ALAD Porphyria Is a Conformational Disease

Eileen K. Jaffe and Linda Stith

ALAD porphyria is a rare porphyric disorder, with five documented compound heterozygous patients, and it is caused by a profound lack of porphobilinogen synthase (PBGS) activity. PBGS, also called "δ-aminolevulinate dehydratase," is encoded by the *ALAD* gene and catalyzes the second step in the biosynthesis of heme. ALAD porphyria is a recessive disorder; there are two common variant *ALAD* alleles, which encode K59 and N59, and eight known porphyria-associated *ALAD* mutations, which encode F12L, E89K, C132R, G133R, V153M, R240W, A274T, and V275M. Human PBGS exists as an equilibrium of functionally distinct quaternary structure assemblies, known as "morpheeins," in which one functional homo-oligomer can dissociate, change conformation, and reassociate into a different oligomer. In the case of human PBGS, the two assemblies are a high-activity octamer and a low-activity hexamer. The current study quantifies the morpheein forms of human PBGS for the common and porphyria-associated variants. Heterologous expression in *Escherichia coli,* followed by separation of the octameric and hexameric assemblies on an ion-exchange column, showed that the percentage of hexamer for F12L (100%), R240W (80%), G133R (48%), C132R (36%), E89K (31%), and A274T (14%) was appreciably larger than for the wild-type proteins K59 and N59 (0% and 3%, respectively). All eight porphyria-associated variants, including V153M and V275M, showed an increased propensity to form the hexamer, according to a kinetic analysis. Thus, all porphyria-associated human PBGS variants are found to shift the morpheein equilibrium for PBGS toward the less active hexamer. We propose that the disequilibrium of morpheein assemblies broadens the definition of conformational diseases beyond the prion disorders and that ALAD porphyria is the first example of a morpheein-based conformational disease.

The porphyrias are diseases resulting from the abnormal functioning of the enzymes of heme biosynthesis.[1] ALAD porphyria (MIM 125270), also known as "Doss porphyria," is a very rare porphyric disorder linked to a profound lack of porphobilinogen synthase (PBGS [EC 4.2.1.24]) activity.[2] PBGS, also known as "δ-aminolevulinate dehydratase" (ALAD), is encoded by the *ALAD* gene.[3] There are two common alleles for *ALAD,* which encode either lysine or asparagine at amino acid position 59,[4] and the relative frequencies of these alleles vary among populations.[5–11] ALAD porphyria is a recessive disorder; the described patients are compound heterozygotes, and there are a total of eight known porphyria-associated *ALAD* alleles.[12–26] Of five documented compound heterozygotes, one was an infant, three were males who became symptomatic during adolescence, and one was a male who received the diagnosis during early adulthood. On the basis of an analysis of a small population, it has been estimated that the prevalence of individuals with 50% of normal PBGS activity, putatively caused by one aberrant *ALAD* allele, is ~2% in the normal asymptomatic population.[27] That study suggested that most instances of compound heterozygosity in *ALAD* result in spontaneous abortions.

Heme biosynthesis in humans is an essential metabolic function controlled predominantly at the first committed step, which is the mitochondrial synthesis of 5-aminolevulinic acid from succinyl–coenzyme A (CoA) and glycine.[28] PBGS catalyzes the asymmetric condensation of two molecules of 5-aminolevulinic acid, to form porphobilinogen.[29] Mammals contain as much as 100-fold more enzyme activity than is necessary for required heme formation,[30] and alternative possible moonlighting functions for PBGS include roles in proteosome-directed protein degradation and in chaperone-assisted protein folding.[31,32] Before the characterization of the porphyria-associated hexameric human PBGS variant F12L, the wild-type protein had been well characterized as a homo-octamer.[33] Crystal structures had been deposited for homo-octameric forms of PBGS from human, yeast, *Escherichia coli,* and *Pseudomonas aeruginosa*; these, along with high sequence conservation for PBGS throughout all kingdoms, led to the hypothesis that all PBGSs are homo-octameric in structure and assembly.[33] However, some size-exclusion chromatography data from the 1960s and 1970s had suggested other possible quaternary assemblies, particularly for the proteins from photosynthetic organisms,[34,35] in which PBGS also functions in chlorophyll biosynthesis. Characterization of the naturally occurring low-activity human PBGS variant F12L revealed its hexameric assembly[36,37] and led to revolutionary new insights into the dynamic flexibility of homo-oligomeric proteins.[38,39] The F12L mutation, which has been documented in two unrelated white individuals,[13,37] was shown, by x-ray crystallographic analysis, to give rise to a dramatic rearrangement in the secondary structure of the N-terminal arm domain of the protein, which resulted in assembly into a hexamer.[36] As illustrated in figure 1, the wild-type protein structure is predominantly a homo-

From the Fox Chase Cancer Center, Philadelphia

Received October 17, 2006; accepted for publication December 4, 2006; electronically published December 21, 2006.

Address for correspondence and reprints: Dr. Eileen K. Jaffe, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111. E-mail: Eileen.Jaffe@fccc.edu

Am. J. Hum. Genet. 2007;80:329–337. 0002-9297/2007/8002-0013$15.00

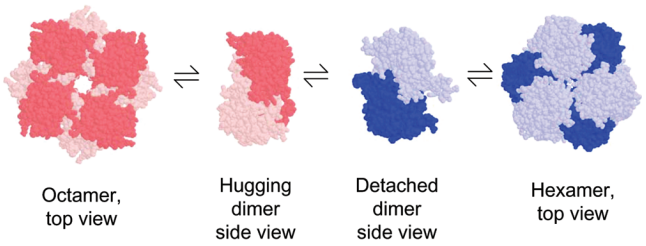

Figure 1. Equilibrium of human PBGS morpheeins. The human PBGS quaternary structure isoforms are the high-activity octamer (wild-type protein [PDB code 1E51] [*shades of red*]), the transient hugging dimer, the transient detached dimer, and the low-activity hexamer (F12L variant [PDB code 1PV8] [*shades of blue*]).[36,38,39] Detailed ribbon diagrams of the assembly of the octamer and hexamer have been published elsewhere.[36,39]

