Serine 19 of Human 6-Pyruvoyltetrahydropterin Synthase Is Phosphorylated by cGMP Protein Kinase II*

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6-Pyruvoyltetrahydropterin synthase (PTPS) participates in tetrahydrobiopterin cofactor biosynthesis. We previously identified in a PTPS-deficient patient an inactive PTPS allele with an Arg16 to Cys codon mutation. Arg16 is located in the protein surface exposed phosphorylation motif Arg16-Arg-Ile-Ser, with Ser19 as the putative phosphorylation site for serine-threonine protein kinases. Purification of recombinant PTPS-S19A from bacterial cells resulted in an active enzyme (kcat/Km = 6.4 × 10^8 M⁻¹ s⁻¹), which was similar to wild-type PTPS (kcat/Km = 4.1 × 10^8 M⁻¹ s⁻¹). In assays with purified enzymes, wild-type but not PTPS-S19A was a specific substrate for the cGMP-dependent protein kinase (cGK) type I and II. Upon expression in COS-1 cells, PTPS-S19A was stable but not phosphorylated and had a reduced activity of ~33% in comparison to wild-type PTPS. Extracts from several human cell lines, including brain, contained a kinase that bound to and phosphorylated immobilized wild-type, but not mutant PTPS. Addition of cGMP stimulated phosphotransferase activity 2-fold. Extracts from transfected COS-1 cells overexpressing cGKII stimulated Ser19 phosphorylation more than 100-fold, but only 4-fold from cGKII overexpressing cells. Moreover, fibroblast extracts from mice lacking cGKII exhibited significantly reduced phosphorylation of PTPS. These results suggest that Ser19 of human PTPS may be a substrate for cGKII phosphorylation also in vivo, a modification that is essential for normal activity.

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* The abbreviations used are: BH₄, (6R)-5,6,7,8-tetrahydrobiopterin; MBP, maltose-binding protein; PK, protein kinase; PTPS, 6-pyruvoyltetrahydropterin synthase; EC 4.6.1.10; cGK, cGMP-dependent protein kinase; 8-Br-cAMP, 8-bromo-cAMP; RT-PCR, reverse transcriptase polymerase chain reaction; CaM PK II, calmodulin protein kinase II; PAGE, polyacrylamide gel electrophoresis; PK, protein kinase.

6-Pyruvoyltetrahydropterin synthase (PTPS) is an essential cofactor for the aromatic amino acid hydroxylases, i.e. phenylalanine hydroxylase, tyrosine hydroxylase, and tryptophan hydroxylase, as well as for all three nitric-oxide synthase isoforms (1). Inherited BH₄ deficiency leads primarily to an increase in plasma phenylalanine, and to dopamine and neurotransmitter deficiency in the brain. Such autosomal recessive inherited defects result either from a biosynthetic or a recycling defect of the BH₄ cofactor (2, 3). Three biosynthetic enzymes are required for de novo cofactor production: GTP cyclohydrolase I, 6-pyruvoyltetrahydropterin synthase (PTPS), and sepiapterin reductase. For recycling of BH₄ following hydroxylase reactions, two enzymatic steps are required that involve the pterin-4a-carbinolamine dehydratase and the dihydropteridinede reductase.

