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Structural and Functional Consequences of Mutations in 6-Pyruvoyltetrahydropterin Synthase Causing Hyperphenylalaninemia in Humans

PHOSPHORYLATION IS A REQUIREMENT FOR *IN VIVO* ACTIVITY*

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Four naturally occurring mutants with single amino acid alterations in human 6-pyruvoyltetrahydropterin synthase (PTPS) were overexpressed and characterized *in vitro*. The corresponding DNA mutations were found in patients with hyperphenylalaninemia and monoamine neurotransmitter insufficiency due to lack of the tetrahydrobiopterin biosynthetic enzyme PTPS. To predict the structure of the mutant enzymes, computer modeling was performed based on the solved three-dimensional structure of the homohexameric rat enzyme. One mutant (ΔV57) is incorrectly folded and thus unstable *in vitro* and *in vivo*, while a second mutant (P87L) has substantial activity but enhanced sensitivity to local unfolding. Two other mutants, R16C and R25Q, form stable homomultimers and exhibit significant activity *in vitro* but no activity in COS-1 cells. *In vivo* ^{32}P labeling showed that wild-type PTPS, P87L, and R25Q are phosphorylated, while R16C is not modified. This strongly suggests that the serine 19 within the consensus sequence for various kinases, R*XX*S, is the site of modification. Our results demonstrate that PTPS undergoes protein phosphorylation and requires additional, not yet identified post-translational modification(s) for its *in vivo* function.

6-Pyruvoyltetrahydropterin synthase (PTPS,[1] EC 4.6.1.10) is required for the second step of the *de novo* biosynthesis of 5,6,7,8-tetrahydrobiopterin (BH_4) starting from GTP. PTPS converts the substrate 7,8-dihydroneopterin triphosphate, the product from GTP cyclohydrolase I (EC 3.5.4.16), into 6-pyruvoyltetrahydropterin, which in turn is metabolized by sepiapterin reductase (EC 1.1.1.153) to the pathway end product BH_4 (1). The BH_4 cofactor is involved in various cellular processes. For instance, it increases the proliferation rate of erythroid cells (2) and is a limiting cofactor for the synthesis of nitric oxide by a number of recently described nitric oxide synthases (3, 4). The role of BH_4 is best understood as redox cofactor for hydroxylation of the aromatic amino acid phenylalanine in liver, but also of tyrosine and tryptophan, which is rate-limiting for the dopamine and serotonin biosynthesis in the brain (5). In newborns, inborn errors of BH_4 metabolism lead to a variant form of hyperphenylalaninemia accompanied by severe monoamine neurotransmitter shortage that results in progressive mental retardation due to limiting cofactor availability for the tyrosine and tryptophan hydroxylases (6). For GTP cyclohydrolase I various autosomal recessive and dominant mutations have been described that lead to a limited BH_4 availability concomitant with mental retardation and dopa-responsive dystonia, respectively (7–9). In contrast, PTPS mutations have only been found to be recessive (10, 11). PTPS deficiency appears in three different phenotypes, a central, a peripheral, and a transient form, which may disappear during infant thriving (12, 13). Patients with the central type of PTPS deficiency exhibit a general lack of BH_4 in all organs and monoamine neurotransmitter shortage in the central nervous system, whereas patients with the peripheral form do not synthesize BH_4 in peripheral organs but have normal BH_4 and neurotransmitter levels in the central nervous system. PTPS deficiency is thus a very heterogeneous disorder with a hitherto unknown molecular basis.

Recent biochemical and structural investigations of the human and rat enzymes have led to a better understanding of PTPS structure-function on a detailed molecular level (14–16). The mammalian PTPS requires two metals for activity, Zn^{2+} and Mg^{2+}, the former being an integral part of each active site of the enzyme. Crystal structure analysis of the rat enzyme confirmed the proposed homohexameric structure and furthermore revealed that the enzyme is composed of two trimers with an overall toroidal shape that has a diameter and height of 60 Å. One subunit folds into a compact $\alpha + \beta$ structure comprising a four-stranded, anti-parallel β-sheet onto which a pair of antiparallel helices is layered. Between strands β-1 and β-2 there is a 23-residue insertion containing a short α-helical segment (α_A; see Fig. 1). Three subunits are related by a 3-fold molecular symmetry axis. Thereby, an unusual 12-stranded anti-parallel β-barrel is formed, which encloses a highly basic pore of 6–12 Å in diameter. The hexamer is formed by face-to-face association of the two trimers. The putative catalytic sites, six per active hexamer, are located at the trimer interface. Three subunits take part in one catalytic site that is formed by two subunits from one trimer and one subunit from the other trimer. The active center contains the binding site for one Zn^{2+} ion and a structural motif that resembles the acceptor site for

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[1] The abbreviations used are: PTPS, 6-pyruvoyltetrahydropterin synthase (EC 4.6.1.10); BH_4, 5,6,7,8-tetrahydrobiopterin; cDMEM, Dulbecco's modified Eagle medium, containing 10% fetal bovine serum, 50 units/ml of penicillin, and 50 μg/ml of streptomycin; MBP, maltose binding protein; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

the pyrimidine ring system of GTP in G-proteins (16).

We previously reported the mutation analysis from two patients with PTPS deficiency (10). In this study, we expanded the mutation analysis to other patients and present the biochemical and structural characterization of four particular mutant PTPS with single amino acid alterations that were found to be responsible for BH_4 deficiency in newborns. The model of the human enzyme structure is based on computational modeling starting from the solved x-ray structure of the rat PTPS. Based on the experimental observations with recombinant mutant and wild-type proteins that suggested the need of certain modification(s), we demonstrate that the native human PTPS is a phosphoprotein potentially modified at serine 19, and we further conclude that PTPS undergoes protein phosphorylation and other post-translational modification(s) for normal *in vivo* function.

EXPERIMENTAL PROCEDURES

Materials—The following oligonucleotide primers used in this study were synthesized on a Gene Assembler Plus DNA synthesizer (Pharmacia Biotech Inc.): PTPS16 (5′-CTCTCCATGTACTGT-Δ-CACAACTTTATAATT-3′), PTPS21 (5′-CGGGATCCGGCCAAGATGAG-3′), PTPS101 (5′-AAGATGTCGACGGAAGGTGGTGGCCGTCGC-3′), PTPS102 (5′-CGGGATCCTATTCTCCTTTATAAACC-3′), PTPS105 (5′-TGAGCACGGAACCCGGGGGCCGTCGCTG-3′), PTPS200 (5′-TTATGATCAAGG**A**GCTGCATAATCG-3′), PTPS201 (5′-AGCACGGAAGGTGGTGG-3′); restriction sites for cloning are underlined; the delta in PTPS16 indicates the position of the triplet deletion (valine codon 57), and the boldface nucleotide in PTPS200 indicates a mismatch for site-directed mutagenesis (proline to leucine exchange). Restriction endonucleases and Moloney murine leukemia virus reverse transcriptase were from Life Technologies, Inc., and Boehringer Mannheim; *Taq* polymerase was from Promega. The oligonucleotide-directed *in vitro* mutagenesis kit, [α-^{32}S]dATP, and [^{32}P]orthophosphate were from Amersham, Corp., and the pMal-c2 expression system was purchased from New England Biolabs. The affinity-purified polyclonal antibody (SZ28) was raised in rabbits against recombinant human PTPS N-terminally fused to the maltose binding protein (MBP), as described elsewhere.[2] Ultragel AcA-44 was purchased from Pharmacia.

