Expression Analysis of Phenylketonuria Mutations

EFFECT ON FOLDING AND STABILITY OF THE PHENYLALANINE HYDROXYLASE PROTEIN

Phenylketonuria is an autosomal recessive human genetic disease caused by mutations in the phenylalanine hydroxylase (PAH) gene. In the present work we have used different expression systems to reveal folding defects of the PAH protein caused by phenylketonuria mutations L348V, S349L, and V388M. The amount of mutant proteins and/or the residual activity can be rescued by chaperonin co-overexpression in Escherichia coli or growth at low temperature in COS cells. Thermal stability profiles and degradation time courses of PAH expressed in E. coli show that the mutant proteins are less stable than the wild-type enzyme, also confirmed by pulse-chase experiments using a coupled in vitro transcription-translation system. Size exclusion chromatography shows altered oligomerization, partially corrected with chaperonins coexpression, except for the S349L mutant protein, which is recovered as inactive aggregates. PAH subunit interaction is affected in the S349L protein, as demonstrated in a mammalian two-hybrid assay. In conclusion, serine 349, located in the three-dimensional structure lining the active site and involved in the structural maintenance of the iron binding site, is essential for the structural stability and assembly and also for the catalytic properties of the PAH enzyme, whereas the L348V and V388M mutations affect the folding properties and stability of the protein. The experimental modulation of mutant residual activity provides a potential explanation for the existing inconsistencies in the genotype-phenotype correlations.

Mammalian phenylalanine hydroxylase (PAH, 1 phenylalanine 4-monoxygenase, E.C. 1.14.16.1) is a non-heme iron and tetrahydrobiopterin-dependent enzyme that catalyzes the hydroxylation of phenylalanine to tyrosine. Defects in the human phenylalanine hydroxylase gene (GenBankTM cDNA reference sequence U49897, MIM 261600) cause phenylketonuria (PKU), a recessive disorder that if not treated from birth leads to mental retardation. PKU is in many ways regarded as a “model genetic disease,” as clinical and biochemical characteristics are well defined, an effective treatment has been successfully implemented, both the gene and the enzyme are well characterized, mutations have been identified, genotype-phenotype correlations have been established, and an animal model has been produced (1). Although PKU is a classical monogenic disorder, the associated features are complex, as pointed out by Scriver and Waters (2). From the genetic point of view, more than 380 mutations have been described associated to different populations (3). After defining the mutational spectrum of PKU in several populations, the aim of the researchers has been the study of the genotype-phenotype correlations (4–6). These studies have addressed the assessment of the severity of the mutations by in vitro expression analysis and examination of the phenotype in homozygous or functional hemizygous patients. Up to now 57 PKU mutations have been expressed in at least one in vitro system (7), and the data obtained have allowed in most cases the prediction of the biochemical phenotype based on the genotype. Nevertheless, some discrepancies have been detected in the genotype-phenotype correlations, especially in patients bearing mutations that result in decreased immunoreactive protein and consequently decreased activity when expressed in vitro (4–6). This is the case of many missense mutations, which are broadly referred to as causing PAH enzyme instability. There are now several reports documenting increased instability and susceptibility toward aggregation and degradation of PKU mutant proteins (8–10), and recently, the thermodynamic stability of native wild-type PAH has been examined (11, 12) analyzing the contribution of instability to PKU compared with other reasons for reduced activity. Currently, knowledge of the three-dimensional structure of PAH is also available (13–15), providing essential information to understand the effect of different mutations on the architecture of the protein. The enzyme is structured in three domains, a flexible N-terminal regulatory domain (residues 1–110), a a-helical rich catalytic domain (residues 111–410), and an oligomerization domain (residues 411–452), which includes a tetramerization motif at the extreme C-terminal end (residues 428–452).

