values reported in rat liver by several other authors, where PAH represents 0.1–0.3% of the total protein [31,32]. As can be seen in the representative Western blots (Fig. 3A), low molecular weight proteolyzed forms of PAH were present in extracts from knockout animals indicating that the low amounts of PAH protein and activity PAH measured in$Pts^{-/-}$ mice result from degradation of the enzyme either in vivo or, less likely, during the quick preparation of the liver extracts. These results strongly suggest that the amount of BH_4 cofactor endogenously synthesized appears to directly affect the conformational stability and consequently the activity of PAH, rather than by influencing PAH gene expression or mRNA stability. Earlier in vitro experiments showed that the binding of BH_4 induces a tighter conformation of the enzyme and reduces the rate and extent of proteolytic degradation of PAH both by trypsin [33] and chymotrypsin [6].

Our in vitro and in vivo results highlight the importance of BH_4 in preserving the enzymatic integrity of PAH. The effect appears to be dramatic as seen in the in vivo situation in the absence of cofactor, but is still observed upon cofactor addition in the in vitro system. In this context, it is also worth mentioning that Kaufman and coworkers observed in the liver biopsy from a patient with PKU due to deficiency of dihydropteridine reductase that the level of PAH was decreased to about 20% of controls [29]. They further speculated that this lack was due to BH_4 deficiency which could lead to an accelerated rate of degradation of PAH, similar to what we find here in our BH_4-deficient mice. A somewhat similar observation was also made for tyrosine hydroxylase in BH_4-deficient mice: the protein in the brain of homozygous newborn mice was reduced in Western blots, whereas the mRNA levels exhibited no difference, as shown by Northern blot analysis [22]. These observations might represent a more general in vivo regulatory mechanism of BH_4 at least for PAH and tyrosine hydroxylase. However, it has to be stressed that tyrosine hydroxylase and dopamine biosynthesis in the brain of $Pts^{-/-}$ mice were not recovered upon administration of BH_4. On the other hand, BH_4 loading led to a reduction of blood phenylalanine levels to normal serum conditions [22,23]. Furthermore, treating adult wild-type mice for 10 days with oral daily doses of 10 mg of BH_4 per kg body weight did not induce any change neither in relative *Pah*-mRNA or PAH expression and activity (not shown), an observation that was recently confirmed regarding PAH activity with a breath test in mice [34].

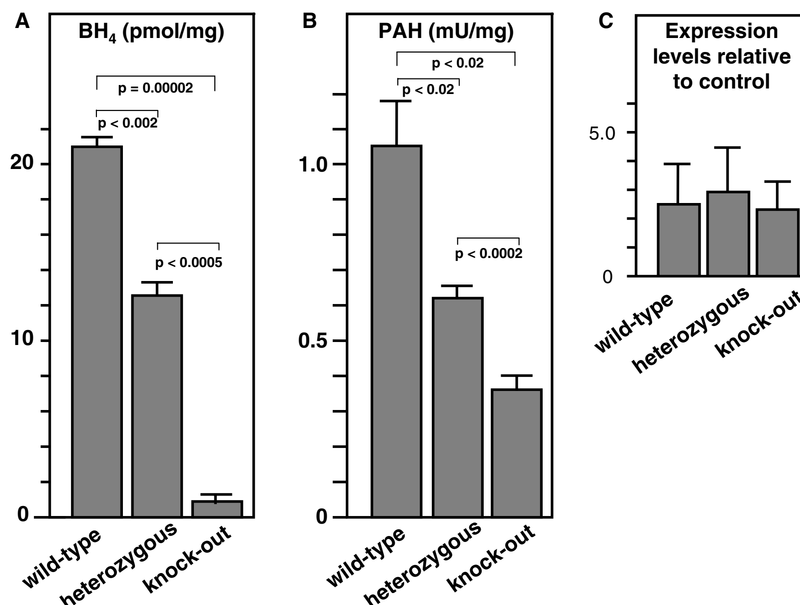

Fig. 2. Comparison of BH_4 content (A), PAH activity (B), and *Pah*-mRNA expression (C) in liver extracts of one-day old mice with different *Pts* genotypes. Values in A and B are given per mg of total protein extract (*p* values for the Student's *t* test are indicated; $n = 3$–6 measurements from at least 3 different animals per group). Note that BH_4 values in A have been published previously ([23] in Table II) and are depicted here for comparison to PAH expression. For TaqMan analysis in C, the liver from 6–8 individual animals was tested for *Pah*-mRNA expression in triplicates and given as relative values compared to the GAPDH control.