Insufficient PTPS activity due to inherited mutations appears to be the most common cause for BH₄ deficiency (4, 5). Among the over 200 individuals identified today with PTPS deficiency, more than 20 different mutant alleles have been found (6). Some of these alterations have been investigated by expression of recombinant proteins for functional studies such as stability, enzymatic activity, or modification(s). From these analyses, which should not be depreciated for the understanding of the wild-type protein, it was discovered that a few of the mutant alleles were defective as protein substrates for potential post-translational modification(s). Thus, a direct link could be inferred from a disease-causing mutation to the requirement of modification for normal in vivo functionality and activity. Such an example is the arginine 16 to cysteine (R16C) PTPS allele that turned out to be reduced in its activity due to an alteration in the protein kinase (PK) recognition site Arg16-Arg-Ile-Ser, with serine 19 as the potential residue for phosphorylation (7). In this context, phosphorylation of BH₄ metabolic enzymes has been proposed to play a role in the regulation or modulation of BH₄ biosynthesis, based on various observations. For instance, GTP cyclohydrolase I was found to be phosphorylated in rat adrenal pheochromocytoma PC-12 cells (8), rat mesangial cells (9), and mouse bone marrow-derived mast cells (10). The primary amino acid sequence of GTP cyclohydrolase I contains numerous consensus sites for phosphorylation, but the site for modification has not been identified. However, in vitro phosphorylation studies indicate that GTP cyclohydrolase I is modified in casein kinase II and protein kinase C (PK-C) (9, 10). Sepiapterin reductase, the terminal enzyme in BH₄ biosynthesis, was found to be stoichiometrically phosphorylated in vitro by calmodulin-dependent PK II (CaM-PK II) and PKC (11). In addition, phosphorylation by both kinases modified the kinetic properties of sepiapterin reductase. However, neither an in vivo relevance nor a modification in cell culture or tissue was reported for sepiapterin reductase. Similarly, dihydropteridinede reductase was also a good substrate for the CaM-PK II in vitro; yet, phosphorylation did not alter its kinetic properties, and so far no in vivo phosphorylation data are available (11). On the cellular level, however, indications for signal transduction pathways that lead to stimulation of GTP cyclohydrolase I expression and/or of BH₄ biosynthetic activity have been reported by several laboratories (9, 10, 12–18). Stimulating agents were cAMP and various growth-promoting cytokines (e.g. interleukins, nerve growth factors, kit ligand, interferon-γ, and lipopolysaccharides). In most cellular assay systems, the addition of these factors led to the de novo biosynthesis of GTP cyclohydrolase I by transcriptional up-regulation. Interestingly, careful investigation of the effect of adding the constitutive activator 8-Br-cAMP to cultured rat dopamine neurons led to the detection of a biphasic time course
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in the increase of cellular BH4 levels (19). Whereas the long-term regulation of BH4 biosynthesis involved an alteration in GTP cyclohydrolase I gene expression, the short-term increase was speculated to be due to phosphorylation of a biosynthetic enzyme capable of modifying BH4 metabolism. Since this induction was cAMP-dependent but no corresponding cAMP-PK site was present in the GTP cyclohydrolase I, it was speculated that PTPS might be activated by phosphorylation. Independently from these observations we recently found the aforementioned non-phosphorylated mutant allele (R16C) in a patient suffering from PTPS deficiency (17). Analysis of the mutated protein pointed toward a regulatory function for such a modification, since the recombinant protein tested under in vitro assay conditions had only a moderately reduced enzymatic activity, but was inactive in vivo although it was expressed as a stable protein in patients’ primary cells. In this work, we addressed the question of which site in the human homologous PTPS is phosphorylated, what effect such a post-translational modification exerts on protein activity in vivo, and which type of PK might be responsible for the specific modification.

EXPERIMENTAL PROCEDURES

Materials and Miscellaneous Methods—The mutagenic primer PTPS205 was synthesized on a Gene Assembler Plus DNA synthesizer (Amersham Pharmacia Biotech): 5′-GCGTGGAGGCTGATCGCCGGG-3′; the underlined nucleotide indicates the mismatch that leads to the serine 19 to alanine exchange (S19A) in the corresponding human PTPS cDNA sequence (20). The oligonucleotides PTPS21, PTPS102, and PTPS201 for PTPS-cDNA amplification have been published before (7). Radioactively labeled [32P]orthophosphate and [γ-32P]ATP (7) were purchased from NEN Life Science Products Inc., and 8-bromo-cAMP and 8-bromo-cGMP from Biomol Research Laboratories Inc. The pMal-c2 expression vector was from New England Biolabs. The protein kinase calmodulin kinase II (CaM-PK II) was assayed in a volume of 50 μl containing 10 μg of the protein substrate (myelin basic protein as control or recombinant PTPS), 50 mM Hepes (pH 7.4), 10 mM MgCl₂, 1.5 mM CaCl₂, 20 μg/ml calmodulin, 1 mM EGTA, 50 μM [γ-32P]ATP (25 Ci/50 μl), and 5 mM dithiothreitol (only for the negative control). The reaction was carried out for 5 min at 30 °C. For quantification, the phosphorylated control peptides were spotted on a Whatman P-81 filter paper, washed 3 times with 75 mM H₃PO₄, rinsed once with ethanol, dried, and counted. All reactions with recombinant PTPS as substrate were stopped with standard “Laemmli sample buffer,” separated on a 12.5% SDS-PAGE, and quantified as described above.

Transient Expression of cGK or PTPS and [32P]Orthophosphate Labeling in COS-1 Cells—COS-1 cell co-transfections with the pSCT1 derivatives plus pSV5-gal (expressing the bacterial β-galactosidase), [25P]orthophosphate labeling, and immunoprecipitation with the ZZZ2 anti-human PTPS antibody were carried out as described (7). PTPS activity was measured and normalized for the β-galactosidase activity co-expressed from the pSV5-gal plasmid. To prevent dephosphorylation, the cell lysis buffer contained the phosphatase inhibitors sodium fluoride (100 mM), sodium pyrophosphate (10 mM), and sodium orthovanadate (2 mM). The protein kinase inhibitors KT5823 and staurosporine were added to cultivated COS-1 cells at the same time point as the [32P]orthophosphate incubation was started. Plasmid pMT3-1cGK or pSCT1-His-cGKII were transfected according to standard procedures (24). Western blot analysis with antisera against the cGK type I or II (kindly provided by H. Hofmann and P. Ruth) was performed according to standard methods (25, 26).