octamer, composed of four hugging dimers, and the F12L protein structure is a homo-hexamer, composed of three detached dimers. The most significant difference between the octameric and hexameric assemblies is the orientation of an $\alpha\beta$-barrel domain (residues 25–330) with respect to an N-terminal arm domain (residues 1–24) of each subunit. Although the F12L mutation is not at the enzyme active site, the quaternary structure change can be seen to destabilize the active-site lid and to account for the low activity of the hexamer.[40] Detailed biochemical and biophysical analysis of the hexamer and octamer of human PBGS showed that these isoforms are in dynamic equilibrium with each other and that catalytic turnover facilitates this equilibration.[39,41] Designed, synthetic single–amino acid changes other than F12L were also shown to alter the equilibrium of human PBGS quaternary structure isoforms[38]; the R240A mutation favors assembly of the hexamer, and the W19A mutation restricts assembly beyond the dimer. These studies led to the introduction of the term "morpheeins."[39,41]

Morpheeins is a recently introduced term used to describe an ensemble of functionally distinct, native quaternary structure assemblies of a given homo-oligomeric protein, in which one functional oligomer can dissociate, change conformation, and then reassociate into a structurally and functionally different oligomer.[38,39,41] Morpheeins were initially described as one possible structural basis for allosteric regulation of protein function.[41] Morpheeins are like prions, in that they are proteins that can exist in more than one quaternary structure assembly. However, unlike the established prion phenomenon, the equilibration of alternate morpheeins is a readily reversible, physiologically relevant mechanism for control of protein function.[41] For human PBGS, the morpheein forms are the high-activity octamer and the low-activity hexamer described above.[36,38,39,41]

The existence of morpheeins of human PBGS raised the question of whether the naturally occurring porphyric mutations in human PBGS also alter the equilibrium of quaternary structure isoforms. The classic conformational diseases, such as Alzheimer disease, occur when a functional protein with a globular structure becomes altered into a structure that oligomerizes into fibers, with disastrous physiologic consequences.[42] Similar phenomena are

Table 1. Characteristics of Human PBGS Variants

Human PBGS Variant	Porphyric in Association with	Activity in CHO Cells[a] (%)	Specific Activity,[b] from Expression in *E. coli*	Octamer:Hexamer Ratio[c]	Yield (mg/g of cells)	Best Fit[d] (mM)	
						Octameric K_m	Hexameric K_m
K59	…	100[e]	62[f]	100:0	2.2	.20	…
N59	…	69.9[e]	41[f]	97:3	3.3	.17	…
N59/C162A	…	ND	42[f] (100)	95:5	3.6	.25	…
F12L[g]	CPO[h]	.5[e]	<.1 (.2)	0:100[i]	6.3	…	17
E89K[g]	C132R	~10[e]	27 (64)	69:31	1.4	.16	6.6
C132R[g]	E89K	~4[e]	.11 (.3)	64:36	3.0	.13	20
G133R[g]	V275M	8.1	~5 (12)	52:48	5.6	.19	8.9
V275M[g]	G133R	1.2	~35 (83)	94:6	4.1	.11	1.4
R240W[g]	A274T	3.5[e]	~2 (4.8)	20:80	~4.0	.07	35
A274T[g]	R240W	19.3[e]	~25 (60)	86:14	2.8	.18	4.0
V153M[g]	818delTC[j]	41[e]	~16 (38)	95:5	7.3	.10	3.1

[a] Taken from previous studies.[12–22,24–26] ND = not determined.

[b] The maximum specific activity (μmol h^{-1} mg^{-1}) seen in the octamer peak eluting from the Sephacryl S-300 column (Q Sepharose column for G133R). This value does not necessarily reflect the specific activity at the optimal pH for each mutant, and it does not necessarily reflect the *V*max values that are associated with the reported K_m values. The values in parentheses are percentage activity.

[c] As estimated during the purification procedures from native gels of the Phenyl Sepharose–purified protein and/or from the elution profile from the Q-Sepharose column.

[d] Fit to either the Michaelis-Menten single hyperbolic equation or the double-hyperbolic model that describes a mixture of two species (fig. 3).

[e] Expression as a fusion protein with glutathione S-transferase.

[f] The specific activities varied from one purification to the next, showing variation up to 30%.

[g] Mutations were prepared in the N59/C162A background.

[h] CPO = coproporphyrinogen oxidase deficiency.

[i] The activity of a small amount of protein (1%–5%) that ran like octamer had characteristics of *E. coli* PBGS, pH 7, activity stimulated by Mg.

[j] Encodes a truncation mutant.

