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Characterization of the wild-type form of 4a-carbinolamine dehydratase and two naturally occuring mutants associated with hyperphenylalaninemia

(phenylalanine hydroxylase-stimulating protein/dimerization cofactor for hepatocyte nuclear factor 1)

GEORG JOHNEN, DEVANAND KOWLESSUR, BRUCE A. CITRON, AND SEYMOUR KAUFMAN

Laboratory of Neurochemistry, National Institute of Mental Health, Bethesda, MD 20892

Contributed by Seymour Kaufman, September 18, 1995

ABSTRACT **The characterization of 4a-carbinolamine dehydratase with the enzymatically synthesized natural substrate revealed non-Michaelis–Menten kinetics. A Hill coefficient of 1.8 indicates that the dehydratase exists as a multisubunit enzyme that shows cooperativity. A mild form of hyperphenylalaninemia with high 7-biopterin levels has been linked to mutations in the human 4a-carbinolamine dehydratase gene. We have now cloned and expressed two mutant forms of the protein based on a patient's DNA sequences. The kinetic parameters of the mutant C82R reveal a 60% decrease in V_{max} but no change in K_m ($\approx$5 μM), suggesting that the cysteine residue is not involved in substrate binding. Its replacement by arginine possibly causes a conformational change in the active center. Like the wild-type enzyme, this mutant is heat stable and forms a tetramer. The susceptibility to proteolysis of C82R, however, is markedly increased *in vitro* compared with the wild-type protein. We have also observed a decrease in the expression levels of C82R protein in transfected mammalian cells, which could be due to proteolytic instability. The 18-amino acid-truncated mutant Glu-87 → termination could not be completely purified and characterized due to minute levels of expression and its extremely low solubility as a fusion protein. No dehydratase activity was detected in crude extracts from transformed bacteria or transfected mammalian cells. Considering the decrease in specific activity and stability of the mutants, we conclude that the patient probably has less than 10% residual dehydratase activity, which could be responsible for the mild hyperphenylalaninemia and the high 7-biopterin levels.**

The cofactor tetrahydrobiopterin (BH_4) is essential for the hydroxylation of the aromatic amino acids phenylalanine, tyrosine, and tryptophan (1). Deficiency in phenylalanine hydroxylase (PAH; EC 1.14.16.1) activity can lead to hyperphenylalaninemia (HPA) or phenylketonuria, whereas enzymatic defects in the biosynthetic pathway of BH_4 can have even more severe clinical consequences because the biosynthesis of neurotransmitters is affected as well (2). Two additional enzymes are involved in the phenylalanine hydroxylating system. BH_4 is oxidized to the carbinolamine 4a-hydroxytetrahydrobiopterin (4a-OH-BH_4) during the hydroxylation reaction. The carbinolamine is then converted to quinonoid dihydrobiopterin (qBH_2). Dihydropteridine reductase (DHPR; EC 1.6.99.7) completes the cycle by reducing qBH_2 back to BH_4. A lack of the regenerating enzyme DHPR can cause HPA and severe neurological impairments, similar to a defective *de novo* synthesis of BH_4 (3). The other enzyme involved in BH_4 regeneration is 4a-carbinolamine dehydratase (EC 4.2.1.-), which catalyzes the dehydration of 4a-OH-BH_4 to qBH_2 (4, 5). The enzyme was originally designated as "phenylalanine hydroxylase-stimulating protein" (PHS) because it could stimulate the hydroxylation at higher concentrations of PAH and at higher pH *in vitro* (6). Initially, the biological role of PHS remained unclear because the dehydration reaction proceeds rapidly nonenzymatically at physiological pH (6, 7). After the discovery of a variant form of HPA in patients who excrete 7-biopterin in their urine (8) and the finding that the dehydratase can prevent the formation of 7-biopterin (9), which is an uncoupling cofactor (10) and inhibitor of PAH (11, 12), it was hypothesized that mutations in the dehydratase gene were the cause of this new form of HPA. Indeed, two mutant alleles of the dehydratase gene have been found in a patient with the described biochemical abnormalities (13). Another biological role of PHS has emerged recently. We and others have demonstrated that PHS is identical to DCoH, the dimerization cofactor for hepatocyte nuclear factor 1 (HNF-1) (14, 15). HNF-1 is a tissue-specific transcription factor that regulates the expression of a variety of mammalian genes and is coactivated by DCoH (16, 17). The temporal and spatial expression pattern during the embryogenesis of *Xenopus* strongly suggests an involvement of PHS/DCoH in the development of amphibian tissues (18), whereas in *Pseudomonas*, PHS/DCoH regulates the expression of a bacterial PAH (19).