The aim of this work has been to provide more information about the effect of three point mutations (L348V, S349L, and V388M) on PAH function, structure, and subunit interaction. Mutation L348V has been reported to have 25–33% residual activity in COS cells (3) and is an example of inconsistent genotype-phenotype correlations, as it is associated with different phenotypes in functionally hemizygous patients (5). V388M is one of the most frequent mutations in Spain, and we have shown that it retains 43% activity in COS cells (16), although normal levels of mutant immunoreactive proteins were detected. This apparent catalytic effect is discrepant with the fact that V388M affects residues located outside the active site in...
the three-dimensional structure of the PAH enzyme (13). On the contrary, S349L is located lining the active site, but expression both in COS cells and in Escherichia coli rendered a highly unstable protein (17). To clarify and extend these results, we have used several complementary expression systems (eukaryotic and prokaryotic) and experimental conditions (co-overexpression with chaperonins, different growth temperatures), providing evidence that the mutations affect directly the folding and stability of the protein. Additionally, we observe that the S349L mutation also affects the catalytic properties of the enzyme, which is attributable to the fact that serine 349 is involved in the iron binding site. Mutant PAH subunit interaction has been examined by the two-hybrid system in mammalian cells, and altered oligomerization is documented by size exclusion chromatography of mutant PAH expressed in the E. coli system. The different experimental approaches employed allow the demonstration of a major folding defect of the mutations causing protein instability, which can be modulated experimentally, revealing a possible mechanism to account for the existing inconsistencies in the genotype-phenotype correlations.

EXPERIMENTAL PROCEDURES

Expression analysis was performed in COS cells using the pRcCMV expression vector (Invitrogen), as described previously (16), and in E. coli using pMALc2 (Biolabs) where PAH is cloned as a fusion protein with MBP under the control of an inducible promoter (17, 18). Mutations were introduced in the PAH cDNA sequence by site-directed mutagenesis using the Gene Editor kit from Promega. COS cells (4 × 10⁵ or 6 × 10⁵), grown at 37 or 27 °C were transfected with the Lipofectin reagent (Life Technologies, Inc). The plasmid pGroESL bearing the GroES and GroEL genes and the chaperonin resistance marker was from DuPont. The plasmids pMALc2-PAH wild-type or pMALc2-PAH mutant were cotransfected with pGroESL into E. coli JM109, and the colonies were selected using LB plates with ampicillin (0.1 mg/ml) and chloramphenicol (0.2 mM). Cells were grown at 37 °C, and expression of MBP-PAH fusion proteins and of GroES and GroEL was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside. At the same time, 0.2 mM ferrous ammonium sulfate was added. Bacteria were harvested 16–21 h after induction and disrupted by sonication in Na-Hepes 20 mM, NaCl 0.2M, pH 7.0, with 1 mg/ml lysozyme and 0.2 mM Pefabloc. After centrifugation, the supernatant (crude protein extract) was used to measure PAH residual activity by monitoring conversion of 14C-Phe to 14C-Tyr (17). Briefly, the standard reaction mixture performed in a 50-µl final volume contained 150–300 µg of total protein, catalase (10 µl at a concentration 100 units/µl), 14C-Phe (0.5 µCi, 0.35 µCi/mmol), and 1 µl of 1 mM 6-methyltetrahydrofolate (synthetic cofactor, added last) in 20 mM Na-Hepes, 0.2 M NaCl, pH 7. At 1 h, the reaction was stopped by boiling for 5 min and centrifugation at 10,000 × g for 5 min. A 6-µl sample of the supernatant was spotted onto a TLC plate, developed two times in chloroform:methanol:ammonia (55:35:10), dried, and visualized by autoradiography.

Wild-type and mutant fusion proteins were purified in an amylose column equilibrated with 20 mM Na-Hepes, 0.2 mM NaCl, pH 7.0, and eluted with buffer containing 10 mM maltose (18). PAH activity was also measured in the purified fraction, using 30–60 µg of protein. Size exclusion chromatography of the purified fusion proteins was performed at 4 °C following the conditions described (18) and using a HiLoad Superdex 200HR column (1.6 cm × 60 cm) prepacked from Amersham Pharmacia Biotech. The fast protein liquid chromatography system, UV monitor, and recorder were all from Amersham Pharmacia Biotech. Assignment of the different enzyme forms to the peaks obtained in the chromatograms was done by comparison with previously published elution positions of tetramers and dimers of the fusion protein (18) and by molecular mass value estimation using calibration curves obtained by running the following standard proteins, obtained from Amersham Pharmacia Biotech: A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), albumin (67 kDa), aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa). Blue dextran and acetonitrile were used to determine the void volume (V₀ = 44.06 ml) and the exclusion volume (Vₑ = 114.9 ml), respectively.