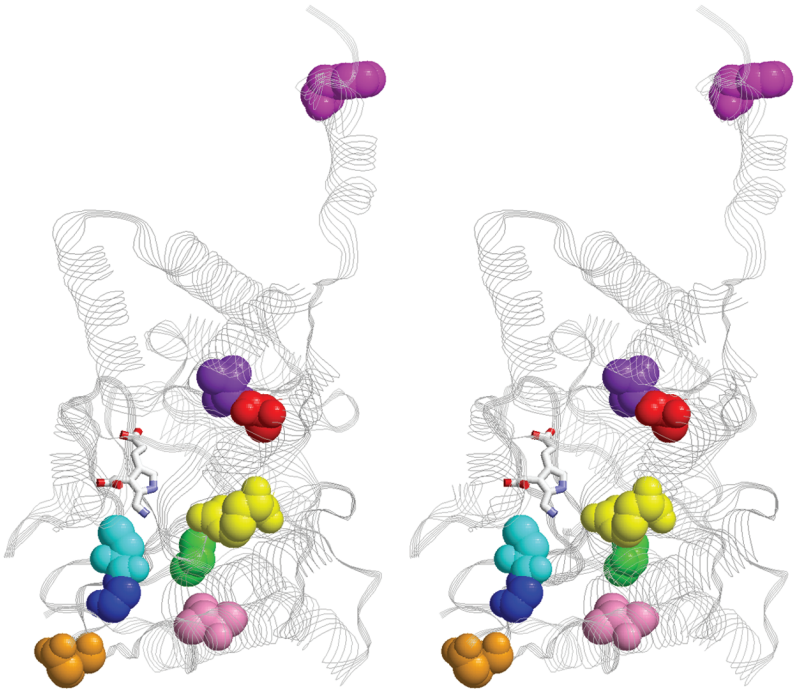

Figure 2. Spatial location of the porphyria-associated mutations of human PBGS. The crystal structure of human PBGS (PDB code 1E51) contains an asymmetric hugging dimer in the unit cell; the two subunits differ predominantly in their regions of disorder. The more highly ordered monomer is illustrated (in stereo) by use of a light-gray strand to illustrate the backbone. Product is seen bound at the enzyme active site (wire-frame representation with Corey, Pauling, Koltun [CPK] coloring), and known human variations are shown using space-filling representation. Lys59, which has some disorder, is green, Phe12 is magenta, Ala274 is red, Gly133 is blue, Arg240 is yellow, Cys132 is cyan, Glu89 is orange, Val153 is pink, and Val275 is purple.

associated with the infectious prion disorders, such as transmissible spongioform encephalopathies in both humans and animals.[43] It has recently been suggested that the disequilibrium of morpheein forms may constitute a broader definition of conformational diseases.[38] Here, we address whether the defective activity of the eight porphyria-associated human PBGS variants,[13–22,24–26,37] which are F12L, A274T, V153M, G133R, R240W, V275M, C132R and E89K, is related to the ability of this protein to interconvert between functionally distinct morpheeins. We asked whether these mutations have a conformational effect that alters the morpheein equilibrium for human PBGS. Figure 2 illustrates the location of these amino acids on one subunit of the wild-type octameric human PBGS crystal structure (Protein Data Bank [PDB] code 1E51). Table 1, which documents the characteristics of these variants, includes the combinations of mutations found in patients with diagnosed porphyria. Our results led us to propose that ALAD porphyria is the first identified morpheein-based conformational disease.

Material and Methods

Plasmid Preparation, Protein Production, and Purification

The plasmids used for the expression of N59, K59, N59/C162A, and F12L were described elsewhere.[36,44,45] The plasmids for the other human porphyria-associated *ALAD* alleles were prepared by mutagenesis with the use of the QuikChange site-directed mutagenesis kit (Stratagene), by starting with a pET3a-based plasmid containing the synthetic gene for human PBGS wild-type variant N59/C162A.[45] The mutagenic primers are listed in table 2. All resultant plasmids were sequenced in both directions throughout the gene. Expression and purification of human PBGS and its variants were as described elsewhere.[38] The purification from the soluble component of the lysed bacterial cells consisted of ammonium sulfate fractionation (20%–45%), hydrophobic affinity chromatography with the use of Phenyl Sepharose (GE Healthcare), anion-exchange chromatography with the use of Q Sepharose (GE Healthcare), and size-exclusion chromatography with the use of Sephacryl S-300 (GE Healthcare). The various human PBGS morpheeins separate from each other on the Q Sepharose column.[36,38,39] For those purified proteins that had a higher-than-expected specific activity, it was further confirmed that they contain the desired mutation by subjecting them to tryptic digestion (for A274T, V275M, and G133R) or AspN protease digestion (for V153M) and mass spectrometric analysis, as we have done elsewhere.[39]

Assessment of the Morpheein Equilibrium for the Resultant Proteins

Initial assessment of the quaternary structure distribution for each protein preparation was based on the elution profile from the Phenyl Sepharose and/or Q Sepharose columns and on the mobility during native-gel electrophoresis[36,38,39] (table 1 and fig. 3). In some cases, individual fractions were analyzed by native

Table 2. Mutagenic Primers for Production of Porphyria-Associated Variants of Human PBGS

	Primer Sequence (5′→3′)	
Mutation	1	2
E89K	CGC GTT CCA AAG GAT aAg CGG GGC AGC GCA GCC	GGC TGC GCT GCC CCG cTt ATC CTT TGG AAC GCG
C132R	CAC GGT CAC cGC GGT CTC CTG AGC GAG	CTC GCT CAG GAG ACC GCg GTG ACC GTG
G133R	CAC GGT CAC TGC cGT CTC CTG AGC GAG AAC	GTT CTC GCT CAG GAG ACg GCA GTG ACC GTG
V153M	CGC CAG CGG TTA GCT GAG aTG GCC TTG GCG	CGC CAA GGC CAt CTC AGC TAA CCG CTG GCG
R240W	CGT GCT GTG GAC tGG GAT GTA CGG GAA GGC	GCC TTC CCG TAC ATC CCa GTC CAC AGC ACG
A274T	GAC TTG CCG CTC aCC GTG TAT CAC GTG AGC	GCT CAC GTG ATA CAC GGt GAG CGG CAA GTC
V275M	GAC TTG CCG CTC GCC aTG TAT CAC GTG AGC	GCT CAC GTG ATA CAt GGC GAG CGG CAA GTC

PAGE. Prior experience with both natural and synthetic mutants established the expected elution time and electrophoretic mobility for the octamer, hexamer, and dimer for those mutations that are neutral (A274T, V153M, and V275M).[36,38] The octameric and hexameric morpheeins of the naturally occurring K59 variant (+1 charge difference) set the expected elution time and electrophoretic mobility for the +1 charged porphyria-associated mutations C132R and G133R. The morpheeins of our previously characterized synthetic mutant R240A (−1 charge difference) set the expected elution time and electrophoretic mobility for the porphyria-associated mutation R240W. A standard was not available for the E89K variant (+2 charge difference), but the two bands observed by native-gel electrophoresis followed the expected pattern (see fig. 3). Molecular size was confirmed during the final purification step, which was a Sephacryl S-300 column; however, such columns cannot easily separate proteins that differ in size by a factor <2. Final assessment was based on a kinetic characterization relative to wild-type human PBGS, which is predominantly an octamer; F12L, which is a stable hexamer; and the previously described synthetic mutant R240A, which is a metastable hexamer.[36,38]

Results

A summation of the results for each of the mutations is given in table 1. The details are described below.

