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Molecular Genetics and Metabolism 86 (2005) S11–S16
Molecular Genetics and Metabolism
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Minireview

Kinetic and stability analysis of PKU mutations identified in BH_4-responsive patients

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Received 29 April 2005; received in revised form 6 June 2005; accepted 7 June 2005
Available online 8 August 2005

Abstract

From all the different molecular mechanisms put forward to explain the basis of BH_4 responsiveness in PKU patients, a clear picture is now emerging based on the results from expression studies performed with a number of missense mutations identified in patients with a positive response in BH_4 loading tests. Two of the proposed mechanisms, namely decreased binding affinity of the mutant proteins for the natural cofactor and stabilization effect of BH_4, have been confirmed for several PKU mutations and the results are reviewed here. The actual view supports a multifactorial basis of the response, highlighting the necessity of detailed in vitro characterization of each mutant PAH protein. Several of the confirmed molecular mechanisms may be operating simultaneously, as exemplified in the data presented, and this may result in different degrees of BH_4 responsiveness.
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Keywords: PKU; Tetrahydrobiopterin; PAH gene mutations; K_M mutants; Chemical chaperon

Introduction

Since the first report on BH_4 responsiveness in patients with PAH gene defects [1], many clinical studies have been undertaken to determine the proportion of patients who could benefit from BH_4 therapy. Inspection of the genotypes of the patients confirmed the involvement in the response of many different missense mutations located all along the structure of the protein and not confined to the cofactor binding regions as initially proposed [2]. At that time, no experimental data supported the prediction of K_M mutants as responsible for BH_4 responsiveness, underlying the need for expression studies and detailed kinetic measurements of mutant PAH proteins with the natural cofactor BH_4. Wild-type and mutant PAH proteins had been effectively expressed in both prokaryotic, eukaryotic, and cell-free systems. However, these studies had focused mainly on the confirmation of the pathological effect as deduced from the reduced relative activity or reduced soluble protein obtained, confirming a folding defect for many of the mutant proteins. In addition, many of these studies were performed using the synthetic cofactor 6-methyl-tetrahydropterin (6M-PH4) which precludes the determination of the activation properties of the enzyme and of the affinity for the natural cofactor used in the in vivo studies.

During the past few years several other mechanisms, among them the chaperon-like activity of BH_4 stabilizing altered conformations of the enzyme, were suggested to explain BH_4 responsiveness. The increasing number of

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doi:10.1016/j.ymgme.2005.06.009

mutations potentially involved made unlikely the explanation of decreased binding affinities for each of them. Again, experimental confirmation of the proposed mechanisms depended on expression analysis of recombinant PAH proteins.

Taking advantage of the expertise of several groups in diverse research fields, a collaborative study was undertaken [3,4] to fill the existing gap in the experimental knowledge of the biochemical characteristics of mutant PAH proteins associated with BH_4 responsiveness and of the effect of the cofactor on these proteins. In this article we review the results from the kinetic analysis of recombinant PAH mutants expressed as MBP fusion proteins in *Escherichia coli* and the results of stability analysis performed in an in vitro transcription-translation assay, which serve for discussion of the molecular basis of BH_4 responsiveness.

Kinetic properties of BH_4-responsive PAH mutants

A total of 18 different point mutations found in the BH_4-responsive patients have been analyzed using MBP-PAH fusion proteins (PAH with maltose binding-protein) in *E. coli* (Fig. 1). From the expressed mutations three of them are located in the regulatory domain (F39L, I65T, and R68S), fourteen in the catalytic domain (H170D, E178G, V190A, P244L, R261Q, A300S, L308F, A309V, A313T, A373T, V388M, E390G, P407S, and R408Q) and one near the tetramerization domain (Y414C). These expressed point mutations have been identified in classical, mild PKU and mild hyperphenylalaninemia (MHP) patients [5] [6]. When expressed in the prokaryotic system, all the mutant proteins have substantial residual activity at standard conditions (1 mM L-Phe, 75 μM BH_4) ranging between 23–120% as compared with wild-type PAH. This is likely a general prerequisite for BH_4 responsiveness, in the sense that at least one of the alleles in a responsive patient should harbour a partially active mutation and severe functionally null mutations do not contribute to the response. Null mutations include those associated with no protein or activity as shown in expression analysis and frameshift or splicing mutations which result in truncated proteins. However, splicing mutations should be considered with caution and their effect characterized precisely, taking into account the recent results of a responsive patient homozygous for the splicing mutation IVS10-3C > T [5], which generates some normal transcript coding for wild-type PAH protein.