Purification and in vitro Phosphotransferase Activity Assays—Human primary skin fibroblasts, human Hep G2 hepatoma, and SK-N-BE neuroblastoma cell lines were cultivated in standard medium (Dulbecco’s modified Eagle’s medium; Life Technologies, Inc.) (7). Embryonal fibroblasts from wild-type and cGKII−/− (25) mice were provided by P. Heier fields and P. Ruth. Cells from confluent plates were washed twice with standard PBS buffer, and incubated for 5 min in ice-cold lysis buffer containing protease inhibitors (50 mM Hepes, pH 7.2, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 200 units/ml aprotinin). Steps beyond this point, unless indicated, were all performed at 4 °C.

The cells were scraped from the plates, and the suspension was centrifuged at 13,000 × g for 2 min to collect the supernatant which was then diluted with 2 volumes of HNTG buffer (20 mM Hepes, pH 7.2, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol). Such a freshly prepared soluble extract from a 100-mm tissue culture plate yielded a volume of approximately 1 ml, and was mixed with recombinant MBP-PTPS fusion protein that was preabsorbed to a commercially available amylase resin suspension (Biolog Inc.). Pre-absorption was performed by mixing 21 μg of purified MBP-PTPS fusion protein with 80 μl of amylase resin suspension (Biolog Inc.). Pre-absorption was performed by mixing 21 μg of purified MBP-PTPS fusion protein with 80 μl of amylase resin suspension, and incubating for 10 min at room temperature, followed by two wash steps each with 0.5 ml of HNTG buffer. The amylase-imobilized MBP-PTPS fusion protein mixed with cell extract was incubated overnight at 4 °C, and washed three times with 0.6 ml of ice-cold HNTG buffer.

For the phosphotransferase reaction, 1 μl of 1 mM ATP, 3.3 pmol of [γ-32P]ATP (10 μCi), and 50 μl of phosphorylation buffer (20 mM Hepes, pH 7.2, 150 mM NaCl, 0.1% Triton X-100) were added and incubated for 20 min at 30 °C. The reaction was then sedimented at 13,000 × g for 1 min, followed by one wash step with 0.2 ml of phosphorylation buffer. For cleavage of human PTPS from the MBP, 3 μg of protease factor Xa was incubated for 20 min at 30 °C. 2 P. Ruth, personal communication.
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Samples were separated by 12.5% SDS-PAGE, stained with Coomassie Blue, dried, and autoradiographed.

RESULTS

Human PTPS Contains Various Consensus Sites for Protein-Serine/Threonine Kinases—The location of all putative protein kinase (PK) phosphorylation site sequences, according to the described consensus sites, are indicated in the amino acid sequence of the human PTPS (Fig. 1), and compiled with the corresponding PKs in Table I. The consensus sites are chosen from a tabulation by Pearson and Kemp (27). A total of 17 combinations for phosphorylation with all possible PKs and the listed sites are found. To identify in a first attempt the potential relevant phosphorylation site(s), we applied and combined selection criteria including (i) data from natural PTPS mutations from patients, (ii) surface accessibility from the threedimensional structure, and (iii) in vitro phosphorylation ability with commercially available PKs and purified PTPS. By scanning the primary amino acid sequence we found six putative phosphorylation sites (Ser\textsuperscript{15}, Ser\textsuperscript{19}, Ser\textsuperscript{21}, Ser\textsuperscript{28}, Ser\textsuperscript{32}, and Ser\textsuperscript{105}), two with overlapping consensus sequences. The first of these clusters with overlapping sequences spans Ser\textsuperscript{15} to Ser\textsuperscript{19} (sites a1, b, and c1, in Fig. 1), and the second Ser\textsuperscript{21} to Ser\textsuperscript{28} (sites a2 and c2, in Fig. 1). In each of these clusters, we identified one functional alteration in the PTPS from patients, i.e. the mutant alleles R16C and R25Q (7). In addition, we knew from previous experiments with COS-1 cells that the R16C, but not the R25Q alteration, was refractory for from previous experiments with COS-1 cells that the R16C, but not the R25Q alteration, was refractory for phosphorylation, we calculated that a 20-fold more efficient labeling (equivalent to 100% phosphorylation of the control peptide) would still result in less than 1% phosphorylation of the PTPS substrate. Thus, from these in vitro experiments the only candidate was cGK (I\textalpha-isoenzyme) suggesting that PTPS might be phosphorylated also in vivo by a cGK-like activity. Furthermore, since several sites are potentially recognized by the cGK (sites a1, a2, b, c1, and c2, in Fig. 1), we tested mutated PTPS to identify the actual phosphorylation site(s).

