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Human pterin-4α-carbinolamine dehydratase/dimerization cofactor of hepatocyte nuclear factor-1α

Characterization and kinetic analysis of wild-type and mutant enzymes

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Pterin-4a-carbinolamine dehydratase/dimerization cofactor for hepatocyte nuclear factor-1α is a protein with two different functions. We have overexpressed and purified the human wild-type protein, and its Cys81Ser and Cys81Arg mutants. The Cys81Arg mutant has been proposed to be causative in a hyperphenylalaninaemic patient [Citron, B. A., Kaufman, S., Milstien, S., Naylor, E. W., Greene, C. L. & Davis, M. D. (1993) *Am. J. Hum. Genet. 53*, 768−774]. The dehydratase behaves as a tetramer on gel filtration, while cross-linking experiments showed mono-, di-, tri-, and tetrameric forms, irrespective of the presence of the single Cys81. Sulfhydryl-modifying reagents did not affect the activity, but rather showed that Cys81 is exposed. Various pterins bind and quench the tryptophan fluorescence suggesting the presence of a specific binding site. The fluorescence is destroyed upon light irradiation. Wild-type and the Cys81Ser protein enhance the rate of the phenylalanine hydroxylase assay ≈10-fold, a value similar to that of native dehydratase from rat liver; the Cys81Arg mutant, in contrast, has significantly lower activity. This is compatible with the hypothesis that the dehydratase is a rate-limiting factor for the *in vivo* phenylalanine hydroxylase reaction. The three proteins enhance the spontaneous dehydration of the synthetic substrate 6,6-dimethyl-7,8-dihydropterin-4a-carbinolamine ≈50−70-fold at 4°C and pH 8.5. The results are discussed in view of the recently solved three-dimensional structure of the enzyme [Ficner, R., Sauer, U. W., Stier, G. & Suck, D. (1995) *EMBO J. 14*, 2032−2042].

Keywords. Pterin-4a-carbinolamine; dehydratase; phenylalanine hydroxylase; tetrahydrobiopterin; hepatocyte nuclear factor-1α.

Pterin-4a-carbinolamine dehydratase (PCD) was first recognized by Kaufman's group as a factor which increases the velocity of the tetrahydrobiopterin (H_4biopterin)-dependent phenylalanine hydroxylase mediated hydroxylation of phenylalanine to tyrosine [1, 2]. PCD dehydrates pterin-4a-carbinolamine which occurs as an intermediate in the reaction mentioned (see Scheme 1 below), a step which was proposed to be rate-limiting in the overall process [3]. The detection of 7-substituted pterins in the urine of certain patients exhibiting a mild hyperphenylalaninaemia led to the assumption that a decreased rate of dehydration by PCD might be responsible for the phenotype [4−6]. Supporting evidence came from oral loading tests with 6-substituted pterins (such as H_4biopterin), resulting in the increased formation of 7-substituted pterins [7]. Furthermore, *in vitro* studies with phenylalanine hydroxylase in the absence of PCD led to the accumulation of 7-substituted pteridine-isomers [8] indicating the occurrence of a defective PCD in these individuals.

In order to investigate whether the role of PCD is indeed the prevention of formation of these rearranged pterins, our group has determined the amino acid sequence of PCD, which was found to be identical for the human and rat proteins [9]. Surprisingly, this sequence is identical to that of a protein involved in the dimerization of hepatocyte nuclear factor-1α (HNF-1α) [9, 10], a homeobox protein regulating the expression of a number of liver genes [11]. This points unambiguously towards a dual role of PCD both in the biosynthesis of biogenic amines/pterin metabolism and modulation of transcription activity by HNF-1α.

These findings have revived the concept, which has surfaced at various occasions in the past 20 years, that pterins are somehow involved in the regulation of gene expression [12]. For instance, the proposal has recently been put forward that pterins are involved in the regulation of melanin biosynthesis in human epidermis, and that the occurrence of 7-pterins resulting from strongly reduced PCD activity in the epidermis may initiate depigmentation in vitiligous patients [13, 14]. It can thus be hypothesized that the interaction of pterins with PCD modulates

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Abbreviations. HNF-1α, hepatocyte nuclear factor-1α; PCD, pterin-4a-carbinolamine dehydratase; H_4biopterin, (6*R*)-5,6,7,8-tetrahydrobiopterin; H_2biopterin, 7,8-dihydrobiopterin; q-6,6-Me_2-H_2pterin, quinonoid 6,6-dimethyl-7,8-dihydropterin; 6,6-Me_2-4a-OH-H_2pterin, 6,6-dimethyl-7,8-dihydropterin-4a-carbinolamine (cf. structure in Scheme 1); [MeOC (NH) $C_2H_4S]_2$, dimethyl-3,3′-dithiobispropionimidate × 2 HCl; Me_2SO_2S, methyl methanethiosulfonate; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid).