We have now generated and characterized the two mutant forms of PHS/DCoH that had been found in the patient and compared them with the wild-type enzyme (GenBank accession no. M83742) to shed more light on the cause of the abnormalities.

MATERIALS AND METHODS

Materials. Restriction endonucleases were purchased from New England Biolabs. Sheep liver DHPR, NADH, glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*), glucose 6-phosphate, phenylalanine, bovine pancreatic trypsin, and bovine spleen cathepsin B were purchased from Sigma. Beef liver catalase was obtained from Boehringer Mannheim. BH_4 was purchased from Schircks Laboratories (Jona, Switzerland). Bovine serum albumin (BSA) was obtained from Pierce. Hepes and 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) were obtained from ICN.

Site-Directed Mutagenesis. The CloneAmp pUC19 mutagenesis system from GIBCO was used to generate the C82R mutant (20). The following sets of primers were used: o331 (5′-AUGGAGAUCUCUCCATGGCTGGCAAGGCA-3′)/ o334 (5′-GCACGUUCGUGAGUGCUC-3′) and o333 (5′-

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Abbreviations: BH_4, tetrahydrobiopterin; 4a-OH-BH_4, 4a-hydroxytetrahydrobiopterin; qBH_2, quinonoid dihydrobiopterin; CHO, chinese hamster ovary; GST, glutathione *S*-transferase; PAH, phenylalanine hydroxylase; PHS/DCoH, phenylalanine hydroxylase-stimulating protein/dimerization cofactor for hepatocyte nuclear factor 1; HNF-1, hepatocyte nuclear factor 1; DHPR, dihydropteridine reductase; BSA, bovine serum albumin; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride.

ACUCACGAACGUGCCGGUCUT-3')/o332 (5'-ACGCGUACUAGUGGATCCTTATGTCATAGACAC-3'). The PCR amplifications (30 cycles) were performed under conditions described previously (13). The Glu-87 → termination (E87Ter) mutant was generated by a simple PCR approach using the primers o308 (5'-TGGCCATGGCTGGCAAGGCA-3') and o349 (5'-TTGGATCCTTAAGAAAGACCGGC-3'). The same PCR conditions as above were used except that the annealing temperature was 65°C. Since human and rat PHS/DCoH cDNA translate into identical proteins (16), we used our rat cDNA clone as the template for both mutagenesis approaches (14).

Subcloning. The PCR products of the C82R mutagenesis were annealed with the plasmid pAMP19 (supplied with the CloneAmp system) and then used to transform competent *Escherichia coli* DH5α cells (GIBCO). Annealing, transformation, and selection were performed according to the supplier's protocol. Plasmids were purified with Qiagen (Chatsworth, CA) kits. After the sequences had been confirmed, the inserts were subcloned in the bacterial expression vector pET3d (Novagen) between the *Nco* I and *Bam*HI restriction sites and in the mammalian expression vector pBJ5 (a generous gift of G. R. Crabtree, Stanford University, Stanford, CA) by using the *Not* I and *Eco*RI restriction sites. The PCR product of the E87Ter mutant was ligated into the pCRII TA cloning vector (Invitrogen). After the sequence had been confirmed, the insert was subcloned in pET3d between the *Nco* I and *Bam*HI restriction sites and in pBJ5 at the *Eco*RI restriction site. The DNA for the glutathione *S*-transferase (GST)-E87Ter fusion protein was prepared by using the pGEX-2T expression vector (Pharmacia) cut with *Bam*HI/*Eco*RI and the E87Ter insert from pCRII cut with *Nco* I/*Eco*RI. Vector and insert were first ligated at the *Eco*RI site, then treated with Klenow enzyme (United States Biochemical) to generate blunt ends, and finally blunt-ligated with T4 DNA ligase (New England Biolabs). The in-frame fusion was performed according to the supplier's protocol and confirmed by sequencing.