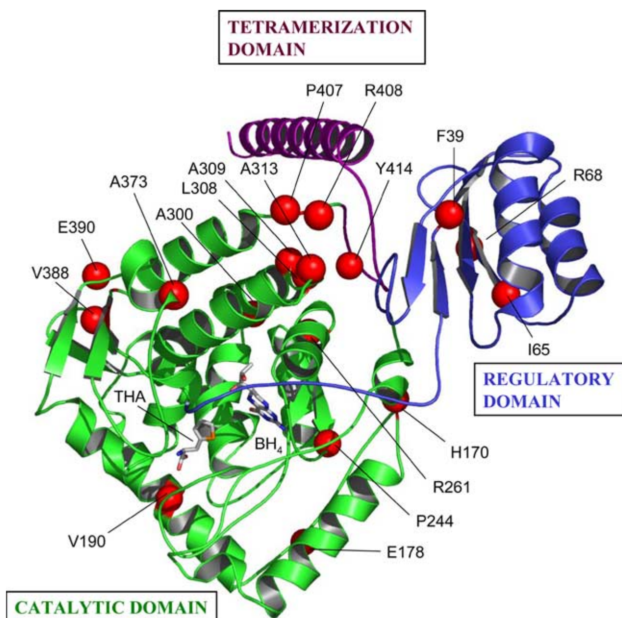

Fig. 1. Localization of the studied mutations in the PAH monomer, complexed with tetrahydrobiopterin (BH_4), 3-(2-thienyl)-L-alanine (THA) and iron (Fe). Protein model was constructed using the published structures of isolated catalytic domain (Protein Data Bank entry: 1KW0 [13]) contact interface between catalytic and regulatory domains (1PHZ, [14]), and tetramer interfaces (2PAH) [15]. Partial models were aligned through their common secondary elements using DALI [16,17] and Spdbv [18] programs to obtain the whole three dimensional representation of PAH protein. The monomer structure is represented as ribbon plot and coloured according to domain organization (catalytic: green, regulatory: blue, tetramerization: purple). Positions of mutation-related residues are indicated as red spheres.

Detailed analysis of the steady-state kinetic parameters of the expressed mutant PAH proteins showed that all have at least some defect in either specific activity, degree of activation by the substrate L-Phe, binding affinity for L-Phe (L-Phe($S_{0.5}$)), for BH_4 ($K_M(BH_4)$), degree of cooperativity (Hill index) for L-Phe or substrate inhibition. The mutations with residual activity close to wild-type values (I65T, R68S, V190A, and E390G) exhibited other kinetic defects i.e., abnormal $S_{0.5}$, K_M or Hill coefficient values, demonstrating in any case that they are loss-of-function mutations (Table 1). Some of these mutations, with such a mild impairment in specific activity have been repeatedly reported to be associated with MHP, the mildest phenotype not requiring dietary therapy.