The cleavage of PAH protein from the MBP fusion partner was performed with Xa factor (ratio protein:Xa factor 1:100) at 4 °C for 3 h. The reaction was further incubated at 4 or 37 °C, and aliquots were removed at different times up to 24 h. After SDS-PAGE and Coomassie Blue staining, MBP and PAH proteins were quantified by laser densitometry.

The ToT-T7 transcription-translation system from Promega was used for pulse-chase experiments. The wild-type and mutant PAH cDNAs cloned in the pRcCMV vector were amplified using a sense primer that introduces the T7 promoter and the consensus Kozak sequences close to the ATG initiation codon (5′-TAATACGACTCACTATAGGGAGCCACCATGTCCACTGCGGTCCTGGAA-3′). Five microliters of the polymerase chain reaction product from the wild type and mutant cDNAs were mixed with the reticulocyte lysate and [35S]methionyl-octylamine (14.3 mM/c). After a 35-min incubation at 30 °C the reaction was stopped with excess cold methionine, RNase (1 mg/ml), and DNase (1 mg/ml). The whole reaction was incubated at 37 °C, and aliquots were removed at different times between 1 and 8 h. All samples were separated by denaturing polyacrylamide gel electrophoresis, and the labeled PAH protein was quantitated by laser densitometry after fluorography.

Thermal stability profiles were performed with purified fusion proteins MBP-PAH expressed in E. coli. Aliquots (20 µl, containing 30–60 µg of purified protein) were incubated at different temperatures for 10 min and chilled on ice. PAH enzyme activity was subsequently measured as described above.

For two-hybrid analysis we have used the Mammalian Matchmaker two-hybrid assay kit (CLONTECH). The plasmid pGL-G5 (kindly provided by P. Staheli) was used as reporter vector containing the luciferase gene under the control of the Gal4 promoter. The full-length human PAH cDNA was excised from pHPA347 (19) with Smal and EcoRI and subcloned into pBluescript. To introduce the normal PAH as fusion protein to the Gal4 (binding domain, BD) and VP16 (activating domain, AD) proteins, the PAH cDNA was excised from pBluescript with BamHI and XbaI and ligated with the AD and BD vectors previously digested with the same enzymes. Both plasmids with the normal cDNA were sequenced using a fimm sequencing kit (Promega) to confirm the in-frame cloning. The GeneEditor in vitro site-directed mutagenesis corresponding to the missense mutations L348V and V388M and all possible combinations of them were introduced into the PAH sequence. COS cells were plated in 6-well plates at a density of 4 × 10⁵ cells/well. In each transfection 1 µg of each plasmid (reporter vector, AD-PAH and BD-PAH vectors) was introduced using Lipofectin reagent (Life Technologies, Inc.). The cells were harvested after 72 h, and luciferase activity was measured. In the transfection experiments using pRcCMVPAH or in the two-hybrid system, PAH proteins were detected by Western blot using PBS anti-PAH monoclonal antibody (20).

For the homology modeling, three-dimensional models of L348V, S349L, and V388M were built on the basis of the human phenylalanine hydroxylase coordinates determined by x-ray crystallography (13, 14, 15) (Protein Data Bank accession codes 1PAH and 2PAH). In the corresponding mutant sites, the corresponding amino acid residues were exchanged for the correct residues. The models were optimized for stereochemistry and refined by energy minimization using X-PLOR (21). The energy-minimized structures were heated at 1000 K and were further refined with a slow cooling simulated annealing molecular dynamics protocol using X-PLOR.