DNA Sequence Analysis. Complete DNA sequences for both strands of each mutant were determined on an automated sequencer as described previously (13).

Expression and Purification of Proteins. Recombinant rat PAH was purified from *E. coli* as described previously (21, 22). Native PHS/DCoH was purified from rat liver according to the procedure of Huang *et al.* (23). Recombinant wild-type and mutant dehydratases were expressed in the *E. coli* strain BL21(DE3) (Novagen), except GST-E87Ter, which was expressed in the strain DH5. Cells were grown and induced as described (14). Proteins were purified by the method of Huang *et al.* (23) followed by an additional gel filtration step (see below). The GST fusion protein was purified by affinity chromatography (14). The purification was monitored by measuring the GST activity with a kit (GST Detection Module) supplied by Pharmacia. Protein was quantitated with the Pierce Bradford assay and BSA as standard. The purity of the proteins was assessed with SDS/PAGE (see below).

Gel Filtration. For additional purification of PHS/DCoH samples and molecular mass analysis, gel filtration on a Superose 12 HR 10/30 column (Pharmacia) was performed. Proteins were eluted in 50 mM sodium phosphate (pH 7.6)/150 mM NaCl with a flow rate of 0.25 ml/min. Protein standards from Pharmacia were used for calibration.

Dehydratase Assays. We used two assays to determine dehydratase activity: (*i*) A direct assay for the kinetic characterization of the purified enzymes (see legend to Fig. 1), and (*ii*) PAH stimulation assay. For the determination of specific activities of crude extracts and purified enzymes, we used a fixed-time assay that measured the formation of extra tyrosine caused by a stimulation of PAH in the presence of the dehydratase. This assay is a modification of a published procedure (23, 24). The assay mixture contained 30 mM potassium phosphate (pH 8.3), catalase at 200 μg/ml, 256 μM NADH, 8 mM glucose 6-phosphate, 4 mM phenylalanine, an excess of both DHPR and glucose-6-phosphate dehydrogenase, PAH at 50 μg/ml, and the PHS/DCoH sample. Reactions were started by the addition of 5 μM BH_4 and incubated for 15 min at 25°C. The tyrosine produced was quantitated with the nitrosonaphthol procedure (25). For the 20 μM BH_4 version of this assay, an equivalent amount of PAH had to be added.

Heat Stability. Samples of wild-type or mutant PHS/DCoH were heated for 5 min at 65°C in a temperature-controlled waterbath. The activity was determined by the PAH stimulation assay (5 μM BH_4).

Analysis of Proteolytic Stability. The experiments are described in the legend to Fig. 2. In a separate experiment under the same conditions, the wild-type protein was incubated for 18 h. Because of self-digestion, trypsin was resupplied every 3 h. Samples were taken at 0, 1, 2, 4, 7, and 18 h. Cathepsin digestions were performed in 25 mM sodium phosphate buffer (pH 6.0) with 0.6 unit of cathepsin per μg of PHS at 37°C.