To obtain maximum specific activity with the natural cofactor BH_4, PAH must be preincubated with its substrate L-Phe, which induces a conformational change displacing the autoregulatory amino terminal sequence from the active site [7,8]. In addition, the enzyme shows positive cooperativity which is physiologically relevant as a mechanism to control blood L-Phe homeostasis [9]. BH_4 acts as a negative regulator blocking L-Phe binding. The kinetic analysis of the 18 mutants showed a disturbance in the regulatory properties for all of them. Regarding L-Phe activation, three patterns were observed: (i) type I mutants which are activated by L-Phe in the same way as the wild type enzyme, (ii) type II mutants which act as if they were already activated (pre-activated mutants), and (iii) Type III mutants which cannot be activated by L-Phe (Table 1). Seven mutants

Table 1
Residual activity, Hill coefficient, and binding affinity for L-Phe of PAH mutant proteins

PAH proteins	Residual activity in prokaryotic system (%)	h	$S_{0.5}$ (L-Phe) (μM)
Wt	100	2.0	145 ± 12
Type I			
H170D	43	2.8	104 ± 3
V190A	110	2.9	139 ± 10
E390G	93	1.5	153 ± 15
A313T	76	1.5	165 ± 18
A373T	56	1.8	144 ± 14
P407S	94	2.1	140 ± 5
Y414C	79	1.5	109 ± 19
Type II-preactivated			
F39L	89	1.4	60 ± 8
I65T	120	1.0	80 ± 10
R68S	92	1.5	73 ± 6
R261Q	78	1.1	610 ± 60
R408Q	67	1.2	38 ± 8
Type III			
E178G	39	1.1	277 ± 38
P244L	20	1.0	160 ± 25
A300S	31	1.1	151 ± 25
L308F	49	1.9	151 ± 13
A309V	44	1.1	150 ± 20
V388M	23	1.1	1200 ± 110

Data from [3,4].

belong to type I, five to type II, and six to type III. Other preactivated PAH mutants had already been reported and the structural changes they induce studied by circular dichroism, fluorescence spectroscopy and molecular dynamics simulations [8]. The authors showed that the mutations analyzed caused a series of conformational changes that mimic those induced by binding of L-Phe, including displacement of the inhibitory N-terminal sequence that covers the active site. This is probably also the case for the preactivated mutants reported here. Three of them (F39L, I65T, and R68S) are located in the regulatory domain in or near the loop R68-Asp75 which interacts with the oligomerization domain and is involved in the activation that accompanies the transition from low-affinity and activity state to a high affinity and high activity state, as proposed for allosteric cooperativity [8].

Nearly all the BH_4 response associated mutants analyzed (the exceptions are H170D, V190A, and P407S) also showed a defect in cooperativity, with Hill coefficients < 2. This is a general observation for many PAH mutants, which in one way or another appear to hinder the transmission of the associated conformational changes. Many mutants also showed a defect in L-Phe binding. Some mutations showed abnormally high affinity for L-Phe such as the regulatory domain mutations F39L, I65T, R68S, and the catalytic domain mutation R408Q, with $S_{0.5}$ values 26–60% lower than the normal PAH protein. Other mutations exhibited a reduced L-Phe affinity such as R261Q and V388M with $S_{0.5}$ values four and eight times higher than the wild type protein (Table 1).

Regarding affinity for BH_4, of the 18 mutant PAH proteins analyzed, 5 show a moderate increase in K_M that means they have a decreased affinity for BH_4 (Table 2). Two of these five mutations are located in the regulatory domain (F39L, I65T) and three are located in the catalytic domain (P244L, L308F, and A309V). Only one mutation (P244L) is close to one of the cofactor binding regions [2].