Enzymes. Dihydropteridine reductase (EC 1.6.99.7); L-phenylalanine hydroxylase, L-phenylalanine tetrahydropterin:oxygen oxidoreductase (EC 1.14.16.1); pterin-4a-carbinolamine dehydratase (EC 4.2.1.–); catalase (EC 1.11.1.6).

the interactions with the $HNF\text{-}1\alpha$ and thereby affects HNF-1α-mediated transcriptional activation.

In agreement with the proposal by Blau et al. [15] that a PCD deficiency is related to the occurrence of 7-pterins, Citron et al. [16] have reported a missense (Cys81Arg) and nonsense (Glu86ter) mutation in the PCD gene of a hyperphenylalaninaemic child who excretes large amounts of 7-pterins.

In the present work we have overexpressed human recombinant PCD in a suitable system yielding the protein quantities required for a detailed biochemical characterization. Cys81 mutants have also been produced in order to study their properties in connection with the genetic defects mentioned above. Attention has been devoted to the interaction of wild-type PCD and its mutants with various pteridines in order to prove the concept that it is an enzyme with a specific binding site. The results are discussed in the context of the three-dimensional structure which has been made available most recently [17]. A final goal of this study is an understanding of the role of PCD in aromatic amino acid metabolism and in hyperphenylalaninaemic patients excreting 7-pterins.

MATERIALS AND METHODS

Enzymes and chemicals. The following chemicals were purchased from the companies indicated: dihydropteridine reductase and L-phenylalanine (Sigma), catalase and NADH (Boehringer Mannheim), H_4biopterin, 7,8-dihydrobiopterin (H_2biopterin), (+)-neopterin [18], (−)-biopterin [19] (Schircks Laboratory), $[MeOC(NH)C_2H_4S]_2$ (dimethyl-3,3′-dithiobispropionimidate × 2 HCl; Pierce), Nbs_2 [5,5′-dithiobis(2-nitrobenzoic acid); Serva], Me_2SO_2S (methyl methanethiosulfonate; Roth), Phenyl-Sepharose (Pharmacia). Phenylalanine hydroxylase was purified from rat liver [20]. 6-Amino-methylpterin was synthesized according to [21]. 7,8-Dihydropterins were obtained by electrochemical reduction [22] of the oxidized forms. 7,8-Dihydroneolumazine is described here for the first time; it was obtained in ≈70% yield by electrolysis of neolumazine at −800 V for 20 h, during which time 2.1 mol electron/mol were consumed (purification by recrystallization from water; M = 254.3 g/mol, $C_9H_{10}N_4O_5$, calc: C 42.19, H 4.72, N 21.87, found: C 41.83, H 4.62, N 21.73). Neolumazine and neopterin were obtained following the general procedure of Viscontini and Leidner [23].

Instrumentation. Ultraviolet/visible spectra were recorded either with a Uvikon 810 or 930 spectrophotometer (Kontron). Fluorescence emission and excitation spectra were recorded with a fluorimeter from Kontron (model SFM-25). Gel filtration was done on a Superose 12 HR column (1 cm × 30 cm; Pharmacia) in 20 mM Tris/HCl, 100 mM KCl pH 7.0. The figures were generated using the program KaleidaGraph, and curve fits generated with appropriate algorithms. The fits of primary kinetic traces (Fig. 5) were obtained with program A (Dr D. Ballou, University of Michigan, Ann Arbor, USA). Kinetic data were simulated with HopKINSIM 1.7 (D. Wachsstock, J. Hopkins Univ.).

Enzyme-coupled assay. Dehydratase activity was measured using a modification of the assay procedure described by Citron and Kaufman [10]. The reaction was performed in 50 mM Tris/HCl pH 8.3 at 25°C, containing 20 µg catalase, 0.5 unit dihydropteridine reductase, 0.2 nmol phenylalanine hydroxylase (from rat liver), 1 µmol L-phenylalanine, 2.9 nmol H_4biopterin, and 100 nmol NADH in 1.0 ml. The consumption of NADH was followed at 340 nm as described by Kaufman [24]. 7-Pterin was analyzed by HPLC according to Adler et al. [28] and Curtius et al. [25]