Chromatographic Behavior

To determine the effect of the mutations on the octamer-hexamer equilibrium for human PBGS, we took advantage of our prior knowledge that these quaternary isoforms can be separated from each other by ion-exchange chromatography.[36,38,39] All the human PBGS variants behaved like the wild-type protein during the Phenyl Sepharose step of the purification, for which a schematic is shown in figure 3*A*. Phenyl Sepharose chromatography provides a partial separation of the octamer (enriched in the earlier part of the peak) and the hexamer (enriched in the later part of the peak). For those samples in which both octamer and hexamer were apparent from a native-gel analysis of the

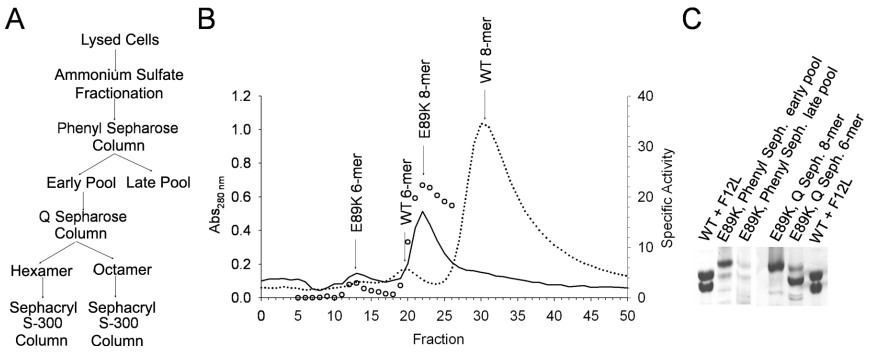

Figure 3. Purification and quantification of the morpheeins of human PBGS. *A,* General purification scheme for human PBGS. There was only one pool collected from the Phenyl Sepharose column for some proteins. *B,* Q Sepharose separation of the hexameric and octameric human PBGS, shown using absorbance (Abs) and specific activity values. For the wild-type (WT) variant N59/C163A (*dotted line*), the single pool from the Phenyl Sepharose column was run on the Q Sepharose column, which separated hexamer (WT 6-mer) and octamer (WT 8-mer). The mobility of the F12L variant is coincident with wild-type hexamer.[36,39] For the porphyria-associated variant E89K (*solid line*), the early pool from the Phenyl Sepharose column was applied to the Q Sepharose column, and the separation of hexamer (E89K 6-mer) and octamer (E89K 8-mer) is shown. Identification of the quaternary states is based on a fully consistent body of data that includes x-ray crystal structures, pH activity profiles, K_m and V_{max} values, dynamic light scattering, and relative mobility on native gels. *C,* A 12.5% acrylamide native PhastGel, showing separation of the morpheeins of human PBGS. The difference in mobility between the wild-type and E89K samples is the surface charge difference caused by the mutation. The E89K hexamer and octamer pools were concentrated by ultrafiltration before the PhastGel was run.

Phenyl Sepharose pooled fractions, it was possible to achieve a near-baseline separation of the hexameric and octameric assemblies by Q Sepharose chromatography, as described elsewhere[36,38] (fig. 3*B*). Chromatographic separation was confirmed by native-gel analysis (fig. 3*C*). The surface charge difference between N59/C162A and the K59 and E89K variants caused both the hexamer and the octamer of the variants to elute earlier in the gradient (at ~22 min and ~31 min, respectively). For all other variants, where the structure suggests that the mutation will not alter surface charge, the chromatographic mobility of the hexamer was fairly tight (214–224 min). For the octamer, however, the elution behavior spanned a larger time range (244–278 min) and suggested that these variants may differ somewhat in the structure of the octamer, perhaps in the relative mobility of charged surface loops. Table 1 includes the observed ratio of octamer to hexamer, based on information from the Phenyl Sepharose separation, the Q Sepharose separation, and native PAGE analysis. The wild-type variants K59, N59, and N59/C162A all purified predominantly as octamer, with 3%–5% appearing as hexamer for the N59-containing variants. For the mutants F12L and R240W, the morpheein equilibrium was shifted far toward the hexameric assembly, at 100% and 80% hexamer, respectively. The variants E89K, C132R, and G133R each showed significantly more hexamer (31%–48%) than did the wild type. These ranges may not be significantly different from each other, since we have established that the octamer-hexamer equilibrium is affected by substrate,[38,39] which is known to be present in the *E. coli* expression host. The variant A274T shows only a slightly higher propensity for hexamer formation (14%) than does the wild type, and the variants V275M and V153M each have little more hexamer than do the wild-type variants N59 and N59/C162A. Hence, from the chromatographic behavior, we can conclude that the mutations F12L, R240W, E89K, C132R, and G133R favor the hexameric assembly significantly more than do the wild-type variants.

Kinetic Behavior of the Naturally Occurring Human PBGS Variants

It is established that the kinetic constants K_m and V_{max} differ substantially between the predominantly octameric wild-type human PBGS and the hexameric F12L variant[36,39] (table 1). The difference in K_m values, in particular, can confirm the octameric or hexameric assembly of a given variant and can provide information about whether both assemblies exist concurrently in the assay mixture. The K_m value for the substrate δ-aminolevulinate (ALA), at pH 7, for the octameric wild-type proteins is in the physiologically relevant range at 0.2 mM. In contrast, for the hexameric F12L variant, the K_m value is 17 mM, which is well above the physiological range for substrate concentration. For the wild-type variants and F12L, when the substrate concentration is varied over a wide range of concentration (from 10 μM to 10 mM), the data fit superbly to the simple hyperbolic Michaelis-Menten equation[36]; data for K59 are included in figure 4. This hyperbolic fit is consistent with the presence of one assembly, either octamer or hexamer, in the assay mixture. However, we have shown that the wild-type proteins, when assayed at elevated pH values (e.g., pH 9) do not show a simple hyperbolic relationship between substrate concentration and reaction rate.[41] Instead, at pH 9, the hexameric form is favored, and the kinetic data fit well to a double-hyperbolic equation indicative of the coexistence of two forms of the protein, one with a K_m in the range of 0.2 mM (octamer) and one with a significantly higher K_m value (hexamer).[36,39] Unlike the wild-type variants, each of the porphyria-associated mutations showed a superior fit to the double-hyperbolic equation, even at the more physiologically relevant pH 7. Figure 4 includes kinetic data at pH 7 for the fraction of protein that purified on the Q Sepharose column as the octamer, for the porphyria-associated variants C132R, V153M, and V275M; they all showed a superior fit to the double-hyperbolic equation. The V153M and V275M variants are chosen for illustration because they did not show a significantly increased amount of PBGS hexamer during the purification (table 1). The derived K_m values at pH 7 for all the human PBGS variants are included in table 1. In all instances, the data for the porphyria-associated human PBGS variants fit best to the double-hyperbolic equation, display a tight K_m value in the range 0.07–0.19 mM ALA, and display a higher K_m