The binding of BH_4 to wild-type and some mutant PAH proteins was also studied at equilibrium using isothermal titration calorimetry (ITC) [4]. This technique which was performed in an anoxic environment at pH 7.0 at 25 °C, optimal conditions which totally prevent oxidation of the cofactor, allows the determination of the binding affinity constant at equilibrium (K_d) and of the thermodynamic properties of the binding process. However, these measurements can only be performed with stable mutants which permit the purification of sufficient amounts of the active tetrameric form of the enzyme. Defective BH_4 binding was observed by this technique for the three regulatory mutations F39L, I65T and R68S. In all cases, there is an entropic penalization to the enthalpically driven BH_4 binding process [4]. When we compare the results of the steady-state analysis and ITC studies a binding defect for F39L and I65T was confirmed (Table 2). The differences with wild type values are smaller for K_M than for K_d and for R68S no significant difference in K_M was measured versus a threefold increase in K_d by ITC. These variations can be attributed to differences in the experimental conditions, as in ITC the formation of the complex ligand-protein (BH_4-PAH) is directly studied at equilibrium in the absence of O_2 and L-Phe, opposite to steady-state kinetic measurements. For the remaining three mutations with an increase in apparent K_M, no ITC studies could be performed because the protein aggregated and not enough protein was obtained (L308F and A309V) or no

Table 2
PAH mutant proteins with decreased binding affinity for BH_4 (K_M or K_d defects)

PAH proteins	K_M[a] (BH_4) μM	K_d[b] (BH_4) μM
Wt	26 ± 3	2.7 ± 0.1
F39L	44 ± 2	8.4 ± 0.8
I65T	39 ± 3	3.9 ± 0.4
R68S	30 ± 3	9.0 ± 1.0
P244L	39 ± 4	ND
L308F	44 ± 8	ND
A309V	38 ± 3	ND

Data from [3,4].
[a] Obtained by steady-state kinetic analysis.
[b] Obtained from equilibrium binding measurements by ITC.
ND, not determined.

calorimetric signal was detected (P244L), suggesting a strong reduction in binding affinity for this mutant [4].

Effect of BH_4 on the stability of BH_4-responsive PAH mutants in a cell-free system

To analyze the effect of the natural cofactor on the stability of PAH proteins, 14 of the above described missense mutants were synthesized in vitro in a coupled transcription–translation (TNT) cell-free system, in the presence or the absence of BH_4. This eukaryotic system has the advantage of allowing easy manipulation of BH_4 levels and rapid analysis of many different mutant proteins, in contrast to standard expression studies using cell culture and transfection. As a measure of the protein stability, the half-lives ($T_{1/2}$) were determined using pulse-chase analysis of radioactively labelled synthesized proteins. At standard conditions (synthesis without added BH_4) all the mutant proteins analyzed except R68S and A300S have decreased $T_{1/2}$ compared to Wt protein, that is they are more unstable and are degraded more rapidly, which correlates with what was previously observed for many other PAH mutants [10]. Most relevant is the increase in $T_{1/2}$ observed for six mutations when the synthesis is carried out in the presence of high amounts of BH_4 (500 μM). In four of them (F39L, A373L, V388M, and E390G), there is a moderate increase in half-life and for A309V and Y414C the effect is evident, with $T_{1/2}$ reaching wild type values (Table 3 and Fig. 2).

Table 3
Half-lives for protein degradation ($T_{1/2}$) for 14 different mutant proteins

Protein	$T_{1/2}$ (h)	
	$-BH_4$	$+BH_4$
Wt	8.7	7.9
No effect of BH_4		
I65T	3.3	3.3
R68S	8.2	8.6
E178G	5.5	5.8
V190A	5.7	5.2
P244L	2.9	2.6
R261Q	4.3	3.2
A300S	8.9	8.8
A313T	4.9	4.9
BH_4 chaperone effect		
F39L	5.6	6.5
A373T	6.1	7.1
V388M	5.5	7.0
E390G	5.6	6.4
A309V	3.6	8.6
Y414C	5.8	8.8

The mutations are separated according to BH_4 effect on stability.
Data from [3,4].

In the TNT system, PAH enzyme activity was also slightly enhanced when the synthesis occurred in the presence of BH_4 [4], results which have been reproduced using different amounts of cofactor in the synthesis pulse [11]. In addition, BH_4 seems to exert a protective role preventing the rapid inactivation in vitro, with $T_{1/2}$ values for inactivation much shorter than those for protein degradation, which is probably related to the generation of reactive oxygen species as the protective effect is partially mimicked by the use of superoxide dysmutase and catalase in the synthesis reaction [4]. This general effect, which also includes the wild-type protein could be specially relevant for mutations with low residual activity.

