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Phenylketonuria as a protein misfolding disease: The mutation pG46S in phenylalanine hydroxylase promotes self-association and fibril formation

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ABSTRACT

The missense mutation pG46S in the regulatory (R) domain of human phenylalanine hydroxylase (hPAH), associated with a severe form of phenylketonuria, generates a misfolded protein which is rapidly degraded on expression in HEK293 cells. When overexpressed as a MBP-G46S fusion protein, soluble and fully active tetrameric/dimeric forms are assembled and recovered in a metastable conformational state. When MBP is cleaved off, G46S undergoes a conformational change and self-associates with a lag phase and an autocatalytic growth phase (tetramers ≫ dimers), as determined by light scattering. The self-association is controlled by pH, ionic strength, temperature, protein concentration and the phosphorylation state of Ser16; the net charge of the protein being a main modulator of the process. A superstoichiometric amount of WT dimers revealed a 2-fold enhancement of the rate of G46S dimer self-association. Electron microscopy demonstrates the formation of higher-order oligomers and linear polymers of variable length, partly as a branching network, and partly as individual long and twisted fibrils (diameter ~145–300 Å). The heat-shock proteins Hsp70/Hsp40, Hsp90 and a proposed pharmacological PAH chaperone (3-amino-2-benzyl-7-nitro-4-(2-quinolyl)-1,2-dihydroisoquinolin-1-one) partly inhibit the self-association process. Our data indicate that the G46S mutation results in a N-terminal extension of α-helix 1 which perturbs the wild-type α–β sandwich motif in the R-domain and promotes new intermolecular contacts, self-association and non-amyloid fibril formation. The metastable conformational state of G46S as a MBP fusion protein, and its self-association propensity when released from MBP, may represent a model system for the study of other hPAH missense mutations characterized by misfolded proteins.

1. Introduction

The human inborn error of metabolism phenylketonuria (PKU; OMIM# 261600) is caused by a dysfunction of the liver enzyme phenylalanine hydroxylase (hPAH; EC 1.14.16.1), inherited in an autosomal recessive fashion. At present > 500 disease causing mutations have been identified in the human *PAH* gene (see PAH Mutation Analysis Consortium database: http://www.pahdb.mcgill.ca/) [1]. Most of them are associated with PKU, and a smaller number has been identified among non-PKU hyperphenylalaninemia (HPA) patients. The current spectrum of alleged PKU/HPA mutations consists of ~60% missense substitutions, ~13% splice variants, ~13% deletions, ~6% nonsense (termination) mutations, and a few insertions, representing a broad spectrum of clinical, metabolic and enzymatic phenotypes [2]. Expression analyses of ~100 missense mutations in complementary *in vitro* systems (for review, see the PAH Mutation Analysis Consortium database) [1] have identified at least three main groups of enzymatic phenotypes which differ in their kinetic behavior and/or stability [3,4], i.e. (i) structurally stable mutations with altered kinetic properties, e.g. mutations at residues involved in substrate (L-phenylalanine, L-Phe) or the pterin cofactor ((6R)-L-*erythro*-5,6,7,8-tetrahydrobiopterin, BH_4) binding; (ii) mutations with normal or almost normal kinetic properties, but reduced stability both *in vitro* and *in vivo*; and (iii) mutations affecting both kinetic and stability properties of the enzyme.

Since a majority of the mutations results in enzyme forms with a propensity to self-associate when expressed as recombinant proteins in *E. coli* or in an *in vitro* transcription–translation system, PKU is often considered as a protein misfolding disease [5]. These mutations are located in different regions of the three-domain structure [4,6–9], and the mechanism of self-association may therefore have a variable structural basis. When overexpressed in *E. coli* several misfolding mutations result in both soluble and insoluble "aggregates", even when expressed and purified as MBP fusion proteins. Although MBP has been shown to have a chaperone like effect [10,11], the soluble mutant

Abbreviations: hPAH, human phenylalanine hydroxylase; rPAH, rat phenylalanine hydroxylase; PKU, phenylketonuria; L-Phe, L-phenylalanine; BH_4, (6R)-L-*erythro*-5,6,7,8-tetrahydrobiopterin; IPTG, isopropyl-thio-β-D-galactoside; MBP, maltose binding protein; SEC, size-exclusion chromatography; WT, wild-type; TM, tetramer; DM, dimer; ANS, 8-anilino-1-naphthalenesulfonic acid; DMSO, dimethyl sulfoxide; EM, electron microscopy