Electrophoresis and Western Blot Analysis. SDS/PAGE was performed under reducing conditions with precast 14% or 16% polyacrylamide gels in a mini gel apparatus (Novex). Protein bands were visualized by using the Fast Stain procedure (Zoion). Blotting was performed in a Novex blotting module according to the manufacturer's recommendations. Immunostaining was performed as described previously by using polyclonal antibodies against PHS/DCoH (24). Isoelectric focusing gels (Novex) in the pH range of 3–10 were used to determine the pI value of the native and recombinant wild-type and the C82R mutant enzymes. Standards (Bio-Rad) in the pI range 4.45–9.6 were used.

Cell Culture. Chinese hamster ovary (CHO) cells (American Type Culture Collection) were grown in Ham's F-12 medium supplied with 10% fetal calf serum (GIBCO) at 37°C under 5% CO_2. Cells were subcultured 20–24 h before transfection. Transient transfections were performed with 3 μg of pSV-β-galactosidase vector (Promega) as an internal control and 10 μg of pBJ5 containing either no insert, wild-type, or mutant insert, using a Gene Pulser apparatus (Bio-Rad) under conditions described previously (16). Cells were harvested 24–28 h after transfection. β-Galactosidase activity of the crude extracts was determined spectrophotometrically with the β-galactosidase enzyme assay system from Promega. The results were used to normalize for transfection efficiency.

RESULTS

Wild-type PHS/DCoH and the mutant C82R were overexpressed in *E. coli* and purified to homogeneity. The expression levels of the mutant were consistently lower (around 50%) than the values for the wild-type protein (Table 1). A subunit molecular mass of 11–12 kDa was determined by SDS/PAGE for both proteins. Gel filtration experiments confirmed that the recombinant wild type, as well as C82R, is a tetrameric protein with a molecular mass of about 44 kDa. Similarity is

Table 1. Expression of wild-type PHS/DCoH and the mutant C82R in *E. coli*

Protein	Expression level, %	Specific activity, μmol/min per mg of protein: Crude extract*	Purified PHS/DCoH*	Purified PHS/DCoH†
Wild type	13.9	2.47	17.78	26.92
C82R	7.3	0.64	8.71	13.23

Expression levels were calculated from the specific activities of the crude extracts and the purified enzymes. The specific activities were determined with the PAH stimulation assay in the presence of 5 μM BH_4 (*) or 20 μM BH_4 (†).

also seen in immunoblotting experiments and stability of both proteins to heat. Supporting evidence for the replacement of Cys-82 by Arg is the mutant's behavior in isoelectric focusing runs. We found a pI of 5.8 for native PHS/DCoH isolated from liver, as reported previously by Hauer *et al.* (15), whereas the recombinant wild-type PHS/DCoH has a pI of 6.2. The tissue enzyme lacks the amino-terminal methionine and is N-acetylated, which explains the increase in negative charge (15). A pI of 6.9 for the mutant C82R reflects an expected decrease in negative net charge over the wild-type molecule.

For the kinetic characterization of wild-type PHS/DCoH and its mutant forms, we developed a spectrophotometric assay that allowed us to measure directly the ability of the enzyme to dehydrate its natural substrate, 4a-OH-BH_4. The latter is generated enzymatically *in situ* from BH_4 via the PAH reaction. The assay is based on a procedure described previously (4, 26). 4a-OH-BH_4 has an absorbance maximum at 246 nm which can be monitored to follow its formation and breakdown. To prevent the formation of interfering amounts of qBH_2 and 7,8-dihydrobiopterin it was important to instantly convert all of the BH_4 into 4a-OH-BH_4, which was then used for the dehydratase assay by promptly adding a PHS/DCoH sample. Therefore, an excess of PAH was used. To use initial rates for the determination of K_m and V_{max}, we first had to quantitate the amount of the 4a-OH-BH_4 formed at a given time. We recorded the spectra of the hydroxylation reaction in short time intervals and deconvoluted them by using the known extinction coefficients of the pure components. A K_m of 5.2 μM and a V_{max} of 25.5 μmol/min per mg of protein were determined for the recombinant wild-type dehydratase at 25°C and pH 8.3. The mutant C82R shows a similar K_m (4.7 μM), whereas its V_{max} is reduced to 7.3 μmol/min per mg, 29% of the wild type (Table 2). The kinetics of both enzymes show sigmoidicity that cannot be fitted to the Michaelis–Menten equation (Fig. 1). The Hill coefficient was about 1.8 for both enzymes. Using the PAH stimulation assay with 5 μM BH_4, which represents a more physiological BH_4 concentration, we obtained a value for the specific activity of the C82R mutant that was about 49% of the wild-type value. Performing the stimulation assay in the presence of 20 μM BH_4, which results in substrate concentrations that are closer to V_{max} conditions, yielded the same result (Table 1). Since we do not know which assay is closer to the conditions *in vivo*, an average of 40% wild-type activity for C82R will be used in the following discussions.