Fibroblast Culturing and Cell Extract Preparation—Human primary skin fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 50 units/ml of penicillin, and 50 μg/ml of streptomycin (cDMEM; Life Technologies, Inc.). Preparation of whole cell lysates for Western blot analysis and PTPS activity measurements was carried out essentially as described (10).

Mutation Analysis of Fibroblasts from Patients—Direct cDNA sequence analysis of reverse-transcribed and PCR-amplified products from previously prepared fibroblast RNA (reverse transcription-PCR sequencing) was carried out as described (10). Sequencing data were collected by using multiple, independent mixtures of PCR products as template for DNA sequencing and by analyzing always both strands with at least five independent primers.

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting—SDS-Polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Laemmli (17) in a mini-gel apparatus (Bio-Rad). As a nonreducing gel-loading buffer, a standard "Laemmli buffer" without the addition of 2-mercaptoethanol was used. The gels were either stained with Coomassie Brilliant Blue or with silver-staining procedures (18). PTPS in crude extracts was analyzed by Western blot. Proteins were blotted onto nitrocellulose sheets (Bio-Rad), incubated with the affinity-purified anti-human PTPS polyclonal antibody SZ28 (1:200 diluted) followed by a second antibody (a goat anti-rabbit IgG alkaline phosphatase conjugate from Bio-Rad), and visualized with the alkaline phosphatase conjugate substrates, nitrotetrazolium blue chloride and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (Promega).

DNA Manipulations and Construction of Expression Vectors—All numbering of human PTPS cDNA and amino acid sequences refers to the published sequence (19). To obtain a recombinant wild-type PTPS lacking the N-terminal methionine, a pMal-c2 derivative was used as described before (14). Briefly, a DNA fragment was amplified by PCR from wild-type cDNA using primers PTPS101, containing a *Sal*I site, and PTPS102, containing a *Bam*HI site. The amplified fragment was cut with *Sal*I, blunt ended by a "fill in" reaction (20), cut with *Bam*HI, and subsequently ligated into the pMal-c2 vector opened with *Xmn*I and *Bam*HI to generate pFPhum. To isolate a recombinant human PTPS lacking the N-terminal five amino acids (hPTPS$_{\Delta 1-5}$) the 5′-site of human liver cDNA was altered by PCR. The cDNA was amplified using the 5′-end primer PTPS105 containing a *Sma*I site and the 3′-end primer PTPS102 (containing a *Bam*HI restriction site). The PCR product was cut with *Sma*I and *Bam*HI and ligated into the expression plasmid pMal-c2 opened with *Xmn*I and *Bam*HI, thereby generating pHSY2002.

To express the mutant proteins R16C (C^{55} to T transition), R25Q (G^{83} to A transition), ΔV57 (triplet deletion of G^{178} to G^{180}), and P87L (C^{269} to T transition), the corresponding fragments were cloned into the pMal-c2 vector in a similar fashion as described above, using the 5′-primer PTSP101 for R25Q and ΔV57, and the 5′-primer PTPS201 for R16C and P87L. The templates for expression of the R16C and R25Q mutant proteins were pHSY27 and pHSY29, respectively, and have been described before (10). Templates for PCR amplifications to construct the corresponding expression plasmids for the mutant proteins ΔV57 and P87L were generated by site-directed mutagenesis. To perform oligonucleotide-directed *in vitro* mutagenesis, the single-stranded bacteriophage M13 derivative pHSY22, an M13 mp19 clone harboring the human liver PTPS cDNA was used as wild-type DNA template (10). Oligonucleotide PTPS200 was used for introducing the C/G^{269} to T/A^{269} transition (P87L mutant), and oligonucleotide PTPS16 was used for introducing the triplet deletion (ΔV57 mutant) into wild-type cDNA. All constructs were confirmed for correct sequences by DNA sequence analysis using the AutoRead sequencing kit in combination with an A.L.F. DNA sequencer (Pharmacia). The following pMal-c2 derivatives were generated: pHSY2003, pHSY2004, pHSY2005, and pHSY2007, expressing the mutant PTPS proteins R25Q, ΔV57, P87L, and R16C, respectively.

To express various PTPS proteins in COS-1 cells, cDNAs were cloned into the eukaryotic expression vector pSCT1 (a derivative of pSCT-GAL556X (21), having the GAL556X fragment replaced by a synthetic polylinker fragment).[3] PCR fragments were generated with the 5′-primer PTPS21 and the 3′-primer PTPS102, each containing a *Bam*HI restriction site, and subsequently cloned into the single *Bam*HI site of plasmid pSCT1. For the standard PCR reactions, the wild-type cDNA or the corresponding mutant DNAs described above were used as templates. The following pSCT1 derivatives were generated: pHSY2009, pHSY2010, pHSY2011, and pHSY2012, expressing the mutant PTPS proteins R16C, R25Q, ΔV57, and P87L, respectively. In addition, the wild-type sequence inserted in the correct orientation (pHSY2013) and in the reverse orientation (pHSY2008) were used as positive and negative controls, respectively.

Expression of PTPS in E. coli and Purification of Recombinant Proteins—The pMal-c2 derivatives, which all express a PTPS fusion protein with the MBP as an N-terminal fusion partner, were transformed into *Escherichia coli* TB1 cells, induced for expression, followed by protein purification as described previously (14).

Protein Analysis—Molecular masses of recombinant proteins purified from *E. coli* were analyzed by electrospray ionization-mass spectrometry using a PE-Sciex Api III instrument equipped with an ion spray source. Protein concentrations were determined by the Bio-Rad assay. Enzyme activities and kinetic assays were performed as described (14). For cross-linking experiments, 10 μg of PTPS proteins were incubated for 2 h at room temperature in 120 μl of phosphate buffer (8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 2.7 mM KCl, 140 mM NaCl, pH 7.4) containing 0.5 mM bis(sulfosuccinimidyl)suberate (Pierce). Reactions were stopped by adding 2.4 μl of 1 M Tris-HCl (pH 8.0), incubated for 30 min at room temperature, and separated by 12.5% SDS-PAGE. Protease cleavage of PTPS with chymotrypsin was performed as described (22), followed by separation of peptides by 17.5% SDS-PAGE. To determine heat stability of proteins, 140 ng of PTPS in 100 mM Tris-HCl (pH 7.4) was incubated for 10 min at different temperatures and chilled on ice, and then the enzymatic activity was determined as described (14).

Transient Expression in COS-1 Cells—Co-transfections of pSCT1-derivatives plus pSVβgal (expressing the bacterial β-galactosidase from the SV40 promoter) were performed by following the DEAE-dextran protocol described by Stratagene, with the following minor modifications: COS-1 cells were treated with the DNA mixture (each plasmid 1

[2] A. Résibois, D. Bürgisser, B. Thöny, and C.W. Heizmann, manuscript in preparation.

[3] M. Pedrocchi, personal communication.