The Recombinant PTPS-S19A Mutant Is Enzymatically Similar to the Wild-type Enzyme but Is Not Phosphorylated by cGK under in Vitro Conditions—Since serine 19 was the only reasonable candidate site for potential phosphoryserine modification in PTPS, we mutated the cDNA to express a recombinant protein with a Ser\textsuperscript{19} to Ala (S19A) exchange. Characterization of the purified mutant PTPS-S19A revealed normal enzyme activity, including thermal stability (not shown) and kinetic parameters akin to that of wild-type enzyme (Table II), confirming that this S19A exchange yielded a correctly folded PTPS. On the other hand, upon incubation with the purified cGK, we observed only background phosphorylation for the PTPS-S19A mutant enzyme. This observation agreed with the fact that the R16C mutant was also not a substrate for this PK, in contrast to the wild-type and PTPS-R25Q that were modified by the cGK. Furthermore, soluble, recombinant cGKI and cGKII were both able to specifically phosphorylate wild-type PTPS in vitro (Fig. 2). In this context it is worth mentioning that we also tested for potential alteration of kinetic parameters of purified wild-type PTPS phosphorylated at serine 19. When the PTPS protein was modified with an in vitro efficiency of approximately three phosphoserines 19 per hexameric com-

\footnote{G. Auerbach and T. Ploom, personal communication.}

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Summary of in vitro phosphorylation data of human wild-type PTPS with putative protein-serine/threonine kinases

| Protein kinase | Motifs | Potential phosphorylation sites in PTPS | Artificial protein substrate | % Phosphorylation of recombinant human PTPS |
|---------------|--------|----------------------------------------|-----------------------------|-------------------------------------------|
| cGK (I-isozyme) | RRXX+ | Arg(16)-Arg-Ile-Ser* (a1) | RKISASEF peptide | 11–32% |
| | RRXX+ | Arg(16)-Leu-Tyr-Ser* (a2) | | |
| | RRXX+ | Arg(9)-Ile-Ser* (b) | | |
| | S*X | Ser*39-Arg (c1), Ser*32-His-Arg (c2) | | |
| cAMP-PK | RRXX+ | Arg(16)-Arg-Ile-Ser* (a1) | Kempede | <1% |
| | RRXX+ | Arg(16)-Leu-Tyr-Ser* (a2) | | |
| | RRXX+ | Arg(9)-Ile-Ser* (b) | | |
| PK-C | RRXX+ | Arg(16)-Arg-Ile-Ser* (a1), Arg(9)-Lys-Tyr-Ser* (a2) | Neurogranin (28-43) | 2% |
| | RRXX+ | Arg(9)-Ile-Ser* (b) | | |
| | S*X | Ser*39-Arg (c1), Ser*32-His-Arg (c2) | | |
| CaMKII | RRXX+ | Arg(16)-Arg-Ile-Ser* (a1), Arg(9)-Lys-Tyr-Ser* (a2) | Myelin basic protein | <1% |
| Casein KII | S*X | Ser*39-Asp-Glu-Glu-Asn (d1), Ser*105-Thr-Thr-Glu-Asn (d2) | RRREEETEE peptide | <1% |

a Abbreviations for protein kinases: cAMP-PK, cyclic AMP-dependent protein kinase; PKC, protein kinase C; casein K II, casein kinase II.

b Asterisks indicate the phosphorylated residue. Only motifs that are present in the human PTPS are shown (see Pearson and Kemp, (27)).

c See Fig. 1 for location of motifs given in parentheses.

d Control assays with peptides were performed as described in the Technical Bulletin for each of the commercially available kinases (filter assays).

In vitro characteristics of purified wild-type and mutant PTPS

| Recombinant PTPS | Specific activity | \( k_{cat} \) | \( K_M \) | \( k_{cat}/K_M \) | % Phosphorylation with cGK |
|------------------|------------------|--------------|-----------|-----------------|--------------------------|
| milliunits/mg | \( s^{-1} \) | \( \mu M \) | \( s^{-1} \) | | | |
| Wild-type | 120 | 0.033 | 8.1 | 4.1 \( \times 10^3 \) | 19 (11)% |
| R16C | 44 | NT | NT | NT | 2 |
| S19A | 129 | 0.035 | 5.5 | 6.4 \( \times 10^3 \) | 0 |
| R25Q | 74 | NT | NT | NT | 17 |

a One unit of enzyme activity catalyzes the production of 1 μmol of BH4 per min at 37°C.

b 100% means that the total of PTPS monomers present was phosphorylated by cGK (I-isoform from Promega).

c In the presence of a 200 molar excess of the PK inhibitor KT5823.

d NT, not tested (for values estimated before, see Table II in Oppeliger et al., Ref. 7).

Vivo Activity and Is a Non-phosphorylated Protein in COS-1 Cells—To test the S19A-PTPS in a eukaryotic cell background, we transiently expressed the mutant proteins in COS-1 cells and measured the enzyme activity. The results of assaying the wild-type and mutant PTPS are shown in Fig. 3. The S19A-PTPS mutant in comparison to the wild-type enzyme had a reduction of activity to 33%. For the S19A and R16C mutants expressed in COS-1 cells, a relative reduction of activity to one-third was observed compared with the purified enzymes. In parallel, we checked the same transiently transfected COS-1 cells following \(^{32}P\)orthophosphate labeling and immunoprecipitation for in vivo phosphorylation of PTPS. As shown in Fig. 4, only the wild-type enzyme but not the S19A-PTPS mutant was significantly phosphorylated. Quantification of the labeled PTPS-monomeric bands showed 12–14% background radioactivity for the S19A and R16C mutants. For control, the R25Q mutant was also included and revealed normal wild-type phosphorylation (107%). This was also expected from in vitro phosphotransferase experiments with cGK, which showed that the PTPS-R25Q mutant was labeled to the same extent as the wild-type enzyme (Table II). To further prove that Ser\(^{19}\) is the only site in human PTPS that is phosphorylated was the analysis by two-dimensional gel electrophoresis analysis of \(^{32}P\)orthophosphate-labeled and immunoprecipitated PTPS enzyme that was transiently expressed in COS-1 cells (not shown) (28).