RESULTS

As described as a preliminary report, we have overexpressed and affinity purified PAH protein as fusion protein with MBP (17). PAH with the S349L mutation produced an unstable protein undetectable in SDS-PAGE. The expression of this mutation in eukaryotic cells showed that the mutant enzyme was not detected by Western blot, and consequently no residual activity in bacteria nor in COS cells could be measured (17). In this work we have extended the expression analysis to two other mutations, L348V and V388M, which have been previously reported to retain residual activity in COS cells, 25–33% for L348V (3) and 43% for V388M (16). Based on the emerging view that many missense mutations in human disease cause defective folding resulting in protein instability (22), we have tested this hypothesis using different experimental approaches known to prevent misfolding of proteins expressed in eukaryotic and prokaryotic systems.

Prokaryotic Expression Studies—In prokaryotes, PAH was
E. coli with (+) or without (−) chaperonin co-expression and purified by affinity chromatography on amylose resin.

Expressed as a fusion protein with MBP. Expression of the mutant proteins compared with the wild type resulted in a variable but lower yield of fusion protein. When we performed the co-overexpression of the plasmid pGroESL, there was a considerable increase in the amount of fusion protein, both for wild type and mutant PAH, although the effect was more pronounced for the mutant proteins, especially for PAH harboring S349L, which as described before, was undetectable without chaperonin coexpression (Fig. 1). Thus, high levels of GroES and GroEL have a clear stabilizing effect on the mutant proteins, revealing a primary defect in folding and/or oligomer assembly. The increase in mutant protein correlated with an increase in residual catalytic activity with chaperonin coexpression, except for the S349L mutation, for which no enzyme activity is rescued (Table I).

The oligomeric state of the fusion proteins was analyzed by size exclusion chromatography. It has previously been described that the oligomeric structure of PAH is similar as fusion protein with MBP and as isolated enzyme (18, 23), and our results also show that the wild-type fusion protein is resolved into three main components, a fraction of high molecular mass aggregates, eluting at the column void volume, a major fraction corresponding to tetramers, and a minor component of dimers. With chaperonin coexpression, there is no substantial change in the oligomeric profile of the wild-type protein (Fig. 2). Regarding the mutant proteins, L348V and V388M fusion proteins show a much lower proportion of tetramers and increased amounts of aggregated forms. When V388M is coexpressed with GroES and GroEL, a major peak corresponding to the tetrameric form is observed with a concomitant decrease in aggregated forms. For L348V with chaperonins the proportion of tetramers also increases, although there is still a considerable amount of aggregates (Fig. 2). In contrast, the S349L protein rescued by chaperonin coexpression is exclusively recovered as aggregates.

In addition to impaired folding, the mutations could also be affecting the stability of the assembled enzyme. Therefore, we analyzed the thermal inactivation profiles for the L348V and V388M fusion proteins purified in the pMalc2 expression system. The curves of the mutant enzymes are clearly shifted with a concomitant decrease in aggregates. For L348V with chaperonins the proportion of tetramers also increases, although there is still a considerable amount of aggregates (Fig. 2). In contrast, the S349L protein rescued by chaperonin coexpression is exclusively recovered as aggregates.

Another approach used to analyze the effect of the PAH mutations on the stability of the protein was performed after digestion with factor Xa of the purified normal and mutant fusion proteins. The cleaved proteins were subsequently incubated at 4 or 37 °C up to 24 h, and the Coomassie Blue-stained bands were quantified by laser densitometry. After cleavage, MBP and wild-type PAH are essentially stable up to 24 h, the ratio PAH/MBP is close to 1 up to 24 h. In contrast, immediately after cleavage, the amount of detectable mutant PAH forms, expressed as PAH/MBP ratio, is reduced to 50% (for V388M), 40% (L348V), and 20% (in the case of S349L). This remaining mutant protein is stable up to 24 h. Similar data are obtained if the fusion proteins are coexpressed with or without chaperonins (data not shown).