TABLE I
Summary of activities and mutations in fibroblasts of four PTPS-deficient patients

Patient (variant)	PTPS activity in fibroblasts[a]	Mutation on DNA level[b]	Predicted amino acid exchange
U.T.[c] (central)	<1%	G^{83} to A (homozygous)	Arg 25 to Gln (R25Q)
J.R.S.[c] (peripheral)	1%	C^{55} to T	Arg 16 to Cys (R16C)
		ΔC^{370} to C^{383}	Lys 120 → Stop (K120X)
L.L.[d] (central)	<1%	ΔG^{178} to G^{180}[e]	ΔVal 57 (ΔV57)[e]
		ΔT^{173} to G^{195}	Lys 54 → Stop (K54X)
S.S.[d] (central)	<1%	C^{269} to T (homozygous)	Pro 87 to Leu (P87L)

[a] Activities in percentage of normal wild-type control.
[b] Numbering of nucleotides refers to the published cDNA sequence by Thöny *et al.* (19); Δ, deletion.
[c] Mutations of patients U.T. and J.R.S. were determined by Thöny *et al.* (10).
[d] Patients' primary fibroblasts supplied by the clinicians; see Oppliger *et al.* (11).
[e] Identical with a deletion G^{175} to G^{177} (ΔV56) due to two consecutive identical codons.

μg) for 15 min, gently rinsed with PBS, treated for 2 min with 15% glycerol/PBS, and rinsed again with PBS, followed by addition of fresh medium (cDMEM) to allow growth under optimal culture conditions. Activity of the reporter gene (β-galactosidase from pSVβgal) was assayed as described in a protocol by Promega and normalized for comparing activity of various expressed PTPS proteins in COS-1 cells.

Labeling of COS-1 Cells with ^{32}P-Orthophosphate and Immunoprecipitation of PTPS from Cell Lysates—COS-1 cells transfected with 2 μg of single plasmid pSCT1-derivatives expressing the various forms of PTPS (pHSY2008–2013; see above) were first grown for 2 days in cDMEM in a 100-mm Petri dish and afterwards supplemented for 4 h with 50 μCi of [^{32}P]orthophosphate/ml of phosphate-free DMEM (Life Technologies, Inc.). Cells were then aspirated and rinsed with PBS, followed by lysis (0.5 ml/dish of PBS containing 1.5 mM $MgCl_2$, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 34 μg/ml of phenylmethylsulfonyl fluoride, 0.7 μg/ml pepstatin, 5 μg/ml leupeptin) and immunoprecipitation with protein A-Sepharose (20 μl of slurry/dish) plus the affinity-purified rabbit anti-human PTPS antibody, SZ28, according to standard protocols (23). Precipitates were separated by SDS-PAGE followed by autoradiography.

Homology Modeling of Human PTPS—Computer modeling was performed with the O molecular graphics program (24).

RESULTS

The aim of this study was to characterize four mutant PTPS proteins deduced from DNA mutation analysis from four patients with the recessive inherited disorder of BH_4 deficiency. A description of the patients and primary fibroblast cell lines is summarized in Table I. More detailed information regarding case reports and clinical symptoms of these patients has been the subject of previous publications (see references in Table I).

Examination and Mutation Analysis of Primary Skin Fibroblasts from PTPS-deficient Patients—PTPS activity from four patients and a healthy control (B. H.) was measured in primary skin fibroblasts (Table I). The patients with the peripheral (J. R. S.), or central type (U. T., L. L., S. S.) of PTPS deficiency showed enzyme activity of 1% or below, respectively, as compared with wild-type activity.

While mutations in the two patients U. T. and J. R. S. had been screened and published before (Ref. 10; see Table I), mutation analysis on cDNA of cells derived from L. L. and S. S. was done during this work. In all cases reverse transcription-PCR analysis using PTPS-specific primers was performed starting from total RNA isolated from cultured primary cells. PCR products were subjected to direct sequence analysis (for experimental details see Ref. 10). Patient L. L. showed on one allele a deletion from G^{178} to G^{180}, thus creating an in-frame deletion of valine codon 57 (ΔV57). The other allele had a deletion of 23 nucleotides extending from T^{173} to G^{195}, leading to a frameshift after lysine codon 54 and subsequent premature stop codon. The putative truncated protein resulting from this allele was named K54X. Patient S. S. had a homozygous C^{269} to T transition, thereby expressing a putative mutant protein having the proline at position 87 replaced by a leucine (P87L; see also Fig. 1).

Immunoreactive material in crude extracts from these fibroblasts was investigated using an affinity-purified anti-human PTPS antibody, SZ28 (Fig. 2). Western blot analysis showed two bands, an upper one at 16 kDa and another band at ~14 kDa. The 16-kDa band is clearly visible in extracts from B. H. (wild-type control) and patient J. R. S. and faintly visible from U. T.; whereas no 16-kDa band is present in extracts from S. S. and L. L. The relative intensity of the 14-kDa band is slightly stronger in all patients as compared with the healthy control. In order to offer some explanations for the nature of the two bands, this pattern was further investigated by first comparing the band sizes with that of the recombinant wild-type PTPS purified from *E. coli* (see below). As can be seen from Fig. 2, the recombinant PTPS comigrated with the upper, 16-kDa band. In another series of experiments, we prepared crude extracts in the *absence* of any protease inhibitors from healthy control cells and tested for the persistence of the two bands by Western blot and for PTPS activity. The two immunoreactive bands remained stable and were still detectable after keeping the extracts for 16 h at room temperature. In parallel, PTPS activity dropped marginally to 63% of the initial activity after 16 h of incubation (data not shown). We also transferred the wild-type PTPS-cDNA by using a retroviral vector into primary fibroblasts from patient S. S. and subsequently regained BH_4 biosynthetic activity. Upon examination by Western blot analysis of crude extracts from these cells, the 16-kDa *and* 14-kDa bands were visible only in cells with restored activity, thus suggesting the upper band was responsible for PTPS activity.[4] From these observations we conclude that the 16-kDa band represents the active form of PTPS and that the 14-kDa band is most probably not a simple protease degradation product of PTPS but rather an unknown protein that interacts with this purified antiserum. Nevertheless, these experiments do not exclude the possibility that the 16-kDa band may represent a modified form of PTPS (see below).

Expression and Purification of Recombinant Wild-type and Mutant PTPS—The mutant enzyme inferred from the 14-base pair deletion in one allele from patient J. R. S., K120X, has previously been shown by recombinant expression to be completely inactive due to the large C-terminal truncation (10). Since it was expected that the mutant protein K54X (patient L. L.) has no activity as well, we did not attempt to further characterize these forms of PTPS.

We overexpressed and purified wild-type and the four mutant PTPS alleles R16C, R25Q, ΔV57, and P87L. The pMal-c2 vector was manipulated in order to express recombinant MBP-

[4] B. Thöny, W. Leimbacher, H. Stuhlman, C. W. Heizmann, and N. Blau, manuscript in preparation.