Neither Staurosporine Nor KT5823, Both cGK Inhibitors, Influence the in Vivo Activation of Human PTPS Expressed in COS-1 Cells—From the initial in vitro experiments with purified enzymes we knew that cGK was able to phosphorylate wild-type PTPS to some extent. In agreement with these findings is the presence of the Phe residue adjacent to the phosphorylation site sequence Arg-Arg-Ile-Ser\(^{19}\)-Phe (see Fig. 1), which is thought to be a negative determinant for cAMP-PK, and a positive for cGK (29). We thus examined the potential of the selective inhibitor KT5823 for cGK in COS-1 cells to see whether our in vivo results with cGK had any relevance in vivo. The inhibitory compound KT5823 is related to staurosporine and has a \( K_I \) of 2.4 nM for the cGK, which is 16- and 33-fold more specific than for the cAMP-PK and PK-C, respectively (30). As a control, we tested the KT5823 inhibitor under our initial in vitro phosphorylation conditions with the commercially available cGK and purified wild-type PTPS, which resulted in not more than a \( \sim 40\% \) inhibition of phosphotransferase activity in the presence of a 200 molar excess of KT5823.

FIG. 2. In vitro phosphorylation of human wild-type and PTPS-S19A with purified cGK type I and II. A typical in vitro phosphorylation experiment is shown with purified cGK (bovine I isoform), recombinant cGKI (bovine I isoform), recombinant cGki (bovine, 0.2 μM), or recombinant cGkiI (murine, 0.2 μM), and PTPS (0.5 μM) purified from bacterial cells. PK autophosphorylation and substrate phosphorylation with purified proteins depending on added cGMP (10 μM) is visible on the autoradiography. Relative phosphorylation of the PTPS-specific band is indicated in % of total subunits. We repeatedly observed no autophosphorylation of the commercially available bovine cGKI. Cyclic-GMP-independent phosphorylation with cGKI type I may be due to a relatively high concentration (200 nM) of recombinant PR in the assay.

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R16C and R25Q mutants were reduced to 37 and 61% activity, respectively. The activity of proteins purified from bacterial cells is given in bars. The purified wild-type and S19A mutant had the same activity under in vitro conditions (100 and 103%), whereas the R16C and R25Q mutants were reduced to 37 and 61% activity, respectively. The activities of the mutants R16C and R25Q have been reported before and are shown here for comparison.

In the next series of experiments, we transfected with a plasmid expressing wild-type PTPS (pHSY2013). After co-incubation of COS-1 with [32P]orthophosphate plus up to 30 μM of the KT5823 inhibitor, the cells were lysed in the presence of phosphatase inhibitors, and immunoprecipitated with a PTPS-specific antibody (S228). The precipitated proteins were subsequently separated by SDS-PAGE and the PTPS-monomeric bands were cut out and quantified for 32P incorporation. From this experiment we observed no inhibitory effect of KT5823 on in vitro PTPS phosphorylation. In parallel experiments, addition of up to 25 μM staurosporine to cultured cells also had no effect on PTPS activity (data not shown). In similar experiments we tested for activation of a cGK that potentially phosphorylated PTPS. This was done by the addition of the cell-permeable activator 8-Br-cGMP. However, this also did not alter the relative phosphorylation of human PTPS. Furthermore, upon co-transfection of COS-1 cells with a vector expressing the wild-type PTPS plus a plasmid expressing the cGKI (pMT3-1c-cGK), we observed a 2-fold stimulation of 32P incorporation into PTPS. This stimulation was again independent of the presence or absence of additional 8-Br-cGMP activator, indicating that cGMP was not a limiting compound in these COS-1 cells (not shown).