**Eukaryotic Expression Studies**—To test the relevance of these results obtained in the E. coli expression system, the mutations were also expressed in COS cells at different temperatures, 27 and 37 °C. At low temperature, S349L mutant protein could be detected by Western blot analysis, reaching near normal levels, although no activity was rescued. Both L348V and V388M showed an increase in residual activity at 27 °C, from 38 to 77% and from 43 to 78%, respectively. Western blot analysis revealed similar levels of immunoreactive protein for the wild-type and mutant proteins expressed at both temperatures (Table I).

**Expression by in Vitro Transcription-Translation**—To confirm a folding defect causing protein instability, we studied the effect of the three mutations on the stability of the PAH protein combining pulse-chase methods with protein expression in a
Expression Analysis Revealing Folding Mutations in PKU

Table I
Expression analysis of PKU mutations in different systems

| PAH          | Expression in E. coli (+GroESL) | Expression in E. coli (+GroEL) | Expression in COS cells | Two hybrid system interaction |
|--------------|---------------------------------|--------------------------------|-------------------------|-----------------------------|
|              | Activity                        | Oligomeric state (proportion)  | Activity                | Protein                     | Activity                  | Protein                     | %                           | %                           | %                           | %                           |
| Wild-type    |                                  |                                | 37 °C                   | 27 °C                      | 37 °C                     | 27 °C                      | 37 °C                     | 27 °C                      | 37 °C                     | 27 °C                      |
| L348V        | 100                             | Aggregates (27%)               | 100                     | 100                        | 100                       | 100                        | 100                       | 100                        | 100                       | 100                       |
| S349L        | 0                               | No protein                     | 0                       | 0                          | 0                         | 0                          | 0                         | 0                          | 0                         | 0                         |
| V388M        | 40                              | Aggregates (59%)               | 43                      | 98                         | 78                        | 82                         | 73                       | 91                       |

FIG. 3. Thermal inactivation profiles of normal and mutant (L348V and V388M) PAH enzymes. Aliquots of the affinity purified fusion proteins were incubated at various temperatures for 10 min and chilled on ice. PAH enzyme activity was subsequently measured. The residual enzyme activities (percentage of the maximum value obtained) are plotted versus the incubation temperature. The mean values from two independent experiments each with fusion protein expressed with (panel B) or without (panel A) chaperons are shown.

Discussion

Currently, most of the PKU missense mutations that have been characterized are believed to destabilize the protein structure. More than 50 mutations have been expressed at least in one in vitro system, and most of them have reduced levels or no immunoreactive protein, correlating with reduced or absent residual activity and not affecting mRNA levels (7). The degradation of PAH proteins harboring missense mutations could be promoted by a defect in folding or in oligomer assembly, as has been suggested in recent studies documenting altered oligomerization and/or increased aggregation of mutant PAH proteins (8, 10, 24). In this work, we report the effect of the L348V, S349L, and V388M mutations on the stability of the PAH protein, using different experimental approaches to reveal possible folding defects.

Our previous work demonstrated that the S349L mutation expressed in E. coli as a fusion protein with MBP produces an unstable protein not detectable in SDS-PAGE. This is in accordance with the situation in COS cells where S349L is also unstable and was not detected by Western blot (17). However,
site as well as the mutated residues are depicted in ball-and-stick mode.

The active site of the native human phenylalanine hydrolase (PAH) and S349L mutant (PAH) enzyme. PAH dimers and therefore the hydroxylation capacity of the enzyme.

The ligands bonded to the iron in the catalytic site as well as the mutated residues are depicted in ball-and-stick mode.

FIG. 5. Comparative front view ribbon of the topology around the active site of the native human phenylalanine hydrolase (A) and S349L mutant (B). The ligands bonded to the iron in the catalytic site are also shown (red) as well as the mutated residues. The three-dimensional structure analysis shows no evident consequences for mutations L348V and V388M, as no drastic change is predicted. However, all the results obtained in both the prokaryotic and the eukaryotic expression systems, as could be predicted from the result obtained after size exclusion chromatography of the fusion protein expressed in E. coli, which consists exclusively of inactive aggregates. The results obtained with L348V and V388M show that they are structural mutations affecting folding and assembly to active tetramers. When expressed in E. coli as fusion proteins, the amount of protein and the residual activity increases with GroES and GroEL coexpression. GroES and GroEL are the prokaryotic homologues of eukaryotic Hsp90/Hsp10, which harbor an ATPase domain and are thought to assist polypeptide folding by partially unfolding nonfunctional conformations that thus escape degradation reinitiating the folding process (30). Increasing the pool of chaperonins will therefore increase the fraction of protein that acquires a functional conformation.