The proteolytic stability of C82R was determined *in vitro* with trypsin. This protease was chosen because of its specificity for positively charged amino acids in substrate sequences. We determined the decrease in dehydratase activity of the wild-type and mutant forms during the course of digestion (Fig. 2). Wild-type PHS/DCoH showed a substantial resistance to digestion, as observed earlier with chymotrypsin (ref. 6; S.K., unpublished results). As anticipated, the mutant exhibited an increased sensitivity towards trypsin, but the severity of the degradation was a surprise. After incubation at 25°C with 0.5 unit of trypsin per μg of dehydratase, the mutant showed less than 2% remaining activity after 1 h. The wild-type remained virtually unaffected over a period of 2.5 h. Further incubation of the wild-type (up to 18 h) showed that it is about 14 times more stable than C82R. Comparable time courses were studied by following the progress of the digestion by SDS/PAGE. The intensity of the wild-type band decreased very slowly and without the appearance of new, low molecular mass bands. The fast digestion of the mutant led to the initial accumulation of lower molecular mass products, the largest corresponding to a molecular mass of 9–10 kDa, that were then rapidly degraded (data not shown). Since trypsin is a pancreatic serine protease not found in normal liver cells, the more abundant lysosomal protease cathepsin was selected for additional studies. The

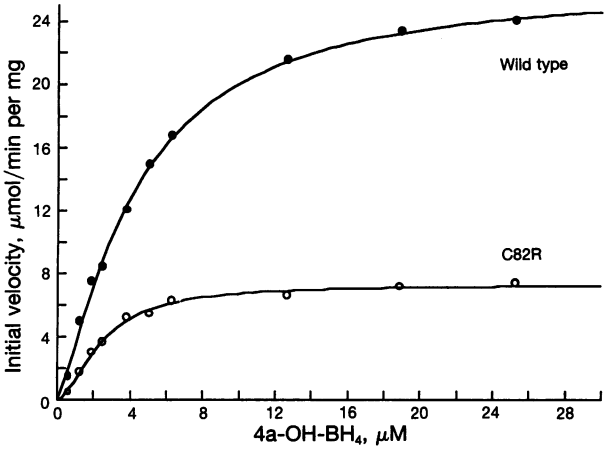

FIG. 1. Dependence of the initial velocity of the PHS/DCoH-catalyzed reaction on the concentration of 4a-OH-BH_4. The curves were obtained with the wild-type enzyme and the mutant C82R. Reactions were performed at 25°C in a final volume of 1 ml, containing the following components: 30 mM potassium phosphate (pH 8.3), 0.3 mM phenylalanine, catalase at 60 μg/ml, and PAH at 52 μg/ml. The reactions were initiated by the addition of BH_4 and followed spectrophotometrically on a Cary 1E (Varian) at 246 nm. An extinction coefficient of 19,600 $M^{-1}\cdot cm^{-1}$ was used to quantitate the amount of 4a-OH-BH_4 formed, which under our assay conditions was, on a molar basis, equal to 95% of the initial amount of BH_4 used. The conversion of BH_4 to 4a-OH-BH_4 was complete within 20 sec; wild type (1.4 μg) or C82R mutant (4.4 μg) was added immediately to the cuvette and the decrease in absorbance was recorded. Initial rates were corrected for the contribution due to nonenzymatic breakdown of 4a-OH-BH_4. Kinetic parameters were computed by fitting experimental data by direct fit to a non-Michaelis–Menten equation with ULTRAFIT version 2.1 (Biosoft).