PTPS Affinity Binding of a Cellular PK That Specifically Phosphorylated the Human PTPS and Is Stimulated by the Addition of cGMP—In the next series of experiments, we wanted to know whether such a PK that specifically phosphorylated serine 19 residue and thereby activated the human PTPS, was present not only in COS-1 cells, but also in human primary fibroblasts, as well as in liver-derived and brain-derived human cell lines where PTPS is expressed. Since we supposed that a specific PK which phosphorylates Ser19 should bind to the PTPS protein sequence Arg16-Arg-Ile-Ser*, we used the recombinant PTPS purified from bacterial cells as bait to capture the corresponding PK from cell extracts. This was achieved by first binding the purified MBP-PTPS fusion to amylose resin, followed by incubation of the complex with soluble protein cell extracts prepared from various cells or cell lines, including human skin fibroblasts, SK-N-BE (human neuroblastoma), Hep G2 (human hepatoma), and COS-1 cells. After gentle washing of these mixtures, the amylose resin-containing complexes were incubated in a phosphorylation buffer in the presence of γ32PATP, followed by separation by SDS-PAGE and autoradiography. A typical example of such an experiment is presented in Fig. 5, A and B, where we showed that, following factor Xa treatment, the PTPS fusion partner was specifically labeled with [32P]phosphate by a PK that bound to the immobilized MBP-PTPS complex. Furthermore, we found that in all cells tested, i.e. in fibroblasts, in COS-1 cells, in liver-derived Hep G2, and in the neuronal cell line SK-N-BE, a soluble PK existed that specifically bound to wild-type PTPS and phosphorylated the Ser19 residue (Fig. 5C). The specificity was proven by the use of the R16C-PTPS mutant as bait that was not phosphorylated, in contrast to the wild-type enzyme (Fig. 5D). In a similar experiment, addition of 50 μM cGMP to this phosphotransferase assay stimulated kinase activity 2-fold (Fig. 6). In contrast, concentrations of up to 8 mM cAMP did not stimulate the PTPS-bound kinase activity. This indicated that the activity of the affinity bound kinase was a cGK. On the other hand, in an accompanying Western blot with antisera directed against the cGK (type I and II) (25), we found no significant cross-reactivity (not shown). This may be due to the apparently low concentration of cGK in these extracts.

Extracts from Transfected COS-1 Cells with Type II but Not Type I cGK Stimulate in Vitro Phosphorylation of Wild-type PTPS More Than 100-fold—The apparently low presence of cGK in the cells examined prompted us to test for increase of phosphotransferase activity in the affinity binding assay with extracts that had overexpressed either cGKI or cGKII. To this end, COS-1 cells were transiently transfected with the cGKI or cGKII expressing plasmids, pMT3-1c-cGK or pSCT1-His-cGKII, respectively. Western blot analysis of cell extracts confirmed overexpression of cGKI and cGKII (not shown). Using the extracts from transfected COS-1 cells, phosphorylation of wild-type and S19A MBP-PTPS fusion proteins and the MBP alone was analyzed. Phosphorylation was quantified after standard factor Xa digestion and separation on SDS-PAGE. Background phosphorylation was determined by using untransfected COS-1 cell extracts and wild-type MBP-PTPS for affinity binding. Extracts with overexpressed cGKI stimulated wild-type PTPS phosphorylation ~4-fold over background (Table III). However, cGKII was able to enhance wild-type PTPS phosphorylation 113–169-fold over background. The S19A MBP-PTPS and MBP controls exhibited the expected phosphorylation of approximately 15 and 1% from background, respectively. Thus, although both cGKI and cGKII could phosphorylate PTPS with purified enzymes in vitro, and cGKII to a limited extent also in vivo using the affinity binding and phosphorylation assay, cGKII appears to be a specific PK for wild-type PTPS phosphorylation, at least under these assay conditions.

Fibroblast Extracts from Mice Lacking cGKII Are Reduced in PTPS-specific Phosphorylation in the in Vitro Affinity Binding and Phosphotransferase Assay—Next we analyzed mice devoid of cGKII, which were recently described (25), for PTPS enzyme activity in liver, kidney, and brain, and for BH4 content in the same organs plus plasma and urine. However, for these parameters, we did not see any significant difference between the cGKII−/− mice and wild-type controls. Therefore, we used our PTPS affinity binding and phosphotransferase assay to test whether cell extracts from cGKII−/− mice showed any difference to those from normal mice. The assay was carried out with extracts prepared from murine embryonal fibroblasts. In the three experiments performed we found reduced phosphorylation capacity specific for wild-type PTPS that was between 39 and 45% of extracts from control mice (Table IV). Again, we observed an effect pointing unequivocally toward cGKII as an important, but not necessarily the only PK for the in vivo phosphoserine modification of PTPS (see also “Discussion”).
DISCUSSION

Our results demonstrate the requirement of phosphoserine 19 in human PTPS for maximal enzyme activity under in vivo conditions. Based on previous observations, as already mentioned under the Introduction, modulation of BH4 biosynthetic activity by phosphorylation was not unexpected. For the recom-

FIG. 4. Test for in vivo phosphorylation of PTPS following immunoprecipitation of proteins expressed in COS-1 cells. A, COS-1 cells transfected for transient expression of wild-type (plasmid pHSY2013) and PTPS-S19A (plasmid pHSY2017) were labeled with [32P]orthophosphate and immunoprecipitated, followed by separation by 12.5% SDS-PAGE and Coomassie Blue staining. In the "control" lane, no plasmid was transfected into COS-1 cells. On a separate lane, 5 μg of recombinant wild-type PTPS (rec. PTPS) was loaded to indicate the position of the PTPS monomer (arrowhead). B, autoradiography of the same gel as in A, showing that only the wild-type PTPS monomeric band is phosphorylated. C, autoradiography of a similar gel as shown in B, expressing in COS-1 cells the wild-type, S19A, R16C (plasmid pHSY2009), and R25Q (plasmid pHSY2010) PTPS, plus no plasmid as control. The Coomassie Blue-stained monomeric PTPS bands were cut out from the gel and quantified in a scintillation counter. The amount of radioactivity is given in percentage of wild-type activity (100%).