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proteins are often recovered mostly as higher-order oligomers [4,6,9]. However, in some mutations, e.g. the missense mutation pG46S in the regulatory (R) domain, the expressed protein is assembled and recovered as stable tetrameric/dimeric forms, in addition to some soluble higher-order oligomers [12]. The MBP-G46S-hPAH tetramer is characterized by a near normal catalytic efficiency, but when the tetramer is cleaved by the restriction protease factor Xa, the G46S tetramer is destabilized and self-associates [12]. When expressed in eukaryotic cells as a non-fusion protein the enzyme is unstable and is rapidly degraded [12], thus explaining the severe PKU phenotype. However, the molecular mechanism of the self-association and the structural properties of the higher-order oligomers remain to be determined.

In the present study, the pG46S mutation was carefully analyzed. This mutation belongs to the second group of enzymatic phenotypes previously defined, with reduced stability both *in vitro* and *in vivo* that results in a severe form of PKU [12]. This group represents a large proportion of the PKU mutations, with a loss-of-function pathogenesis due to reduce stability [13]. The mutation pG46S is also located in the regulatory domain of hPAH, and there is growing evidence that the R-domain of hPAH is largely involved in the instability of hPAH mutant proteins [9]. Therefore, the recombinant WT-hPAH and the G46S-hPAH mutant form were isolated as MBP-$(pep)_{Xa}$-PAH fusion proteins with the goal to: (i) characterize the self-association process of G46S-hPAH induced *in vitro* by factor Xa cleavage and how the solvent conditions affect its propensity to self-associate; (ii) examine the effect of substrates (L-Phe and BH_4), phosphorylation of Ser16 (Ser16 is, together with substrate and cofactor, one of the main regulators of hPAH function, involved in the activation of the enzyme [14] and causing conformational changes in the regulatory domain [15,16]) and molecular/pharmacological/chemical chaperones on the propensity to self-associate; (iii) investigate if the self-association results in the formation of any stable polymeric structures such as fibrils, and (iv) gain some insight into the molecular mechanism by which such structures are formed. To answer these questions, we used a combined approach of real-time light-scattering, thioflavin-T and ANS fluorescence and structural analyses by electron microscopy (EM). Additionally, complementary studies on a MBP fusion protein with the regulatory domain, comprising residues 2–120, were performed on the WT and G46S mutant form.

2. Material and methods

2.1. Materials

TB1 cells, the prokaryotic expression vector pMAL-c2/pMAL-hPAH and the amylose resin were obtained from New England Biolabs (USA). The restriction protease factor Xa was obtained from Protein Engineering Technology ApS (Aarhus, Denmark). The catalytic *C*-subunit of cAMP-dependent protein kinase (PKA) was from Biaffin GmBH & Co KG (Kassel, Germany). SDS molecular mass standard (low M_r range) was delivered by Bio-Rad. The pterin cofactor tetrahydrobiopterin (BH_4) was obtained from Schircks Laboratories (Jona, Switzerland) and glycerol, trimethylamine *N*-oxide (TMAO) and (−)-epigallocatechin gallate (EGCG) were from Sigma-Aldrich (St. Louis, MO, USA). 3-amino-2-benzyl-7-nitro-4-(2-quinolyl)-1,2-dihydroisoquinolin-1-one was purchased from Maybridge (Tintagel, Cornwall, UK). The recombinant human molecular chaperones Hsp40 (catalog number SPP-400), Hsp70 (catalog number ESP-555) and Hsp90 (catalog number SPP-776) were provided by Assay Designs (Ann Arbor, MI, USA).