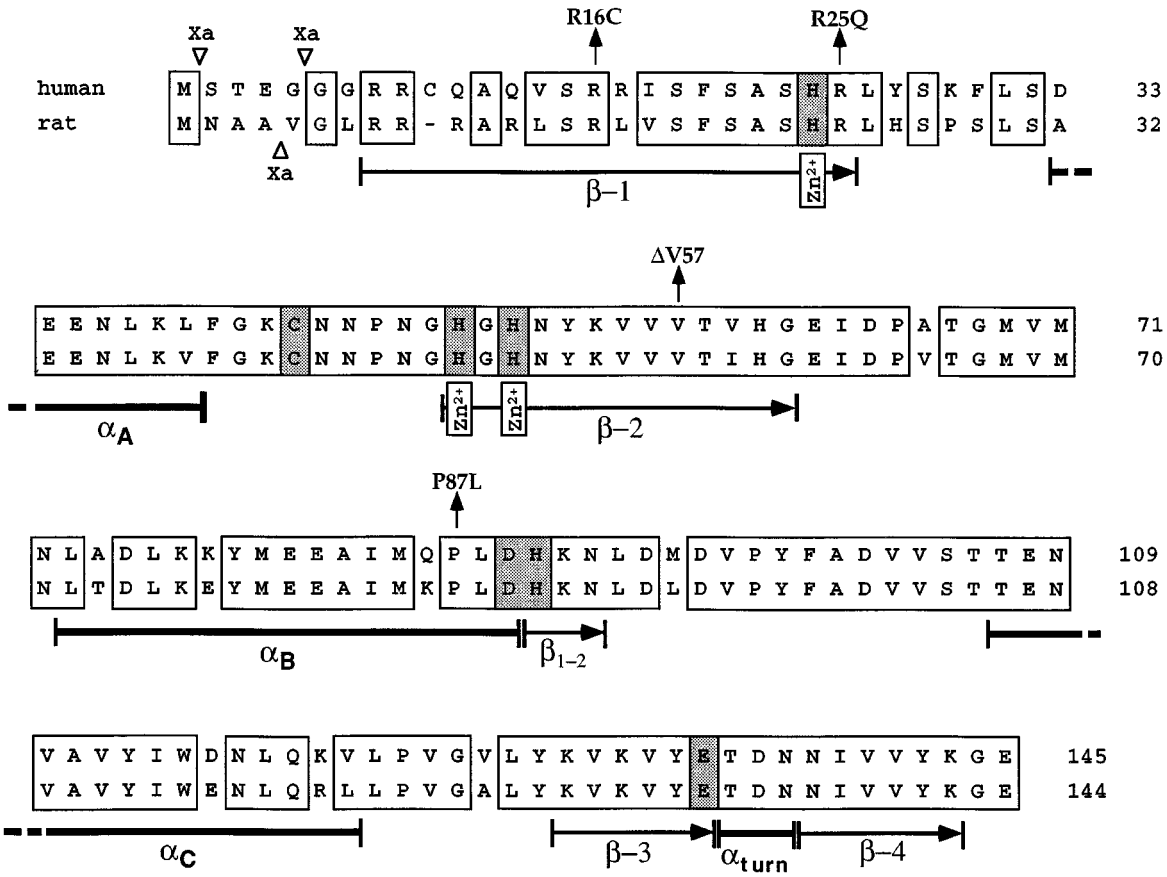

FIG. 1. **Fold of the human and rat PTPS monomers.** The amino acid sequences of human and rat PTPS are *aligned*, and identical amino acid residues or conservative valine-leucine-isoleucine substitutions are *boxed*. Extensions of the secondary structural elements deduced from the solved crystal structure of rat PTPS are indicated, and numbering of α-helices and β-strands is given *below* the amino acid sequence. Residues directly participating in the active center and/or Zn^{2+}-ion binding are dotted (His^{23}, Cys^{42}, His^{48}, His^{50}, Asp^{88}, His^{89}, and Glu^{133} for the rat monomer). Single amino acid exchanges found in human mutant PTPS that are described in this work are indicated by *arrows* (R16C, R25Q, ΔV57, and P87L). Positions of introduced factor Xa protease recognition sites for cleavage of PTPS from the N-terminal MBP partner following purification of recombinant MBP-PTPS fusion proteins are marked by *triangles*.

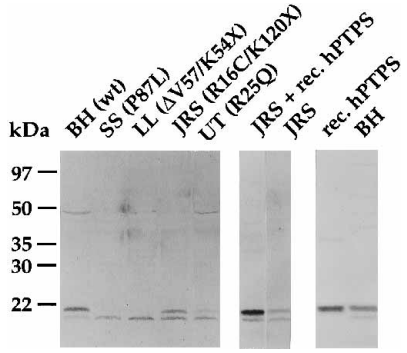

FIG. 2. **Immunodetection of PTPS in primary fibroblasts from various patients.** Western blot analysis of crude extracts from cultured fibroblasts is shown (see "Experimental Procedures"). Proteins were separated under reducing conditions by 12.5% SDS-PAGE. Each *lane* contains approximately 0.1 mg of total protein. Names of patients and predicted amino acid exchanges for PTPS are indicated for each *lane* (see also Table I). Purified human recombinant PTPS (1 ng) was added to crude extracts from patient J. R. S. (*JRS + rec. hPTPS*) or loaded on a separate *lane* (*rec. hPTPS*). Cross-reacting material was detected by using the affinity-purified rabbit anti-human PTPS antibody SZ28. *BH*, wild-type control; *SS*, *LL*, *JRS*, and *UT*, patients.

PTPS protein fused by a factor Xa protease recognition site (tetrapeptide IEGR; for details see "Experimental Procedures"). The MBP-PTPS proteins were affinity-purified, followed by protease factor Xa treatment, and were subsequently separated from the MBP fusion partners as described (14). All PTPS proteins purified by this procedure had the N-terminal sequence NH_2-STEGGGR (with the exception of $hPTPS_{\Delta 1-5}$; see Fig. 1) and were checked by SDS-PAGE analysis (Fig. 3) and electrospray mass spectrometry.

The measured average compound masses of the wild-type (16,255.08 ± 3.71) and mutant proteins R16C (16,199.9 ± 1.42) and R25Q (16,224.24 ± 8.63) were in good agreement with the calculated masses of 16,256 (wild-type), 16,203 (R16C), and 16,228 (R25Q), respectively. Wild-type PTPS revealed the monomeric as well as the dimeric forms when inspected by SDS-PAGE under reducing conditions, as shown before (14). The same was true for the mutants with single amino acid exchanges, R16C, R25Q, and P87L. The additional bands below the 16.3-kDa monomer of mutant P87L were repeatedly found and are due to moderate proteolytic degradation in the course of purification. Separation by SDS-PAGE under nonreducing conditions revealed for P87L the presence of a single monomeric band, two dimeric bands, and some bands with higher molecular weight (not shown). These observations were partially confirmed by mass spectrometry analyses, where three major peaks of averaged compound masses were detected, one of 32,541 ± 6.15 which is the dimer of two full-length monomers (calculated mass of 32,544) and a second peak of 30,966.11 ± 2.76, which is interpreted as a heterodimer of a full-length protein and a putative truncated form lacking the first 15 amino acids (calculated mass of the heterodimer is 30,970; putative PTPS derivative starting with ^{17}RISFSA; see Fig. 1). A third peak with the mass of 29,391.94 ± 4.68 might

be due to dimer formation of the N-terminally truncated form (calculated mass of 29,395). Taken together, wild-type, R16C, and R25Q exhibit a very similar behavior upon overexpression and purification, with a yield of ~50 mg of protein from 0.5 liter of *E. coli* culture. Although overexpression of the P87L allele yielded the same amount of protein, this mutant exhibited a higher sensitivity with regard to degradation during the purification procedure.