Conclusions

As deduced from the data reviewed here, the response to BH_4 is multifactorial and several mechanisms may be acting in concert for each mutant protein. An example is A309V as we observe a slight elevation in K_M and also a stabilization in vitro with BH_4 preventing protein degradation. Both effects may be related. Structural localization of the A309 residue (Fig. 3A) shows that the side chain of alanine 309 is within 4 Å of distance from Ala 316, Leu 321, Ala 403, and Ile 406, conforming a hydrophobic cluster that maintains the local spatial structure. The change of alanine to valine at position 309 of PAH introduces a large side chain in the same environment, probably leading to structural changes and/or instability, which can be propagated to the active centre. This region is close to the cofactor BH_4 and the change could hinder

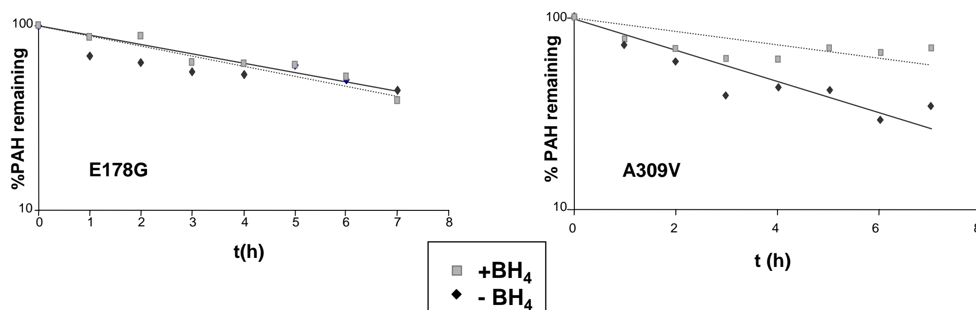

Fig. 2. Semilogarithmic representations of the results obtained after pulse-chase experiments in the TNT system of a mutation with no effect of BH_4 (R178G) and a mutation with evident effect of BH_4 (A309V).