FIG. 5. PTPS affinity binding of a specific Ser19 phosphotransferase activity present in various human cells. A, phosphorylation reactions separated by 12.5% SDS-PAGE and stained with Coomassie Blue. Soluble protein extracts from human skin fibroblasts were incubated overnight at 4 °C with amylose resin in the absence (lanes 1 and 2) or presence of amylose-bound, recombinant MBP-PTPS fusion protein (lanes 3 and 4). After washing, a phosphorylation reaction was carried out with buffer containing [γ-32P]ATP. Subsequently, the mixture was washed and samples in lanes 2 and 4 were incubated with protease factor Xa for cleavage of the MBP-PTPS fusion protein. On lane 5, 5 μg of recombinant wild-type PTPS was loaded (rec. PTPS). B, autoradiography of the same gel as in A, showing specific labeling of the MBP-PTPS fusion protein (lane 3), or the PTPS monomer cleaved off from the MBP partner by the protease factor Xa treatment (lane 4). C, a comparable experiment is presented as in A and B, lane 4, using cell extracts from fibroblasts, SK-N-BE, Hep G2, and COS-1 cells. Following the phosphorylation reaction, the MBP-PTPS fusion protein was digested with protease factor Xa. The PTPS fusion partner is depicted on SDS-PAGE (upper panel) and on the corresponding autoradiography (lower panel). D, autoradiography of a similar gel as shown in A, lane 4. The MBP-PTPS fusion was incubated with extracts from fibroblasts (lanes 1 and 3) or COS-1 cells (lanes 2 and 4). The amylose resin was preabsorbed with either recombinant MBP-wild-type-PTPS (lanes 1 and 2) or the MBP-PTPS-R16C mutant (lanes 3 and 4). All samples were incubated with phosphorylation buffer containing [γ-32P]ATP, followed by protease factor Xa treatment.
Phosphorylation of PTPS by cGMP Kinase II

Fig. 6. Stimulation of kinase activity with cGMP in the PTPS affinity binding and in vitro phosphotransferase assay. The experiment was performed with extracts from skin fibroblasts basically as shown in Fig. 5, A and B (lane 4), by adding increasing amounts of cGMP to the phosphorylation reaction. Phosphotransferase activity was determined by quantification of $^{32}$P incorporation after cutting out the PTPS protein band from the gel.

Table III

| Transfection | Recombinant substrate | Absolute cpm | Phosphorylation % |
|--------------|-----------------------|--------------|------------------|
| MBP-PTPS (wild-type) | 58 (1-fold) | <1 | |
| MBP-PTPS-S19A | 19 | <1 | |
| MBP | 1 | <1 | |
| cGKI MBP-PTPS (wild-type) | 217 (4-fold) | 2 | |
| cGKI | 27 | <1 | |
| cGKI-MBP | 3 | <1 | |
| cGKII MBP-PTPS (wild-type) | 9815 (169-fold) | 100 | |
| cGKII | 177 | <2 | |
| cGKII-MBP | 191 | <2 | |

$^a$ cGKI was expressed from plasmid pMT3-1a-cGK, and cGKII from plasmid pSCT1-His-cGKII.

Table IV

| Fibroblasts from mouse strain | Recombinant substrate | Phosphorylation % |
|-------------------------------|-----------------------|------------------|
| cGKII+/− (wild-type) | MBP-PTPS (wild-type) | 100 | |
| cGKII+/− (knock-out) | MBP-PTPS-S19A | 13–17 | |
| cGKII−/− (wild-type) | MBP-PTPS (wild-type) | 39–45 | |
| cGKII−/− (knock-out) | MBP-PTPS-S19A | 13–23 | |

$^a$ Results are from two independent experiments.

biminent enzymes from bacterial cells, where such post-translational modification does not take place, we found the same enzymatic activities for wild-type PTPS and the S19A mutant. In contrast, under in vitro conditions, PTPS needs Ser$^{19}$ for phosphorylation to become fully active. However, under the assay conditions tested, we found a relative activation of “only” a factor of three of the COS-1 cell enzyme. It is conceivable that activation in vivo is actually severalfold higher, since the phosphoserine in PTPS might be unstable and subject to rapid enzymatic dephosphorylation during preparation of soluble protein extracts for enzymatic assays. We wanted to test this hypothesis by using phosphatase inhibitors during cell extract preparations. Unfortunately, we found with recombinant protein from bacterial cells that the bona fide PTPS activity was ≥17-fold inhibited in the presence of phosphatase inhibitors added under in vitro enzyme assay conditions (not shown). This was not entirely unexpected as the PTPS enzyme carries out an internal redox reaction and a phosphate-elimination step, which is probably suppressed in the presence of phosphatase inhibitors (31, 32).