Table 2. Kinetic parameters for the dehydration of 4a-OH-BH_4 by PHS/DCoH at 25°C and pH 8.3

Protein	V_{max}, μmol/min per mg	K_m, μM	Hill coefficient
Wild type	25.5 ± 0.5	5.2 ± 0.3	1.7 ± 0.2
C82R	7.3 ± 0.4	4.7 ± 0.3	1.9 ± 0.2

The parameters were determined as described in the legend to Fig. 1. Results are the means ± SD of two experiments.

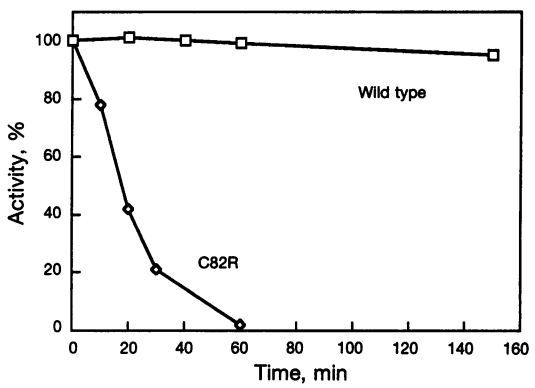

FIG. 2. Proteolytic stability of C82R and wild-type PHS/DCoH. In a final volume of 30 μl, 4.08 μg of mutant or wild-type protein was incubated at 25°C with 0.204 μg (2.04 units) of trypsin in 40 mM Hepes (pH 7.4)/10 mM $CaCl_2$. At different time intervals (wild type: 0, 20, 40, 60, and 150 min; C82R: 0, 10, 20, 30, and 60 min) 5-μl samples were taken, the reaction was stopped by adding 5 μl of 2 mM AEBSF and BSA at 2 mg/ml, and the remaining dehydratase activity was measured with the PAH stimulation assay (5 μM BH_4). The activities are expressed as percent of the initial activity for both mutant and wild type.

results obtained with cathepsin (0.6 unit/μg of dehydratase) were similar to the trypsin digestions (data not shown).

The truncated mutant E87Ter could not be overexpressed in *E. coli*. Only very small amounts could be detected with immunoblotting of supernatants and pellets from crude extracts, and no dehydratase activity was detectable. Purification of the protein was not pursued. Instead, we constructed a GST fusion protein version of the mutant. Although the fusion protein of the mutant, GST-E87Ter, was overexpressed in *E. coli*, after extraction of the bacterial cells most of the protein was located in the insoluble fraction. The small amount of soluble fusion protein was purified and analyzed on SDS/PAGE. The purified protein was a mixture of the full-length fusion protein, several low molecular mass degradation products, and free GST. The full-length GST-E87Ter fusion protein and its low molecular mass forms showed bands after staining with polyclonal antibodies raised against wild-type PHS. Again, no dehydratase activity could be detected, whereas transferase activity was detectable, suggesting that at least the GST part had folded properly. Free GST or GST fused to wild-type PHS/DCoH does not interfere with the dehydratase activity (14). Attempts to cleave the GST part from the fusion protein with thrombin led to complete digestion of its E87Ter portion. Finally, E87Ter DNA was subcloned in the mammalian expression vector pBJ5 and used for the transfection of CHO cells. No dehydratase activity could be detected in extracts from transfected CHO cells. The extracts also showed negative results in immunoblotting experiments. In the same transfection experiments, activity and expression of C82R and the wild-type enzyme were easily detected. As in *E. coli*, the expression level of the mutant C82R was about 50% lower than that of the wild type. A comparison is shown in Table 3.