A completely different behavior was observed for mutant ΔV57. In comparison with wild-type PTPS, this mutant protein eluted earlier from the gel filtration column and was poorly cleaved by factor Xa protease, and only a minor fraction was recovered upon size exclusion followed by affinity chromatography. Consistent with these observations, purified ΔV57 appears as a smear on the SDS-gel, with a major fraction migrating as monomer plus several different aggregation and/or degradation products (see Fig. 3). Thus, mutant ΔV57 appears not to be stable as a hexamer and has a tendency to aggregate into higher molecular structures because it is probably not correctly folded during the biosynthesis of the recombinant fusion protein or after cleavage from the MBP partner. Since purification of this mutant protein ended up repeatedly with a poor yield, we did not perform mass spectrometry analysis.

Kinetic Characterization of the PTPS Enzymes—The kinetic parameters of wild-type and mutant enzymes were examined using 7,8-dihydroneopterin triphosphate as substrate (Table II). The specific activity and apparent K_m value of recombinant wild-type PTPS was 120 ± 8.5 milliunits/mg and 11.6 ± 2.4 μM, respectively, comparable with the specific activity of 100 milliunits/mg and a K_m of 10 μM determined for the human PTPS isolated from liver biopsy (25). The recombinant mutant enzymes R16C, R25Q, and P87L had reduced but, to our surprise, significant activity compared with wild-type PTPS, *e.g.* R25Q exhibited over 60% of wild-type activity. In contrast, ΔV57 had no activity. The K_m value of P87L was similar to that of wild-type, whereas for R16C and R25Q a 2–3-fold increase in K_m was observed. Thus, substrate binding for these two enzymes seems to be lowered. The turnover numbers for mutants R16C, R25Q, and P87L were altered by a factor of 1.5–3, reflecting a marginal reduction of enzymatic efficiency.

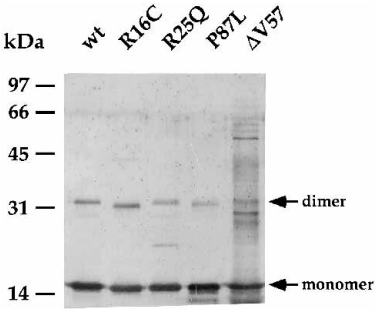

FIG. 3. **Electrophoretic analysis of purified recombinant wild-type and mutant PTPS.** Silver staining of 1.5 μg of wild type (*wt*) and of each mutant PTPS (R16C, R25Q, P87L, and ΔV57) was performed after separation of proteins by 12.5% SDS-PAGE in the presence of 2-mercaptoethanol (5% v/v) in the loading buffer. The *arrowheads* indicate the position of the 16.3-kDa monomer, and of the 32.5-kDa dimeric form of PTPS.

Bearing in mind the very low activities detected in primary fibroblasts from patients U. T., J. R. S., and S. S. (≤ 1%; see Table I), it was unanticipated to find such significant activities for the overexpressed alleles R25Q, R16C, and P87L, respectively. These mutant alleles needed to be further characterized in order to explain the different PTPS activities between fibroblasts *in vivo* and recombinant enzymes *in vitro*.

Investigations of the Folding and Stability of Wild-type and Mutant PTPS—Next, we examined hexamer assembly, protease sensitivity, and thermostability under *in vitro* conditions as a means to investigate correct folding of wild type and the mutationally altered PTPS. Protein cross-linking in solution, followed by SDS-PAGE analysis, was performed to see whether the mutant enzymes assemble spontaneously into dimer, trimer, and hexamer, in a similar fashion as the wild-type enzyme does (14). As shown in Fig. 4*A*, the multimer pattern of R16C, R25Q, and P87L was indistinguishable from that of the wild-type. As expected from results of previously performed gel filtration and SDS-gel analyses, ΔV57 did not form these homomultimers in solution, supporting the conclusion that this mutant may not be correctly folded.

We then analyzed the degradation pattern of the three mutant proteins with single amino acid exchanges following incubation with chymotrypsin (Fig. 4*B*). Upon protease digestion for 60 min, only P87L showed a relatively enhanced degradation pattern by chymotrypsin, thus indicating a reduced protein stability due to differential sensitivity to unfolding.

Since it was described earlier that PTPS is relatively heat-stable (25), susceptibility to heat pretreatment of mutant proteins R16C, R25Q, and P87L was assayed and compared with wild-type activity (not shown). Whereas wild type, R16C, and R25Q had still more than 80% of their initial activities following incubation at 50 °C, a different behavior was observed for P87L. For this mutant, activity dropped very sharply already when incubated at 50 °C, specifying enhanced thermolability at least under *in vitro* conditions.

Examination of Wild-type and Mutant Alleles Expressed in COS-1 Cells—To determine enzyme activities of single mutant proteins in a eukaryotic cell background, the different alleles were transfected into COS-1 cells for transient expression. The eukaryotic expression plasmid pSCT1 (see "Experimental Procedures") was manipulated to harbor either wild-type or mutant cDNAs encoding the R16C, R25Q, ΔV57, or P87L forms of PTPS. Enzyme activity was measured in a standard assay using the COS-1 cell extracts, plotted in percentage of wild-type activity, and compared with the relative activity measured for purified enzymes from *E. coli* (Fig. 5*A*). Activities were normalized to β-galactosidase activity expressed from a co-transfected plasmid, and expression was checked by Western blot analysis of crude extracts using the polyclonal rabbit anti-human PTPS antibody (SZ28; Fig. 5*B*). The background PTPS activity in COS-1 cells was below the detection minimum (<0.1

TABLE II
Kinetic parameters of wild-type PTPS and its mutant derivatives

Protein	Specific activity[a,b]	Turnover number	K_m[b]	Relative activity
	milliunits/mg	s^{-1}	μM	*% of wild type*
Wild type	120.0 ± 8.5	0.032	11.6 ± 2.4	100 ± 7
R16C	44.0 ± 3.0	0.012	30.8 ± 0.7	37 ± 3
R25Q	73.5 ± 2.5	0.020	30.5 ± 2.6	61 ± 2
ΔV57	≤0.5	ND	ND	<0.1
P87L	36.0 ± 4.5	0.010	13.0 ± 7.6	30 ± 4

[a] One unit of enzyme activity catalyzes the production of 1 μmol of BH_4/min at 37°C.
[b] The mean values and standard deviations were determined from three independent experiments. ND, not determined.