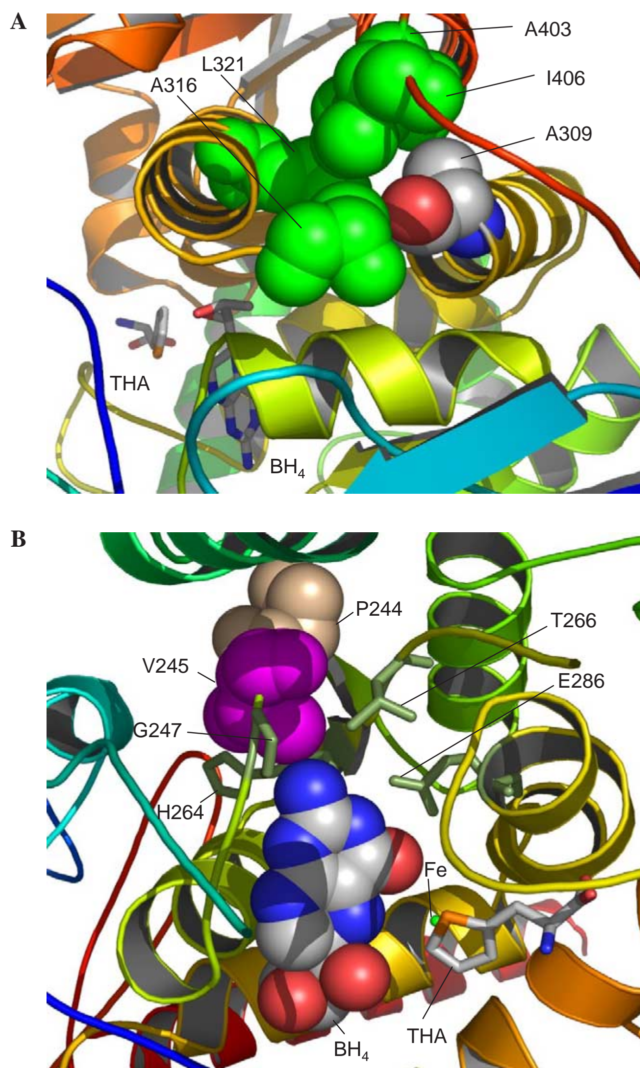

Fig. 3. (A) Closer view of A309 and nearby residues. Protein secondary elements are depicted as in Fig. 1. Atoms of A309 are represented as spheres and coloured by element. Atoms of surrounding residues A316, L321, A403, and I406 are coloured in green. Positions of THA and BH_4 are also indicated. (B) The neighbouring residues of P244 residue and the contact between BH_4 and V245. Protein secondary elements are depicted as in Fig. 1. BH_4 cofactor atoms are represented as spheres and coloured by element. P244 and V245 atoms are coloured in pale brown and purple respectively. Positions of residues at less than 4.0 Å of the interface between BH_4 and V245, as well as the THA molecule and Fe atom are also shown.

correct binding of BH_4, resulting in an increase of the K_M and a decrease of the stability of the PAH protein.

A second example is P244L (Fig. 3B). Proline residues confer strong constrains of conformational freedom to the bond angles of the protein backbone, necessary to maintain proper residue contacts. In the vicinity of Pro 244, the carbon atom in position γ-2 of Val 245 is located at only 3.2 Å of N_2 of BH_4. Mutation of Pro 244 to Leu relaxes locally the active centre conformation, implying instability. BH_4 bound at the cofactor site would establish appropriate contacts with Val 245 as well as with other residues in the neighbourhood —less than 4.0 Å— of the interface between Val 245 and BH_4 (Gly 247, His 264, Thr 266, and Glu 286) restoring correct protein backbone stability.

Taking into account complementary results from different authors, the emerging view considers stabilization of PAH protein the major mechanism contributing to BH_4 responsiveness. In transgenic mice with complete or partial deficiency in cofactor biosynthesis the rate of hepatic PAH enzyme activity and amount of protein increased significantly with BH_4 content without affecting gene expression or PAH mRNA levels [11]. This and preliminary results in hepatoma cells [12] rules out the hypothesis of an effect of BH_4 on PAH gene transcription or PAH mRNA stability. On the other hand, decreased binding affinities for BH_4 are modest in nature and affect only a few mutations. BH_4 appears to have a general chaperon-like protective effect for PAH, stabilizing the functional forms of PAH and preventing proteolytic degradation. This may explain the high number of mutations involved in BH_4 responsiveness, as most missense mutations have already been described to affect folding and stability of the functional tetrameric form of the enzyme. Future investigations need to be carried out in mice or cell lines in more physiologically relevant conditions to definitely delimitate the molecular basis of BH_4 responsiveness.

In summary, although these results provide insights about the molecular mechanism underlying the response it may not be possible to select or predict the genotypes associated with a possible positive response to cofactor treatment, because the majority of the responsive patients are compound heterozygous for two different missense mutations each one with a different molecular behaviour. In addition, interindividual differences in pharmacokinetics properties could explain the different degree of responsiveness associated to identical or similar genotypes.

Acknowledgments

This work received support from the Instituto de Salud Carlos III, Ministerio de Sanidad y Consumo (REDEMETH, G03/05, RECGEN C03/07, and PI020117) and from Dirección General de Investigación, Ministerio de Educación y Ciencia, grant SAF2004-06843 (to P. Gomez-Puertas). The financial support of Fundación Ramón Areces to the Centro de Biología Molecular Severo Ochoa is gratefully acknowledged.

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