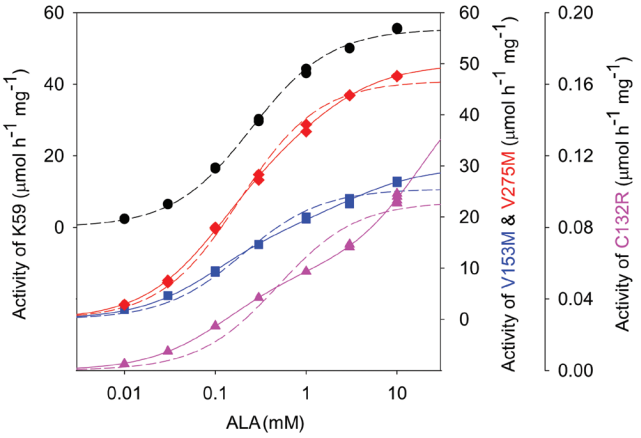

Figure 4. Determination of K_m values at pH 7 for the variants of human PBGS. Data are fitted to the hyperbolic Michaelis-Menten equation (*dashed lines*) and to the double-hyperbolic equation (*solid lines*); the latter suggests a mixture of two species with two different K_m values. Data are presented for the wild-type variant K59 (*black circles*); for C132R (*magenta triangles*), which shows a significant propensity to exist as a hexamer during the purification (see main text); and for V153M (*blue squares*) and V275M (*red diamonds*), which did not show significant hexamer in the purification. The superior fit to the double-hyperbolic equation is shown for C132R, V153M, and V275M.

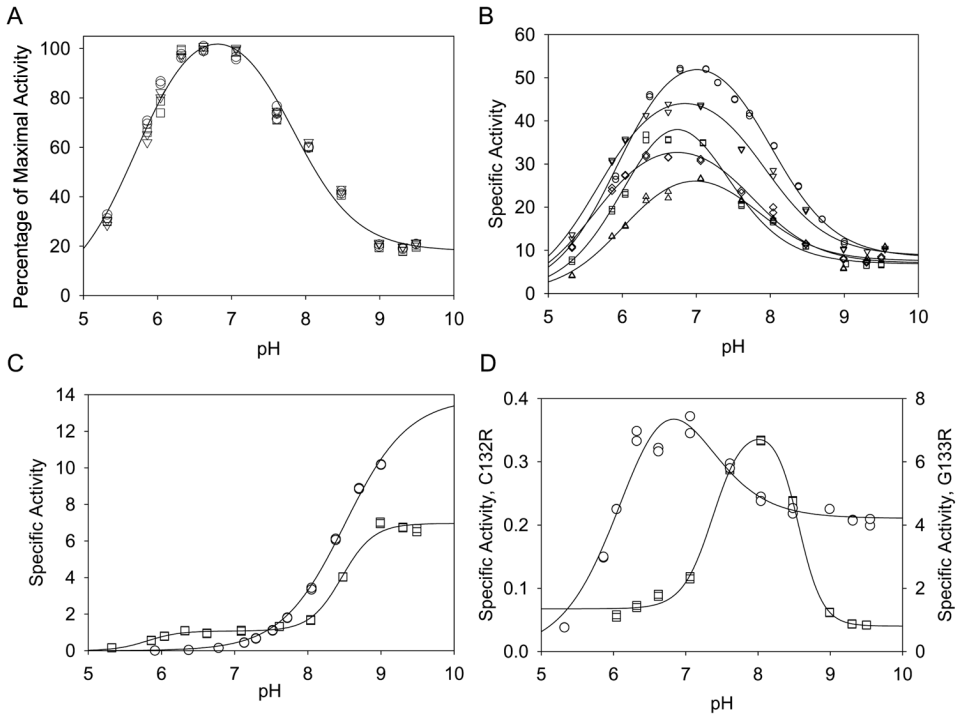

Figure 5. pH activity profiles for human PBGS variants. *A,* Wild-type variants N59 (*squares*), K59 (*downward-pointing triangles*), N59/C162A (*circles*), and K59/C162A (*diamonds*). Data are presented as percentage of maximal activity, to emphasize the similarity of these three curves. *B,* Variants E89K (*squares*), V153M (*upward-pointing triangles*), A274T (*diamonds*), and V275M (*downward-pointing triangles*), compared with the wild-type parent protein N59/C162A (*circles*). *C,* The predominantly hexameric variants F12L (*circles*) and R240W (*squares*). *D,* Variants C132R (*squares*) and G133R (*circles*). Cys132 is a ligand to the essential catalytic zinc ion.

value in the range 1.5–35 mM ALA. These kinetic data can be interpreted as evidence that all the porphyria-associated human PBGS variants have a morpheein equilibrium that is shifted toward the hexamer, relative to that of the wild-type variants.