Assay with 6,6-dimethyl-7,8-dihydropterin-4a-carbinolamine (6,6-Me_2-4a-OH-H_2pterin). The procedure first described by Bailey et al. [26] was modified to obtain the 6,6-Me_2-4a-OH-H_2pterin (Fig. 4). A solution of 6*N*-(2-amino-2-methyl-propyl)-2,5,6-triamino-(3*H*)-4-oxo-pyrimidine (20 mM) in methanol/water (1:1) was made anaerobic by flushing with argon. A threefold excess of bromine was added to this solution under constant stirring at 29°C. At pH 2 the amino group at C5 is readily oxidized to an imino group. After stirring for 12 min, 50-µl aliquots were rapidly frozen in liquid nitrogen. For assays, aliquots were thawed on ice and the pH was adjusted with 30 µl 0.5 M Tris/HCl pH 9.5 to ≈pH 8.5. All assays were performed in 1 ml 10 mM Tris/HCl pH 8.5 at 4°C unless specified otherwise.

Oligonucleotides. The following oligonucleotides were synthesized on a Gene Assembler DNA synthesizer (Pharmacia LKB): PCDH15 (5′-CG*GAATTCATA***TG**GCTGGCAAAGCACACAG-3′), containing an *Eco*RI and a *Nde*I restriction site (italics) and the ATG start codon (bold) plus the codons for the first six residues of the mature form of human and rat PCD; PCDH20 (5′-CG*GGATCC***CTA**<u>GTGGTGGTGGTGGTGGTG</u>TGTCATGGACACTGCTACTTG-3′), encoding the C-terminal seven amino acids of PCD followed by six consecutive histidine residues (underlined) plus an amber stop codon (bold) and a *Bam*HI restriction site (italics); PCDH24 (5′-AGCACCCATGAG<u>AGT</u>GCCGGCCTTTC-3′) and PCDH26 (5′-AGCACCCATGAG<u>CGT</u>GCCGGCCTTTC-3′), for PCR-directed mutagenesis, exchanging the Cys81 codon to Ser (C81S) and to Arg (C81R), respectively (base changes are in bold; codon changes are underlined).

Construction of expression vectors. Oligonucleotides PCDH15 and PCDH20 were used to amplify by PCR a DNA fragment coding for 110 amino acids; i.e. the 104 residues encoding PCD starting with methionine and having an additional six histidine codons at the C-terminus. The template for standard PCR amplification was a plasmid-subclone harboring the cDNA for PCD generated from the previously isolated λgt11-clone from a human liver cDNA library [27]. The PCR product was treated with *NdeI/BamHI* endonucleases and ligated into the expression vector pGEMEX-2-Nde cut with *NdeI/BamHI* to generate pHDH14. The single *Nde*I site overlapping with the ATC start codon of phage T7 gene 10 was used for generating inframe fusions in plasmid pGEMEX-2-Nde. The second *Nde*I site in the original pGEMEX-2 vector (Promega) was removed by site-directed mutagenesis (D. Bürgisser, personal communication). To express mutant PCD proteins, PCR-directed mutagenesis was performed using the primers PCDH24 (for C81S) or PCDH26 (for C81R) in combination with primer PCDH20. The ≈100-bp DNA products from the first PCR were purified over a 2.5% agarose gel and used in a second round of PCR amplification as primers in combination with PCDH15. The products from the second PCR were cloned into the pGEMEX-2-Nde vector as described above. The generated plasmids pHDH14, pHDH26 and pHDH37 expressed the wild-type, C81S-PCD and C81R-PCD proteins, respectively, each containing a COOH-terminal histidine tag. The nucleotide sequence of all constructs was confirmed by DNA sequence analysis, performed by the dideoxy chain-termination method [28], using the AutoRead sequencing kit and an automated laser fluorescent DNA sequencer (Pharmacia LKB Biotechnology).

Purification of recombinant PCD. Expression of histidine-tagged wild-type and mutant PCD was achieved in *Escherichia coli* BL21 (DE3) pLysS (Promega) harboring the different pGEMEX-2-Nde derivatives. Histidine-tagged proteins were isolated in a one-step affinity purification using a Ni^{2+} resin column according to a protocol by Novagen. Briefly, *E. coli* was grown in 500 ml Luria-Bertani medium [29], containing 25 µg/ml chloramphenicol and 50 µg/ml ampicillin, to an absorbance