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In addition to impaired folding, the thermal inactivation experiments reveal that the half-denaturation temperatures of the mutant proteins are clearly shifted to lower temperatures, showing a reduced stability of the assembled enzyme and irrespective of whether the protein has been expressed with or without chaperonins. The half-denaturation temperature obtained for the wild-type fusion protein (59 °C) correlates with the results obtained by IR spectroscopy for the isolated PAH enzyme (11).

Size exclusion chromatography demonstrate an oligomerization defect as most of the mutant protein is present as aggregates, and the proportion of tetramers, which are the most active form (specific activity ~2.5-fold compared with dimers) (23), are clearly diminished compared with the wild-type protein. The in the case of V388M, coexpression of chaperonins clearly shifts the oligomeric profile to a predominant fraction of tetramers. For L348V, the effect of chaperonins appears to be mainly an increase in the amount of protein. In COS cells we observe near normal amounts of mutant immunoreactive protein at 37 °C, which is more sensitive to reveal folding mutations, as has been argued in medium chain acyl-CoA dehydrogenase deficiency (31). In COS cells, mutant PAH proteins are apparently trapped in a relatively stable conformation, which could represent a folding intermediate or, in any case, not a correctly assembled fully active tetrameric form. Lowering the cultivation temperature has a clear positive effect in the yield of mutant protein acquiring a functional conformation, as reflected by the increase in residual activity to near normal levels. The three-dimensional structure analysis shows no evident consequences for mutations L348V and V388M, as no drastic change is predicted. However, all the results obtained in both the prokaryotic and the eukaryotic expression systems, as could be predicted from the result obtained after size exclusion chromatography of the fusion protein expressed in E. coli, which consists exclusively of inactive aggregates. The results obtained with L348V and V388M show that they are structural mutations affecting folding and assembly to active tetramers. When expressed in E. coli as fusion proteins, the amount of protein and the residual activity increases with GroES and GroEL coexpression. GroES and GroEL are the prokaryotic homologues of eukaryotic Hsp90/Hsp10, which harbor an ATPase domain and are thought to assist polypeptide folding by partially unfolding nonfunctional conformations that thus escape degradation reinitiating the folding process (30). Increasing the pool of chaperonins will therefore increase the fraction of protein that acquires a functional conformation.

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systems are clear and point to a genuine effect on folding for the L348V and V388M mutations.

We have also used the two-hybrid assay in mammalian cells as a powerful approach for the analysis of the effect of PKU mutations on assembly/oligomerization of the PAH dimers. In this experiment the reporter gene expression implies the association between two different PAH subunits. The dramatic reduction in luciferase activity when the COS cells were transfected with the mutant S349L protein indicates the effect of this mutation on the oligomerization of the enzyme. On the contrary, L348V and V388M show no effect on the protein-protein interactions in the two-hybrid assay, consistent with the presence of some dimers and tetramers in the prokaryotic expression system. The results correlate with the observations in the eukaryotic system where there is some residual activity present.

In conclusion the present study demonstrates that the L348V, S349L, and V388M mutations affect the folding properties of the PAH protein, although S349L also shows a catalytic effect, because this residue is directly involved in the iron binding site. In addition, we demonstrate that L348V and V388M also affect the stability of the enzyme once assembled. Most significantly, we show that experimentally the amount of protein can be modulated depending on chaperonin and temperature conditions, providing clues to explain the genotype-phenotype inconsistencies described for some mutations. These results obtained with a cytoplasmic protein extend similar in vitro studies of mitochondrial enzymes (25–27), revealing a common mechanism of many genetic disorders.

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