DISCUSSION

The enzymatic characterization of recombinant wild-type PHS revealed non-Michaelis–Menten kinetics. A Hill coefficient of 1.8 and the sigmoidal curve shape indicate cooperativity between subunits of the tetrameric enzyme. This is in contrast to the reported characterization of PHS/DCoH with a synthetically generated substrate, where normal Michaelis–Menten kinetics had been observed (27). However, a conformational change in the enzyme upon binding of pterins had been reported by Rebrin *et al.* (28) and Ficner *et al.* (29). This change might go beyond the active center and influence other subunits or might even have consequences for the transcriptional activity of PHS/DCoH. It is possible that components of our assay, which includes a complex mixture of enzymes and their substrates, may influence the kinetic behavior of the dehydratase. On the other hand, most of the enzymes and substrates of our assay mixture would also be present in a living cell. K_m and V_{max} values are similar to the values published by Rebrin *et al.* (27). A K_m of 3.0 μM and a V_{max} of 16.5 μmol/min per mg (3.3 sec^{-1}) have been reported with chemically synthesized 4a-OH-BH_4 at pH 8.4 and 10°C (compare Table 2). Except for the different pI value, all other properties of the native wild-type enzyme, such as kinetic parameters, heat stability, molecular weight, and proteolytic stability, are very similar to the properties of the recombinant wild-type enzyme (data not shown). Therefore, we conclude that the recombinant mutant C82R we examined is also not significantly different from the native mutant present in the patient.

Table 3. Expression of wild-type PHS/DCoH and the mutants C82R and E87Ter in transfected CHO cells

Protein	Specific activity, μmol/min per mg	Expression level, %
Wild type	0.50 ± 0.08	2.8 ± 0.4
C82R	0.12 ± 0.02	1.4 ± 0.2
E87Ter*	0	0

Expression levels were calculated from the specific activities of the crude extracts (CHO) and the purified proteins (see Table 1). The specific activities were determined with the PAH stimulation assay (5 μM BH_4) and corrected for transfection efficiency. The transfections were performed in duplicate and represent a typical experiment. Results are means ± SD.

*An activity equivalent to 5–10 ng of wild-type PHS/DCoH could have been detected; 55 μg of E87Ter crude extract was used for the assays.

We assume general acid *and* base catalysis at the catalytic site of the dehydratase. To learn more about the mechanism of dehydration, conserved amino acids in the active center have to be targeted for site-directed mutagenesis. PHS/DCoH from *Pseudomonas* has kinetic properties similar to those of the human enzyme, with about 30% sequence identity (19). It is an ideal candidate to search for conserved amino acids that could be of importance for catalysis. For example, the sole cysteine in the human protein is not conserved in the *Pseudomonas* protein. As expected, replacement of this residue in the human protein by arginine does not lead to severe inactivation. Therefore, it is unlikely to be a crucial residue for catalysis. We looked for suitable residues that are conserved in the amino acid sequence of both proteins and are located in the carboxyl-terminal half of PHS/DCoH, where the mutations of our patient have been found. Striking is a highly conserved region that includes His-62, His-63, and Pro-64. Other invariant residues are Lys-72, His-80 (the latter being close to the sole Cys), and Asp-89, which lies in the domain that is missing in E87Ter. A catalytic Asp–His dyad, like the structural element in serine proteases, has been proposed to act as a general base in the active center of scytalone dehydratase (30). On the basis of the recently obtained crystal structure of PHS/DCoH, it had been suggested that a dyad consisting of Asp-89 and His-62 is involved in a general base catalysis to dehydrate 4a-OH-BH_4, as well as His-63, which might act as a general acid (31). Preliminary results from ongoing mutagenesis experiments indicate that His-62, -63, and (partially) -80, as well as Asp-89, are important for catalysis (G.J. and S.K., unpublished work). Therefore, at least one residue crucial for catalysis would be missing in E87Ter and render the mutant inactive, even if it would be expressed in the cell. The crystal structure of PHS/DCoH reveals that a subunit consists of three helices packed against one side of a four-stranded β-sheet with the proposed active center located between the loops that connect the helices and the sheet (29, 31). The central one of the three helices is formed by the 18 carboxyl-terminal amino acids of the protein, exactly those residues that are missing in the E87Ter mutant. It is likely that the removal of such a structural element would cause a collapse of the three-dimensional structure of PHS/DCoH or prevent a proper folding of the protein. Consequently, a rapid proteolytic degradation can be anticipated. A direct proof is not possible because tissue samples of the patient are not available, but our expression experiments in *E. coli* and CHO cells support these assumptions. We can conclude that no dehydratase activity arises from the paternal (E87Ter) allele of the patient.