One unexpected kinetic result was the relatively high specific activity for some of the purified proteins, relative to activities reported when these variants were first expressed in Chinese hamster ovary (CHO) cells (table 1).[12,17,18,37] Unlike in previous studies, we used a bacterial heterologous expression system to produce significant quantities (~20–50 mg) of each of the porphyria-associated human PBGS. Those variants characterized by unexpectedly high activities were A274T, E89K, and V275M, which had threefold, sixfold, and eightfold more activity, respectively, than was reported for CHO cell expression. Potentially significant differences between these two expression systems include the expression temperature, which is 15°C for the bacteria, and the cellular concentration of the substrate ALA, for which there is tight control in mammalian tissues.[46] Since we have shown that enzymatic activity can shift the human PBGS morpheein equilibrium toward the octamer,[38,39] the ready availability of ALA in the bacterial expression system may have favored formation of the more active octameric assembly.

pH Activity Relationships for the Naturally Occurring Human PBGS Variants

Previous studies established a dramatic difference between the pH activity relationship of the predominantly octameric wild-type human PBGS and the hexameric variant F12L.[36,39] It was established that the altered pH activity relationship was a result of the quaternary structure change, not the specific F12L mutation.[36,38] To expand on these studies, the specific activity of the remaining naturally occurring human PBGS variants was determined as a function of pH by use of a fixed substrate concentration of 10 mM and an assay time of 5 min for V275M, A274T, R240W, V153M, and G133R; 6 min for K59; 7 min for E89K, N59, and N59C162A; and 3 h for C132R (fig. 5). Because the K_m for the substrate ALA varies dramatically between the octameric and hexameric assemblies of human PBGS, it is important to point out that the data in figure 5 reflect only specific activity and not V_{max} values. Furthermore, using the synthetic mutant R240A, we have established that the equilibrium between the low-K_m octamer and the high-K_m hexamer is pH dependent.[38]

Although the absolute specific activity of the wild-type variants is not constant (table 1), the shape of the pH activity profile is identical for N59, K59, and N59C162A

(fig. 5*A*). The pH activity profiles were obtained for the purified octameric components of the high-activity variants E89K, V153M, A274T, and V274M; these profiles are all similar to that of the wild-type variant (fig. 5*B*), where there is a pK_a value of pH ~6 and a second pK_a value of pH ~8. The most notable variation from this trend is in the second pK_a value for E89K, which is shifted to pH <7.5. The pH activity profile for the predominant hexameric component of the R240W variant is comparable to that for the hexameric F12L variant (fig. 5*C*). On the basis of our work with the synthetic mutant R240A,[38] we interpret the activity of R240W in the pH range 6–7 as the result of the propensity of the substrate ALA to pull the equilibrium toward the octameric form.[38] According to this interpretation, longer assay times would be predicted to result in higher activity at this slightly acidic to neutral range of pH values. The pH activity profiles for C132R and G133R—for both, it has been proposed that they disrupt binding of the essential catalytic zinc ion—are also compared (fig. 5*D*), and both are quite different from the pH activity profiles for the other natural human PBGS variants. In these cases, because the catalytic zinc is compromised, we cannot expect to interpret the pH activity relationship on the basis of the quaternary structure assembly alone.

Discussion

D. Shemin's early studies on the enzymes of heme biosynthesis revealed that PBGS, then called "δ-aminolevulinate dehydratase," was present in tissues at levels far greater than what appeared to be required for its function in heme biosynthesis.[30] This is consistent with the later clinical observations that patients with ALAD porphyria have more than one genetic aberration.[12–15,17,18,21,25,26] When parents or siblings of these patients were available for analysis, those relatives with only one aberrant gene were found to have ~50% normal PBGS activity in blood, but these relatives did not have porphyria. The F12L mutation was first discovered in an asymptomatic heterozygous infant during a screen for hereditary tyrosinemia.[37] The screen is based on the fact that a metabolic by-product in patients with tyrosinemia is succinylacetone, which is a potent inhibitor of PBGS. The infant showed 12% normal PBGS activity. F12L is now known to produce a low-activity hexameric form of PBGS that is dramatically different from the wild-type octameric assembly.[36] Coexpression of wild-type human PBGS and F12L demonstrated the ready assembly of heteromeric oligomers comprising two different kinds of human PBGS subunits—some that contained phenylalanine at position 12 and some that contained leucine. Such heteromeric oligomers would be expected to exist in compound heterozygous patients with ALAD porphyria. In our previous studies, the hetero-octamers composed of wild type and F12L exhibited kinetic behavior like that of the wild-type octamer, and the heterohexamers were like the F12L homohexamer, but both were shown to exist in a dynamic equilibrium. Similar dynamic equilibria of heteromeric oligomers would be expected to exist in compound heterozygous patients with porphyria, and this may relate to the episodic nature of the disease. The work with coexpression of wild-type human PBGS and F12L, as well as the synthetic variant R240A, proved that homo-oligomers of PBGS can come apart, change shape, and reassemble into a structurally and functionally different homo-oligomer.[38,39] Because F12L and the designed synthetic human PBGS variants R240A and W19A had shown that single–amino acid mutations could dramatically alter the folding and assembly of human PBGS,[36,38,39] we addressed whether the porphyria-associated *ALAD* alleles encode a PBGS with an altered quaternary-structure (morpheein) equilibrium.

The present study demonstrated that each porphyria-associated mutation shifts the human PBGS morpheein equilibrium toward the hexamer. This demonstration provides a new way to think about genetic alterations that compromise function but are not obviously at the enzyme active site. Genetic mutations that shift a morpheein equilibrium favor a distinct alternate quaternary assembly, rather than an amorphous, "misfolded" state. Knowledge of the structural difference between the more-active and the less-active morpheein assemblies may provide a foundation for correcting the structural and functional imbalance. Other human proteins have been suggested to exhibit morpheein-like characteristics,[41] and it is possible that natural genetic variations or disease states are related to shifts in these morpheein equilibria. The human enzymes that, so far, have been suggested to exist as morpheeins are the mitochondrial enzyme succinyl-CoA transferase that functions in the metabolism of ketone bodies; purine nucleoside phosphorylase, which is an important component of the purine salvage pathway; ribonucleotide reductase, which is responsible for balancing the production of deoxyribonucleotides for DNA replication; and the peroxiredoxins, which function in cell-signaling pathways. Having established that the *ALAD* alleles associated with ALAD porphyria all perturb the morpheein equilibrium for human PBGS, we suggest that the genetic diseases succinyl-CoA transferase deficiency (MIM 245050),[47] purine nucleoside phosphorylase deficiency (MIM 164050),[47] and those related to ribonucleotide reductase or peroxiredoxins may also reflect perturbation of the morpheein equilibria for these proteins and constitute conformational diseases. A conformational disease resulting from perturbation of a morpheein equilibrium may be suspected in cases in which a homo-oligomeric protein structure is known and a disease-causing missense mutation is known to be distant from the enzyme active site.