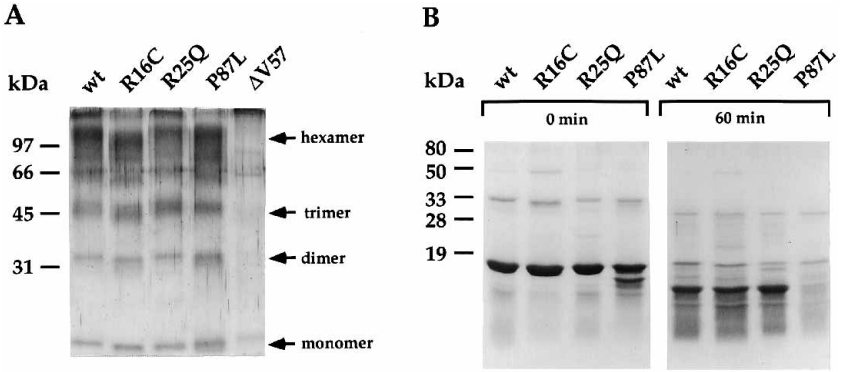

FIG. 4. **SDS-PAGE of purified wild-type and mutant PTPS upon (*A*) cross-linking in solution and (*B*) chymotrypsin digestion.** *A*, 1.6 μg of each recombinant protein, treated with the cross-linker bis(sulfosuccinimidyl)suberate in solution, was separated by 12.5% SDS-PAGE under reducing conditions, and silver-stained to visualize multimerization of PTPS monomers. *B*, 11.6 μg of the different PTPS proteins were untreated (0 min) or treated (60 min) with chymotrypsin, separated by 17.5% SDS-PAGE, and stained with Coomassie Brilliant Blue.

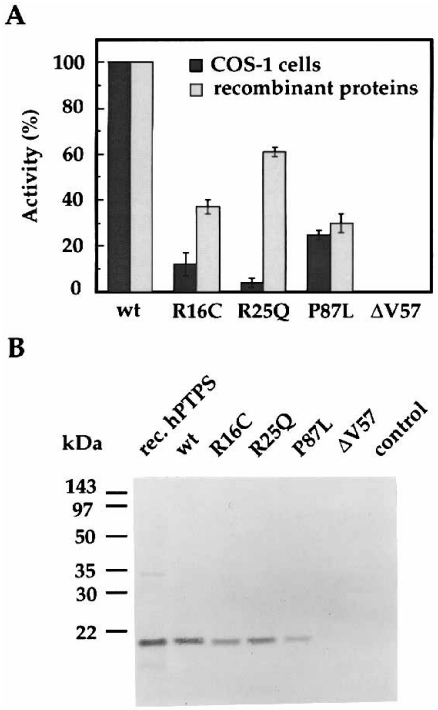

FIG. 5. **Relative activities of recombinant proteins and of transfected alleles in COS-1 cells (*A*) and visualization of PTPS cross-reacting material in crude extracts from the transfected COS-1 cells (*B*).** *A*, PTPS activity was measured of affinity-purified PTPS proteins isolated from *E. coli* (*open bars*), and crude extracts from transiently transfected COS-1 cells expressing various PTPS alleles (*filled bars*). *B*, Western blot analysis of crude extracts from COS-1 cells expressing the indicated PTPS proteins used in *panel A*. The amount of total protein loaded in each *lane* was normalized to equal amounts of β-galactosidase activity (see "Experimental Procedures"). *control*, COS-1 cell extract containing the wild-type PTPS in the reverse orientation (pHSY1008).

microunits/mg), and no cross-reacting material was visible by Western blot analysis of extracts, thus offering COS-1 cells as a useful system to determine activity of single PTPS alleles. Except for P87L, all mutant alleles had significantly reduced PTPS activities in COS-1 cells as compared with wild-type activity. Mutant P87L exhibited a marginal reduction of activity to 26%, similar to the relative activity found for the purified protein (30%). Upon examination by Western blot, only ΔV57 seemed to be rapidly degraded in COS-1 cells because it was not visible in the extracts tested, whereas from all other alleles a single band at 16 kDa was present. The *relative* reduction of activity of mutants R16C and R25Q in COS-1 cells as compared with the corresponding mutant alleles purified from *E. coli* suggested the potential occurrence of some eukaryotic cell-based modification(s) that does not take place in the *E. coli* background and might activate the wild-type PTPS. Inspection of the primary amino acid sequence of PTPS revealed the presence of several consensus sequences for potential phosphorylation by protein kinases (see also "Discussion"). Interestingly, the two arginine mutants, R16C and R25Q, are both located in an amino acid motif R*XX*S*, representing the consensus recognition sequence for several protein kinases that phosphorylate the serine residue (asterisk; see also Fig. 1) (26). Further experiments to investigate potential phosphorylation were thus performed.

^{32}P-Phosphorylation of PTPS in COS-1 Cells—COS-1 cells transfected for transient expression with the different PTPS alleles (see above) were labeled with [^{32}P]orthophosphate, and PTPS was immunoprecipitated with the affinity-purified antibody SZ28 and analyzed by 12.5% SDS-PAGE. After Coomassie Blue staining, only a monomeric band at 16 kDa was additionally visible for wild type, R16C, R25Q, and P87L, as compared with the untransfected COS-1 control cell lysate and ΔV57 (Fig. 6*A*). The absence of ΔV57, possibly due to rapid degradation, is consistent with the Western blot analysis of COS-1 cell extracts shown in Fig. 5*B*. Upon autoradiography of the gel, radioactively labeled PTPS monomers were specifically visible for wild type, as well as for R25Q and P87L (Fig. 6*B*). No ^{32}P modification was observed for R16C, suggesting that Arg^{16} might be part of the protein kinase recognition motif ^{16}RRIS (Fig. 1) that is perturbed by the amino acid exchange of arginine to cysteine. Therefore, serine 19 may not be phosphorylated. In contrast, Arg^{25} appears not to be required for phosphorylation, since mutant R25Q was labeled with ^{32}P as well as wild-type PTPS at the putative Ser^{19} site. Although we cannot yet rule out the possibility that wild-type PTPS is phosphorylated at multiple sites, another type of modification may be prevented by the arginine 25 to glutamine substitution that would explain the observed differences in activity shown in Fig. 5*A* (see "Discussion").

Structure of the Human Wild-type and Mutant PTPS—A prerequisite for predicting the structural consequences of single amino acid alterations is knowing the three-dimensional structure of human PTPS. A comparison of the human and rat PTPS sequences revealed that the most variable patch is located at the N terminus. In the rat PTPS x-ray crystal structure, the N terminus is not defined and, therefore, is probably not an integral part of the subunit fold (15). Thus, we attempted to crystallize two different recombinant human PTPS, one having only the N-terminal methionine deleted and another one lacking the N-terminal five amino acids (hPTPS$_{\Delta 1-5}$;