A possible explanation for the surprising sensitivity of C82R to proteolytic digestion and its loss of enzymatic activity might also be found in the crystal structure of wild-type PHS/DCoH. Ficner *et al.* (29) report that the solvent-accessible side chain of Cys-82 is not part of the pterin binding site but is in its vicinity. A decrease in activity after replacement with arginine was proposed to be due to a structural change affecting the position of His-80. The amino acids around position 82 are in a loop that connects the sheet with the above-mentioned carboxyl-terminal helix which is missing in the mutant E87Ter. Apparently, the loop is accessible, especially after a conformational disturbance by the bulky and positively charged arginine, to proteases that are able to digest the helix from the

remaining structure, and subsequently C82R would meet the same fate as the truncated mutant. The lower expression levels of C82R in CHO cells and bacteria support the hypothesis that a similar decrease might also occur in the patient. Other reasons for the lower expression levels could be a lower solubility of the mutated protein or a negative influence of the amino acid substitution on protein folding. An effect of the altered genetic code of C82R on transcription and translation—e.g., through methylation and codon usage—is less likely since the decrease is similar in mammalian and bacterial cells.

If we assume that each of the PHS/DCoH alleles contributes half of the protein and no compensation of expression levels occurs, then the total dehydratase activity present in the patient should be about 20% compared with normal individuals. This calculation is based on completely inactive E87Ter and 40% active C82R. Despite a lack of *in vivo* data on the quantitative relationship between amounts of PHS present during hydroxylation and amounts of 7-biopterin formed, it is questionable whether 20% residual activity is low enough to explain the large amounts of 7-biopterin found in the patient's urine. If the increased sensitivity to proteolytic degradation occurs *in vivo*, the cellular activity of C82R might drop by another 40–60%, so that the resulting total dehydratase activity in the patient could be as low as 10%. Our dehydratase assays have been performed at pH 8.3 and 25°C. A different response of wild-type and C82R at physiological pH and 37°C cannot be ruled out.

Although the patient and his heterozygous parents are asymptomatic, we cannot preclude the possibility that a total lack of PHS/DCoH would lead to serious clinical manifestations. It is even possible that the patient, now 3 years old, will show signs of illness as he gets older. PHS/DCoH binds to the transcription factor HNF-1 and enhances transcription (16). Previously, we found that the patient has apparently normal functions associated with HNF-1-regulated gene products (13), suggesting that the other function of PHS/DCoH is not significantly affected by the mutations. This is supported by preliminary results in collaboration with G. Crabtree's group that show a normal binding of C82R to HNF-1 in gel retardation experiments (L. Hansen, W. Wang, and G. Crabtree, personal communication). It has to be noted, however, that binding of PHS/DCoH to HNF-1 is not likely to be the only cause for its transcriptional effect (16, 17, 31). Elucidating connections between the transcriptional activity of PHS/DCoH and dehydratase activity, pterin binding, binding of transcription factors, and a postulated binding to nucleic acids (31) will be one of the more important challenges in the near future of pterin biochemistry.

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