Acknowledgments

This research was supported by grants from the National Institutes of Health, ES03654 (to E.K.J.) and CA006727 (to the Institute

for Cancer Research, Fox Chase Cancer Center), by an appropriation from the Commonwealth of Pennsylvania, and by the Pennsylvania Department of Health (Tobacco Funds). The Pennsylvania Department of Health specifically disclaims responsibility for any analyses, interpretations, or conclusions. This research used the mass spectroscopy capabilities of the Biochemistry and Biotechnology Fox Chase Cancer Center Core Facility; we thank Dr. Yibai Chen for her assistance with the mass spectral identification of the purified proteins. We thank our colleagues Drs. Warren Kruger and Alfonso Bellacosa for critical review of the manuscript before submission.

Web Resource

The URL for data presented herein is as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for ALAD porphyria, succinyl-CoA transferase deficiency, and purine nucleoside phosphorylase deficiency)

References

1. Sassa S (2002) The porphyrias. Photodermatol Photoimmunol Photomed 18:56–67
2. Doss M, Schmidt A (1972) Zwei Suchteste fur Porphyrein. Z Klin Chem Klin Biochem 10:230–231
3. Battistuzzi G, Petrucci R, Silvagni L, Urbani FR, Caiola S (1981) δ-Aminolevulinate dehydrase: a new genetic polymorphism in man. Ann Hum Genet 45:223–229
4. Wetmur JG, Kaya AH, Plewinska M, Desnick RJ (1991) Molecular characterization of the human δ-aminolevulinate dehydratase 2 ($ALAD^2$) allele: implications for molecular screening of individuals for genetic susceptibility to lead poisoning. Am J Hum Genet 49:757–763
5. Montenegro MF, Barbosa F Jr, Sandrim VC, Gerlach RF, Tanus-Santos JE (2006) Ethnicity affects the distribution of δ-aminolevulinic acid dehydratase (ALAD) genetic variants. Clin Chim Acta 367:192–195
6. Parra EJ, Ribeiro JC, Caeiro JL, Riveiro A (1995) Genetic structure of the population of Cabo Verde (west Africa): evidence of substantial European admixture. Am J Phys Anthropol 97: 381–389
7. Shen XM, Wu SH, Yan CH, Zhao W, Ao LM, Zhang YW, He JM, Ying JM, Li RQ, Wu SM, et al (2001) Delta-aminolevulinate dehydratase polymorphism and blood lead levels in Chinese children. Environ Res 85:185–190
8. Suzen HS, Duydu Y, Aydin A (2004) Molecular analysis of δ-aminolevulinic acid dehydratase (ALAD) gene polymorphism in a Turkish population. Biochem Genet 42:461–467
9. Wetmur JG, Lehnert G, Desnick RJ (1991) The delta-aminolevulinate dehydratase polymorphism: higher blood lead levels in lead workers and environmentally exposed children with the 1-2 and 2-2 isozymes. Environ Res 56:109–119
10. Weuve J, Kelsey KT, Schwartz J, Bellinger D, Wright RO, Rajan P, Spiro A III, Sparrow D, Aro A, Hu H (2006) Delta-aminolevulinic acid dehydratase (ALAD) polymorphism and the relation between low-level lead exposure and the Mini-Mental Status Examination in older men: the Normative Aging Study. Occup Environ Med 63:746–753
11. Zheng Y, Song W, Wang Y (2001) The gene polymorphism of delta-aminolevulinate dehydratase (ALAD) in 530 cases of Chinese Han population [in Chinese]. Zhonghua Yu Fang Yi Xue Za Zhi 35:16–18
12. Maruno M, Furuyama K, Akagi R, Horie Y, Meguro K, Garbaczewski L, Chiorazzi N, Doss MO, Hassoun A, Mercelis R, et al (2001) Highly heterogeneous nature of δ-aminolevulinate dehydratase (ALAD) deficiencies in ALAD porphyria. Blood 97:2972–2978
13. Akagi R, Inoue R, Muranaka S, Tahara T, Shigeru T, Anderson KE, Phillips JD, Sassa S (2006) Dual gene defects involving δ-aminolaevulinate dehydratase and coproporphyrinogen oxidase in a porphyria patient. Br J Haematol 132:237–243
14. Akagi R, Kato N, Inoue R, Anderson KE, Jaffe EK, Sassa S (2006) δ-Aminolevulinate dehydratase (ALAD) porphyria: the first case in North America with two novel ALAD mutations. Mol Genet Metab 87:329–336
15. Doss MO, Stauch T, Gross U, Renz M, Akagi R, Doss-Frank M, Seelig HP, Sassa S (2004) The third case of Doss porphyria (δ-amino-levulinic acid dehydratase deficiency) in Germany. J Inherit Metab Dis 27:529–536
16. Sakamoto D, Kudo H, Inohaya K, Yokoi H, Narita T, Naruse K, Mitani H, Araki K, Shima A, Ishikawa Y, et al (2004) A mutation in the gene for δ-aminolevulinic acid dehydratase (ALAD) causes hypochromic anemia in the medaka, *Oryzias latipes*. Mech Dev 121:747–752
17. Akagi R, Nishitani C, Harigae H, Horie Y, Garbaczewski L, Hassoun A, Mercelis R, Verstraeten L, Sassa S (2000) Molecular analysis of δ-aminolevulinate dehydratase deficiency in a patient with an unusual late-onset porphyria. Blood 96:3618–3623
18. Akagi R, Shimizu R, Furuyama K, Doss MO, Sassa S (2000) Novel molecular defects of the δ-aminolevulinate dehydratase gene in a patient with inherited acute hepatic porphyria. Hepatology 31:704–708
19. Gross U, Sassa S, Jacob K, Deybach JC, Nordmann Y, Frank M, Doss MO (1998) 5-Aminolevulinic acid dehydratase deficiency porphyria: a twenty-year clinical and biochemical follow-up. Clin Chem 44:1892–1896
20. Yano Y, Kondo M (1998) ALAD deficiency porphyria (ADP) [in Japanese]. Ryoikibetsu Shokogun Shirizu 19:139–140
21. Sassa S (1998) ALAD porphyria. Semin Liver Dis 18:95–101
22. Fujita H, Ishida N, Akagi R (1995) delta-Aminolevulinate dehydratase deficiency [in Japanese]. Nippon Rinsho 53:1408–1417
23. Sithisarankul P, Schwartz BS, Lee BK, Kelsey KT, Strickland PT (1997) Aminolevulinic acid dehydratase genotype mediates plasma levels of the neurotoxin, 5-aminolevulinic acid, in lead-exposed workers. Am J Ind Med 32:15–20
24. Muraoka A, Suehiro I, Fujii M, Murakami K (1995) delta-Aminolevulinic acid dehydratase deficiency porphyria (ADP) with syndrome of inappropriate secretion of antidiuretic hormone (SIADH) in a 69-year-old woman. Kobe J Med Sci 41:23–31
25. Ishida N, Fujita H, Fukuda Y, Noguchi T, Doss M, Kappas A, Sassa S (1992) Cloning and expression of the defective genes from a patient with δ-aminolevulinate dehydratase porphyria. J Clin Invest 89:1431–1437
26. Sassa S, Ishida N, Fujita H, Fukuda Y, Noguchi T, Doss M, Kappas A (1992) Cloning and expression of the defective genes in delta-aminolevulinate dehydratase porphyria: compound heterozygosity in this hereditary liver disease. Trans Assoc Am Physicians 105:250–259
27. Thunell S, Holmberg L, Lundgren J (1987) Aminolaevulinate