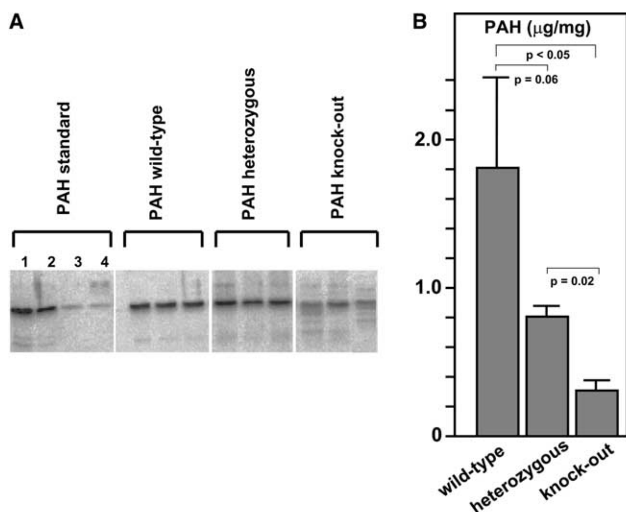

Fig. 3. Quantification of PAH in liver homogenates of one-day old mice with different *Pts* genotypes. Values are given per mg of total protein extract. (A) Quantitative Western blot analyses. PAH standards; lanes 1, 2, 3 and 4: 100 ng, 25 ng, 6.3 ng, and 3.3 ng purified human recombinant PAH [26]. Three μl of liver extract (about 30 mg total protein/ml) was separated on the gels for the *Pts* wild-type, heterozygous and knockout mice. (B) Averaged quantitative data from (A).

3.3. Implications for BH_4-responsive hyperphenylalaninemia

PAH forms a low activity and high stability complex with BH_4 at metabolic stages characterized by low concentration of L-Phe [30]. In turn, L-Phe is known to elicit an activating conformational change on PAH that surpasses the closed conformation induced by BH_4 [4]. Formation of the PAH-BH_4 complex appears to be an important regulatory mechanism maintaining the integrity of the enzyme, and the lack of BH_4 would thus turn out to be deleterious for PAH, in agreement with the results shown here. These results also seem to have implications to understand the regulatory role of BH_4 also in disease situations, notably for BH_4 responsiveness in a subclass of mild PKU patients. The PAH mutations leading to this phenotype are spread throughout the 3D-structure [12]. A common feature, however, is that the mutants are all characterized by a considerable level, although insufficient, of remaining PAH activity. The reason for why BH_4 supplementation therapy is beneficial might be related to the fact that BH_4 appears to be sub-saturating in vivo. Kure et al. have recently discussed the implications of cofactor sub-saturation, related to a lower hepatic concentration of BH_4 (5–10 μM) with respect to the K_m for the cofactor in the PAH reaction (25 μM), to explain the increase in enzyme activity upon supplementation [34]. Also relevant is our finding that BH_4 appears to be sub-saturating with respect to PAH content in order to form the stabilizing PAH-BH_4 complex. Thus, wild-type mice contain about half molar concentration of BH_4 (21 pmol/mg protein) with respect to PAH (40 pmol PAH subunit/mg protein) (see above). Mitnaul and Shiman [30] reported stoichiometric amounts of cofactor and enzyme in rat hepatocytes, while to our knowledge the values for this relation are not known for human liver. Nevertheless, and because BH_4 is also a cofactor for other liver enzymes, not all BH_4 is expected to be available for complex formation with PAH and it seems predictable that supplementation will further extend the protective mechanisms that BH_4 exerts on the enzyme as we describe here and elsewhere, notably in non-homeostatic disease conditions, as PKU.

In conclusion, our results support that BH_4 supplementation, in addition to increase in PAH activity by increasing the sub-saturating in vivo hepatic concentration of the cofactor [34], will significantly increase PAH activity by a rise in the amount of

active and more stable enzyme that would reach the threshold value for normal L-Phe hydroxylation levels in the patients.

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