2.2. Site-specific mutagenesis

The WT regulatory domain (WT-hPAH (2–120)) and the mutant G46S regulatory domain (G46S-hPAH (2–120)) were obtained by introducing a stop signal in codon 121 of hPAH, by site-directed mutagenesis (QuikChange^R II, Stratagene), using the pMAL-WT-hPAH vector [17] and pMAL-G46S-hPAH vector [12] as template, respectively. Primers 5′-GACACAGTGCCCTGGT**AA**CCAAGAACCATTCAAGAGC-3′ (forward) and 5′-GCTCTTGAATGGTTCTTGG**TT**ACCAGGGCACTGTGTC-3′ (reverse) used for mutagenesis were provided by Eurogentec, Seraing, Belgium (the mismatch nucleotides are shown in bold type). The authenticity of the mutagenesis was verified by DNA sequencing as described previously [12].

2.3. Expression and isolation of fusion proteins

Expression of both the WT and the mutated form G46S of hPAH and the R-domain of hPAH (residues 2–120) was performed in *E. coli* as fusion proteins (MBP-$(pep)_{Xa}$-hPAH) [17]. The bacteria were grown at 37 °C and the induction of hPAH by 1 mM isopropyl-thio-β-D-galactoside (IPTG) was performed for 24 h at 28 °C. The fusion proteins were purified by affinity chromatography (amylose resin) and centrifuged in a TL-100 Ultracentrifuge (Beckman, USA) for 20 min at 50,000 *g* before size-exclusion chromatography (SEC) as described [17]. SEC was performed at 4 °C using a HiLoad Superdex 200 HR column (1.6 cm × 60 cm) prepacked from Amersham Biosciences (GE Healthcare, Uppsala, Sweden). The mobile phase consisted of 20 mM Na-Hepes and 0.2 M NaCl, pH 7.0 and the flow rate was 0.38 ml min^{-1}. The tetrameric/dimeric hPAH fusion proteins and the R-domain of hPAH also as a fusion protein were collected and concentrated by Centriplus 30 filter (Amicon, MA, USA). The concentration of purified fusion proteins was measured by the absorption coefficient A_{280} (1 mg ml^{-1} cm^{-1}) = 1.63 [17] for the full-length enzymes and A_{280} (1 mg ml^{-1} cm^{-1}) = 1.34 for the truncated form hPAH (2–120). A colorimetric method [18] was also used in some cases to measure enzyme concentrations, with bovine serum albumin as the standard.

T427P-hPAH [19] and ΔC24-hPAH [20] were expressed as fusion proteins with MBP in *E. coli* and isolated essentially as the G46S mutant protein, except that before SEC the proteins were cleaved for 4 h at 4 °C by factor Xa protease, using a protease to substrate ratio of 1:200 (by mass) to remove the MBP partner.

2.4. Phosphorylation of hPAH fusion protein

The phosphorylation of the fusion protein (tetrameric form) was performed in a reaction mixture containing 0.1 mM ethylene glycol bis-(α-amino ether)-N,N,N′N′-tetraacetic acid, 0.03 mM EDTA, 1 mM DTT, 10 mM MgAc, 60 μM ATP in 15 mM Na-Hepes, pH 7.0, at 30 °C for 1 h [21]. The enzyme concentrations were 20 μM (hPAH) and 100 nM (catalytic subunit) of PKA. The degree of phosphorylation was measured by the electrophoretic mobility shift [6]. The non-phosphorylated fusion protein control was obtained by incubating the enzyme in the absence of added PKA catalytic subunit, under otherwise identical conditions.

2.5. Cleavage of MBP-hPAH fusion proteins and assay of self-association by light scattering

In order to study the time-course for the cleavage of the tetrameric and dimeric MBP-hPAH fusion proteins and the self-association of the released enzyme, the fusion proteins were incubated in a medium containing 20 mM Na-Hepes, 0.1 M NaCl, pH 7.0 at 25 °C, except when stated otherwise. Before assay the tetrameric or dimeric fusion proteins were subjected to high-speed ultracentrifugation at 210,000 *g* for 15 min at 4 °C. In the standard assay the concentration of the fusion protein was 0.74 mg ml^{-1} and the concentration of factor Xa was adjusted to give a final ratio (by weight) of 1:150 relative to the fusion protein. Parameters as pH, neutral salt concentration, temperature, protein concentration and the presence of substrates (L-Phe and BH_4) varied in different experiments as described in the Results section. Self-association of the factor Xa released enzyme was followed in real-time by light scattering, as measured by the increase in apparent absorbance