dehydratase porphyria in infancy: a clinical and biochemical study. J Clin Chem Clin Biochem 25:5–14
28. Shemin D, Russell CS, Abramsky T (1955) The succinate-glycine cycle. I. The mechanism of pyrrole synthesis. J Biol Chem 215:613–626
29. Shemin D, Russell CS (1953) δ-Aminolevulinic acid, its role in the biosynthesis of porphyrins and purines. J Am Chem Soc 75:4873–4874
30. Shemin D (1972) δ-Aminolevulinic acid dehydratase. In: Boyer PD (ed) Enzymes. Academic Press, New York, pp 323–337
31. Guo GG, Gu M, Etlinger JD (1994) 240-kDa proteasome inhibitor (CF-2) is identical to δ-aminolevulinic acid dehydratase. J Biol Chem 269:12399–12402
32. Gross M, Hessefort S, Olin A (1999) Purification of a 38-kDa protein from rabbit reticulocyte lysate which promotes protein renaturation by heat shock protein 70 and its identification as δ-aminolevulinic acid dehydratase and as a putative DnaJ protein. J Biol Chem 274:3125–3134
33. Jaffe EK (2000) The porphobilinogen synthase family of metalloenzymes. Acta Crystallogr D Biol Crystallogr 56:115–128
34. Liedgens W, Grutzmann R, Schneider HA (1980) Highly efficient purificaton of the labile plant enzyme 5-aminolevulinate dehydratase (EC 4.2.1.24) by means of monoclonal antibodies. Z Naturforsch [C] 35:958–962
35. Van Heyningen S, Shemin D (1971) Quaternary structure of δ-aminolevulinate dehydratase from *Rhodopseudomonas spheroides*. Biochemistry 10:4676–4682
36. Breinig S, Kervinen J, Stith L, Wasson AS, Fairman R, Wlodawer A, Zdanov A, Jaffe EK (2003) Control of tetrapyrrole biosynthesis by alternate quaternary forms of porphobilinogen synthase. Nat Struct Biol 10:757–763
37. Akagi R, Yasui Y, Harper P, Sassa S (1999) A novel mutation of δ-aminolaevulinate dehydratase in a healthy child with 12% erythrocyte enzyme activity. Br J Haematol 106:931–937
38. Tang L, Breinig S, Stith L, Mischel A, Tannir J, Kokona B, Fairman R, Jaffe E (2006) Single amino acid mutations alter the distribution of human porphobilinogen synthase quaternary structure isoforms (morpheeins). J Biol Chem 281: 6682–6690
39. Tang L, Stith L, Jaffe EK (2005) Substrate-induced interconversion of protein quaternary structure isoforms. J Biol Chem 280:15786–15793
40. Jaffe EK (2004) The porphobilinogen synthase catalyzed reaction mechanism. Bioorg Chem 32:316–325
41. Jaffe EK (2005) Morpheeins—a new structural paradigm for allosteric regulation. Trends Biochem Sci 30:490–497
42. Carrell RW, Lomas DA (1997) Conformational disease. Lancet 350:134–138
43. Fishbein L (1998) Transmissible spongiform encephalopathies, hypotheses and food safety: an overview. Sci Total Environ 217:71–82
44. Jaffe EK, Martins J, Li J, Kervinen J, Dunbrack RL Jr (2001) The molecular mechanism of lead inhibition of human porphobilinogen synthase. J Biol Chem 276:1531–1537
45. Jaffe EK, Volin M, Bronson-Mullins CR, Dunbrack RL Jr, Kervinen J, Martins J, Quinlan JF Jr, Sazinsky MH, Steinhouse EM, Yeung AT (2000) An artificial gene for human porphobilinogen synthase allows comparison of an allelic variation implicated in susceptibility to lead poisoning. J Biol Chem 275:2619–2626
46. Ponka P (1999) Cell biology of heme. Am J Med Sci 318:241–256
47. Hamosh A, Scott AF, Amberger J, Valle D, McKusick VA (2000) Online Mendelian Inheritance in Man (OMIM). Hum Mutat 15:57–61