When PTPS was modified in vitro resulting in approximately three phosphoserines 19 per hexameric active complex, we did not see any alteration of enzymatic characteristics. However, it is not known whether modification maximally of three subunits per hexamer would have been enough to detect any potential alteration of the kinetic properties. Furthermore, it is also possible that PTPS phosphorylation does not directly modulate the in vivo enzymatic properties, but may influence either stability and/or folding or interaction with another essential cellular partner. In the latter case, the enzymatic assay under our in vitro conditions with purified components would not reflect the in vivo situation. In any case, the molecular mechanism by which PTPS enzymatic activity is stimulated by the phosphoserine modification remains to be elucidated.

Besides the in vitro phosphorylation studies with purified enzymes, the results with the protein affinity assays and transfection studies corroborated that PTPS phosphorylation is carried out in vivo by cGK. In this context, Kapatos and collaborators (19) reported that 8-Br-cAMP is a stimulating factor for the activation of BH$_4$ biosynthesis in cultured rat dopamine neurons. In their work, they observed a short-term increase in cellular BH$_4$ content and it was speculated that this effect might be attributed to PTPS phosphorylation and thus activation. Unfortunately, the constitutive activator 8-Br-cGMP was not tested in these cell culture experiments with dopamine neurons. Cyclic-GMP could thus not be completely excluded as an alternative activator, especially since it is known that cGK can be activated also by cAMP. On the other hand, a cGK may be responsible for constitutive phosphorylation of a fraction of rat PTPS present in the cell, whereas upon stimulation of BH$_4$ biosynthesis with 8-Br-cAMP, additional phosphorylation of the remaining fraction may further enhance PTPS activity. In any case, it is likely that the stimulating effect of rat PTPS may involve a similar post-translational modification at the corresponding site, i.e. rat phosphoserine 18 modification. Inasmuch as this site, Arg-X-X-Ser$^{7}$, is found to be conserved among all described primary amino acid sequences for PTPS, including human, rat, mouse, salmon, and the Drosophila enzymes, corresponding serine or threonine phosphorylation in this motif might be generally required in PTPS (33, 34).

In mammalian tissues two types of cGK have been identified, a soluble type I of 75 kDa and a membrane-associated type II of 85 kDa. Type I cGK, consisting of α and β isoforms, is more generally expressed in various peripheral tissues, with several substrates identified, and is thought to act as a regulator of cardiovascular homeostasis. Type II cGK is abundant in at least kidney, bone, many brain regions, and epithelial cells of the intestine. It is a regulator of the cystic fibrosis transmembrane conductance regulator the only substrate identified except for the PTPS presented here. Due to the abundance of cGKII in the brain, it is suggested to play a role in nitric oxide/cGMP signaling in the central nervous system (35). With the experiments presented here, the BH$_4$ biosynthetic enzyme PTPS may be another physiological substrate for cGKII. Consequently, the cGKII responsible for phosphoserine 19 modification must be expressed in cell types where PTPS phosphorylation is required. Even though we could not detect any cross-reactive material with cGKII antibodies in extracts from cells used in this study, the more sensitive RT-PCR analysis revealed that cGKII is expressed in all these cell types, i.e. fibroblasts, COS-1, Hep G2, SK-N-BE (data not shown; for references, see also Refs. 36 and 37).

Although both types of cGK have distinct tissue distribution
and/or subcellular localization, they could both phosphorylate cystic fibrosis transmembrane conductance regulator (38) and PTPS in vitro with purified, soluble enzymes. Thus far, interchangeability of substrate phosphorylation by cGKI and cGKII seems not to take place under in vivo conditions. Nevertheless, it may be found to some extent in a situation with a complete lack of cGKII. Under such physiological conditions, a compensatory activity by cGKI or an unknown PK could take over to some extent at least for the PTPS substrate. On the other hand, two different PK one of which is the cGKII, could be responsible for maximal Ser19-PTPS phosphorylation in vivo (see also above). Besides such a hypothetical compensatory reaction as suggested by the reduced but still significant phototransferase activity for PTPS in extracts from cGKII−/− fibroblasts, another effect could be responsible for the non-detectable Phe-cGKII.

Regulator protein, PTPS may thus be another substrate for reduction. After the cystic fibrosis transmembrane conductance regulator, choline and serotonin biosynthesis as well as for NO production in neurological tissues, which require the cofactor for catecholamine oxidation and dephosphorylation of metabolite neopterin rise significantly in primates at least after stimulated expression of GTP-cyclohydrolase I (for a recent discussion, see Ref. 34).

In conclusion, cGKII was identified as PK for modification of PTPS at the single serine site. PTPS participates in BH4 generation in peripheral organs such as liver and kidney, but also in neurological tissues, which require the cofactor for catecholamine and serotonin biosynthesis as well as for NO production. After the cystic fibrosis transmembrane conductance regulator protein, PTPS may thus be another substrate for cGKII.

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