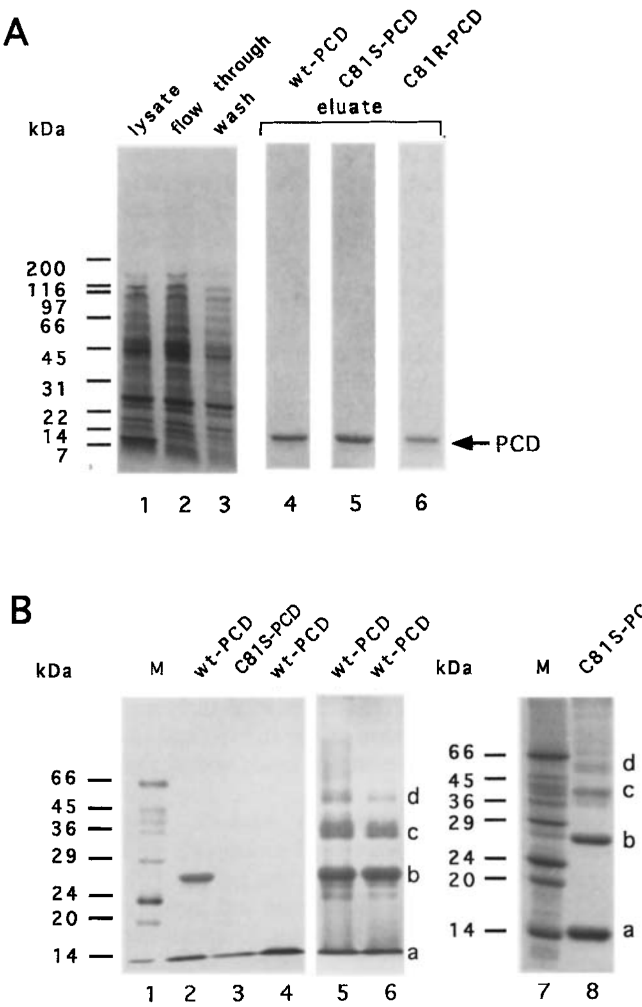


Fig. 1. SDS/PAGE analysis of wild-type, C81S- and C81R PCD at different stages of isolation, and under various conditions affecting oligomerization. (A) Visualization of purification on 8–18% gradient SDS/PAGE stained with Coomassie blue. Lane 1, 19 μg protein lysate from *E. coli* containing wild-type (wt) PCD; lane 2, 21 μg protein from flow-through following loading wt-PCD-containing fractions onto nickel (II)-nitriloacetic acid-agarose column; lane 3, 9 μg wash with 60 mM imidazole (wt-PCD); lanes 4–6, purified wt, C81S and C81R PCD, respectively, 1–2 μg each lane. The sample buffer in all lanes contained 100 mM dithiothreitol. (B) Lanes 1 and 7, protein standards; lane 2, 7 μg wt PCD without reducing agent; lane 3, 5 μg C81S PCD without reducing agent; lane 4, 7 μg wt PCD with mercaptoethanol; lane 5, 13 μg PCD cross-linked with 2 μg $[MeOC(NH)C_2H_4S]_2$; lane 6, 13 μg wt PCD cross-linked with 0.4 μg $[MeOC(NH)C_2H_4S]_2$; lane 8, 13 μg C81S PCD cross-linked with 18 μg $[MeOC(NH)C_2H_4S]_2$. (a–d) indicate the positions expected for the mono-, di-, tri- and tetrameric forms of cross-linked wt (lane 5 and 6) and C81S PCD (lane 8). See Materials and Methods for further details.

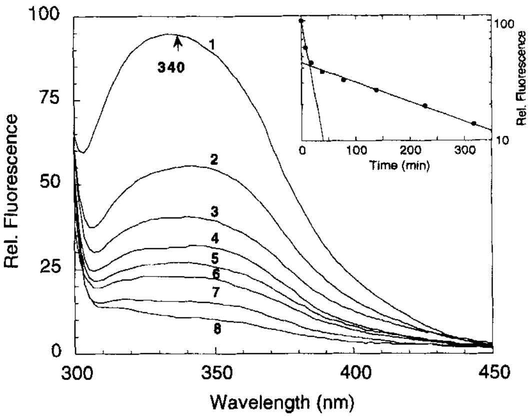


Fig. 2. Effect of ultraviolet irradiation on the tryptophan fluorescence of PCD. Wild-type PCD at a concentration of 1.1 μM in 100 mM Tris/HCl pH 8.4 was irradiated in the fluorimeter cell compartment with a $\lambda_{exc.}$ = 280 nm (bandwidth 10 nm). Curve 1, first emission spectrum recorded. Curves 2, 3, 4, 5, 6, and 7, were recorded upon irradiation for 8, 18, 38, 78, 138 and 228 min, respectively. Curve 8 is the emission spectrum recorded after a total of 318 min irradiation. The inset is the logarithmic time plot of the fluorescence intensity at 340 nm. The spectra are not corrected for instrumental artifacts.