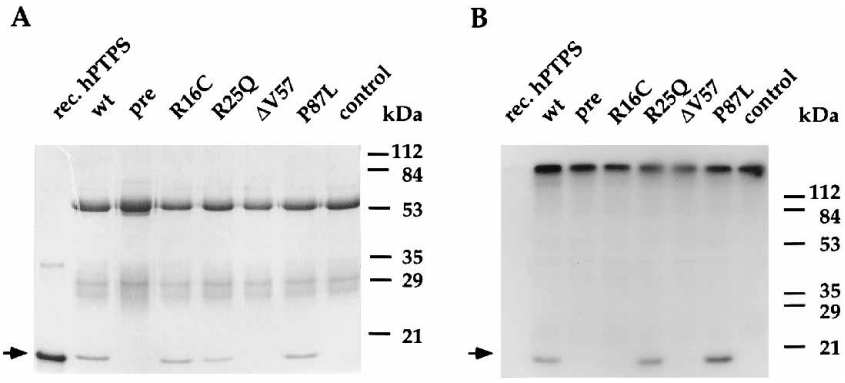

FIG. 6. **Immunoprecipitation of phosphorylated human PTPS expressed in COS-1 cells.** *A*, transiently transfected COS-1 cells expressing the indicated forms of PTPS were labeled with [^{32}P]orthophosphate, immunoprecipitated with the anti PTPS affinity-purified antibody (SZ28), separated by 12.5% SDS-PAGE, and stained with Coomassie Blue. The following controls were loaded: 5 μg of recombinant wild-type PTPS purified from *E. coli* (*rec. hPTPS*), lysate treated with the preimmune serum (*pre*), and precipitate from COS-1 cells harboring pHSY2008, containing the wild-type PTPS sequence in the reverse orientation (*control*). *B*, autoradiography of the same gel as in *panel A* showing the *in vivo* ^{32}P-labeled wild-type (*wt*), R25Q, and P87L PTPS. The *arrowhead* indicates the position of the PTPS monomer.

see Fig. 1); however, these two active, human forms were refractory to crystallization.

In order to obtain a *model* of human PTPS, we started out with the structure of a rat liver PTPS subunit and replaced the relevant 15 amino acids using a molecular graphics system (24). The modeling was restricted to the positioning of amino acid side chains in sterically reasonable conformations. We did not try to minimize the resulting structure, because the modeling did not produce strains in the structure with regard to the modified side chain positions. The following description of the mutation sites is based on the model of human PTPS (see Figs. 1 and 7).

The side chain of residue Arg^{16} lies at the entrance to the putative active site pocket and could possibly bind the triphosphate moiety of the substrate during catalysis. Since we do not know the exact nature of this interaction, it was impossible to estimate from modeling the effect of the R16C mutation. The distance of Cys^{16} to the active site residue Cys^{43} is 12 Å, which makes a disulfide bond formation unlikely.

Arg^{25} is located close to the hexamer equator region on the protein surface and is only sequentially close to the Zn^{2+}-binding residue His^{24}. Mutant R25Q should exhibit no gross structural differences to wild-type PTPS and should behave accordingly in catalysis.

Val^{57} is located on β-strand 2 and points toward the hydrophobic interior of the subunit. In the deletion mutant ΔV57, the in-out β-strand pattern is perturbed, which definitely will affect protein folding. Indeed, we did not see expression of a correctly folded mutant protein.

Helix α_B is an irregular four-turn helix in that it contains a kink between the third and fourth turns. The latter is enlarged due to the breakage of the H-bonding pattern through the presence of Pro^{87}. Exchange of this residue into leucine might lead to structurally localized conformational changes, *e.g.* formation of a regular four-turn helix. This could have an effect on the positions of Asp^{89} and His^{90}, which are involved in an intersubunit catalytic triad and thus implicated in the catalytic process (16).

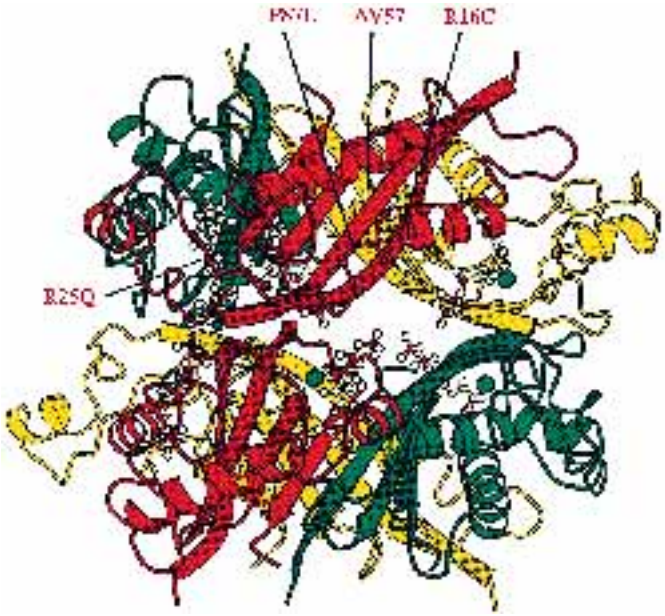

FIG. 7. **Stereo picture of the human PTPS hexamer.** The subunits are shown in a ribbon-type presentation from the N-terminal residue 8 to the C-terminal amino acid 145. The monomers of each trimer are shown in *different colors*. The positions of the amino acids affected in the mutants that were detected and analyzed are indicated for one monomer in *red*. The positions of the relevant active centers are indicated by the *green* spheres that represent the Zn^{2+}-ions. The 7,8-dihydroneopterin substrate located in each active center is shown in a *stick and ball* representation.

DISCUSSION

Although PTPS deficiency is well characterized in terms of screening for metabolites based on the established BH_4 biosynthesis pathway, the molecular consequences *in vivo* of mutant PTPS, and the different phenotypes of PTPS deficiencies are not understood. Here we expressed recombinantly and characterized four naturally occurring mutant PTPS with single amino acid alterations that were detected in the PTPS-cDNA from four patients with hyperphenylalaninemia due to BH_4 deficiency. Following, we will first discuss some aspects of human wild-type PTPS in comparison with the rat enzyme, followed by the consequences of the single amino acid exchanges and implication of *in vivo* PTPS phosphorylation.

Structural prediction of the human PTPS by homology modeling is based on the high conservation of the primary sequence as compared with the rat enzyme. Moreover, human and rat PTPS share not only the homohexameric property as shown experimentally, but also all the residues involved in active site formation and Zn^{2+}-ion binding (Fig. 1) (15, 16, 19). The only distinction is located at the very N-terminal part of the proteins, which was not determined in the rat x-ray crystal structure (15). Nonetheless, attempts to crystallize the recombinant 144-amino acid-containing human PTPS counterpart failed. We also expressed and tried without success to crystallize a

truncated form of the active human enzyme, $hPTPS_{\Delta 1-5}$. The rationale behind expressing this truncated version of PTPS was 2-fold. On the one hand, we argued that a structurally nonlocalized domain such as the N terminus may prevent hexamer assembly and growth of crystals. On the other hand, we performed a computer search with the human PTPS for potential protein patterns by using the PROSITE program (Wisconsin GCG software, version 8). As a result of this pattern search, a myristoylation site was predicted at glycine residue 6. However, an internal glycine as a potential *N*-myristoylation site would require a proteolytic processing to expose the Gly^6 as the N-terminal residue (27, 28). The rat PTPS isolated from liver tissue was shown to be N-terminally truncated by having cleaved off the first four amino acids and therefore exposing a valine at the N terminus (29), which is not a substrate for *N*-myristoylation. Whether the differences in the amino termini between the human and rat enzyme have some functional meaning and whether the human PTPS undergoes proteolytic processing too, followed by potential modification(s), remains to be clarified. In this context it is worth mentioning that the cysteine residue only present in the human protein sequence at position 10 has been shown not to influence *in vitro* enzyme activity by assaying a PTPS mutant with a cysteine to alanine substitution.[5]

Only those PTPS mutants with a single amino acid difference as compared to wild-type PTPS were chosen for recombinant expression and characterization, since the C-terminally truncated allele K120X (patient J. R. S.) has previously been shown to be completely inactive, and therefore K54X (patient L. L.) must be inactive as well (10). Overexpression and purification of the second allele from patient L. L., ΔV57, revealed that this mutant form is incorrectly folded, and it was expected to be unstable *in vivo*. Structure prediction and biochemical and enzymatic tests, as well as the COS-1 cell expression, are in agreement with the absence of any cross-reactive 16-kDa PTPS in primary fibroblasts from patient L. L. due to rapid degradation of ΔV57 (Fig. 2).

Patient S. S. with the homozygous P87L allele exhibits no 16-kDa cross-reactive PTPS in primary fibroblasts, and consistently weaker bands were observed with Western blots from extracts of overexpressing COS-1 cells (Fig. 5*B*). This suggests that a Pro^{87} exchange to leucine induces modest instability, and P87L is thus more sensitive to degradation *in vivo*. A structurally localized conformational change was predicted from computer modeling. Experimental evidence for local differences in tertiary structure is the altered protease digestion pattern (Fig. 4*B*) and the enhanced sensitivity to thermal denaturation of the otherwise normal hexameric mutant protein. Interestingly, the P87L mutant has significant enzymatic activity as recombinant protein *in vitro* as well as in COS-1 cells (Fig. 5*A*) and is phosphorylated in the eukaryotic cell background. In contrast, the activity measured in fibroblast extracts prepared from patients' primary cells was below the level of detection. One explanation for this discrepancy is that a slightly altered susceptibility to protease due to the single Pro^{87} to leucine substitution leads to an enhanced degradation *in vivo*, which is not compensated by the *de novo* biosynthesis of this mutant PTPS. Further investigations toward characterizing the Pro^{87} function in human PTPS will be of interest at least due to the fact that from eight PTPS-deficient patients analyzed for DNA mutations so far, four patients where found to have one or both alleles with alterations at the Pro^{87} site (11, 30).

The arginine 16 and 25 substitutions, found in patients J. R. S and U. T., respectively, are the most surprising PTPS alleles in terms of *in vivo versus in vitro* enzymatic activities. In primary fibroblasts, R25Q seems to be less stable than R16C and wild-type PTPS, based on Western blot analyses, whereas all three proteins are equally stable when expressed in COS-1 cells. Both recombinant arginine mutants exhibit similar biochemical properties as compared with the purified wild-type PTPS, including the significant activity in the *in vitro* assay with the substrate 7,8-dihydroneopterin triphosphate (Fig. 5*A*). Only substrate binding seems to be somewhat lowered in these two mutants as revealed by the increased K_m. However, in fibroblasts as well as in the COS-1 cells, the activity of both arginine mutants is absent or drastically reduced. This suggested the occurrence of protein modification(s) of wild-type PTPS that takes place in a eukaryotic cell background only and is impaired in these two mutant PTPSs. Modification by protein phosphorylation was an attractive hypothesis, because the other BH_4 biosynthesis enzymes, GTP cyclohydrolase I and sepiapterin reductase, have recently been shown to be phosphorylated (31, 32). Moreover, the residues Arg^{16} and Arg^{25} are both located in potential consensus recognition sequences for various kinases (26); *i.e.* residue Arg^{16} overlaps with the motif R*XX*S*, and Arg^{25} overlaps with the motifs R*XX*S* and S**X*R, where the asterisk indicates the phosphoserine (see also Fig. 1). Upon *in vivo* incubation with [^{32}P]orthophosphate, wild-type PTPS and the R25Q were found to be phosphorylated, whereas the R16C mutant was not modified by ^{32}P. This has several implications. Since the R16C mutant appears not to be a substrate for phosphorylation in COS-1 cells and the ^{16}RRIS site is the only motif that overlaps with a common consensus phosphorylation site (R*XX*S*) described for many kinases (26), we conclude that serine 19, which is located within this motif and is on the protein surface (not shown), must be the site of phosphorylation. Direct proof for this has yet to be provided, especially since *in vitro* attempts to phosphorylate with various commercially available protein kinases, including cAMP-dependent protein kinase, calmodulin-dependent protein kinase, and protein kinase C, revealed that purified wild-type PTPS is *not* a substrate for any of these enzymes.[6] All these protein kinases tested have the common consensus phosphorylation site R*XX*S*, which overlaps with the ^{16}RRIS site altered in mutant allele R16C. Another consequence of modification by P_i, as we speculate, is that the phosphorylated form of PTPS might be more active than the nonmodified protein. This is not only based on our measurements with *in vitro* purified enzymes compared with activity in COS-1 cells (Fig. 5*A*), but it is also suggested by Zhu *et al.* (33). In their report, they treated cultured rat dopamine neurons with 8-bromo-cAMP, which stimulated BH_4 biosynthesis. This observation led them to suggest that BH_4 metabolism might be regulated through phosphorylation via cAMP-dependent protein kinase. Since GTP cyclohydrolase I, the first enzyme for BH_4 biosynthesis, has no consensus sequence for this kinase, PTPS was speculated to be a substrate for the cAMP-dependent protein kinase due to the presence of such consensus sites (33). Although based on our observations up-regulation of PTPS through phosphorylation is also favored, the cAMP-dependent protein kinase seems not to be the *directly* interacting kinase, because PTPS was not labeled with ^{32}P by this kinase in *in vitro* assays (see above). Thus, the corresponding protein kinase that phosphorylates wild-type PTPS remains to be identified.

The present results are complicated by the observation that,

[5] T. Oppliger, B. Thöny, D. Bürgisser, C. W. Heizmann, and N. Blau, unpublished observation.

[6] T. Oppliger, B. Thöny, C. W. Heizmann, and N. Blau, unpublished results.

although Arg^{16} is needed in PTPS for modification such as phosphorylation, this modification may not be sufficient, as shown by the inactive R25Q mutant that was found to be labeled with ^{32}P, presumably at Ser^{19}. Arg^{25} is located in the motifs SHR^{25} and $^{25}RLYS$ (Fig. 1) with the potential corresponding phosphorylation sites serine 23 and/or serine 28, respectively. Although we cannot completely rule out additional phosphorylation at sites other than the putative phosphoserine at position 19, it seems unlikely that mutant R25Q is partially phosphorylation-defective. Additional phosphorylation would imply that the presence of Arg^{16} is a prerequisite for the phosphorylation of Ser^{23} and/or Ser^{28}, because R16C was shown not to be labeled at all with ^{32}P. We postulate that other not yet specified post-translational modification(s) and/or interactions of wild-type PTPS may be required for maximal *in vivo* activity and stability. Such additional alterations or interactions are somehow prevented by the absence of Arg^{25} and not only lead to instability *in vivo* but also render PTPS completely inactive. We are currently testing various possibilities for additional post-translational modifications of PTPS.

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