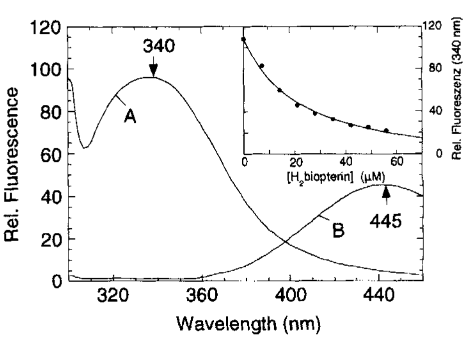
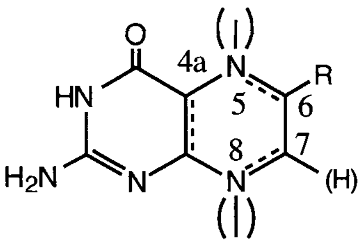
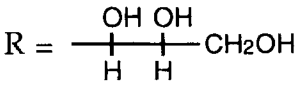
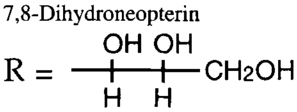
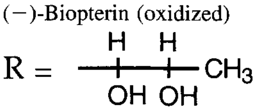
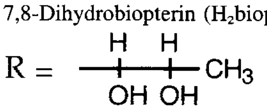
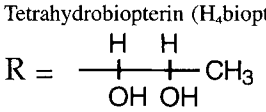
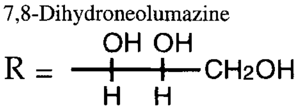


Fig. 3. Effect of H_2biopterin on the fluorescence emission of PCD. Wild-type PCD, 1.1 μM in 100 mM Tris/HCl pH 8.4, has the fluorescence emission spectrum of trace (A) in the absence of ligand, when excited at 280 nm. Addition of H_2biopterin at the increasing concentrations shown in the inset leads to essentially complete quenching of the tryptophan fluorescence emission (curve B, [H_2biopterin] = 580 μM). The emission of curve (B) is due to the chromophore of H_2biopterin. The fluorescence values shown in the inset were obtained by reading the emission intensity at fixed excitation (λ = 280 nm) and emission (λ = 340 nm) wavelengths for a short period (≈5 s) in order to minimize the photodecomposition described in Fig. 2. The spectra are not corrected for instrumental artifacts. The line through the experimental points in the inset is the best fit for a monophasic saturation curve (R^2 = 0.997, K_d apparent ≈20 μM). The pterins listed in Table 1 show similar behavior. Note that the concentrations of PCD is that of the monomer.

of 0.6 at 600 nm, and subsequently induced for expression for 3 h by adding isopropyl β-D-thio-galactopyranoside to a final concentration of 0.4 mM. Cells were harvested by centrifugation, resuspended in 5 mM imidazole, 0.5 M NaCl, 20 mM Tris/HCl pH 7.9, and 10% glycerol (buffer B), and frozen in liquid nitrogen. The frozen cells were lysed by gently thawing at 4°C and centrifuged for 20 min at 40000 rpm in a Sorvall T-1270 rotor. The supernatant was directly applied to a 10-ml nickel (II)-nitriloacetic acid-Sepharose (Quiagen) equilibrated in buffer B, washed with six column volumes of buffer B plus 60 mM imidazole. The histidine-tagged proteins were eluted with a linear gradient of 60–1000 mM imidazole in buffer B. The major protein peak, eluted between 200–400 mM imidazole, was pooled, dialyzed against 5 mM Tris/HCl pH 8.0, concentrated to 10 mg/ml protein using an Amicon YM3 membrane. Wild-type PCD and mutant proteins were stored at −70°C.

Protein analysis and modification. The N-terminal amino acid sequences were determined by Edman degradation and the molecular masses were defined by electrospray-ionization mass spectrometry (PE-Sciex API III instrument equipped with an ion-spray source; analyses performed by Drs P. Hunziker and D. Hess, Department of Biochemistry, University of Zürich). Protein concentration was determined by the BioRad assay and by the ultraviolet absorption of tyrosine and tryptophan residues. To visualize proteins, precast 8–18% SDS/PAGE gels and ExcelGel buffer strips were used from Pharmacia, and stained with

Table 1. Binding constants of various pterin analogs to wild-type (wt), C81S and C81R PCD. The K_d values were estimated as exemplified in Fig. 3. $\{h\}$ are the estimated Hill coefficients and refer to the equivalents of ligand bound to PCD monomer.

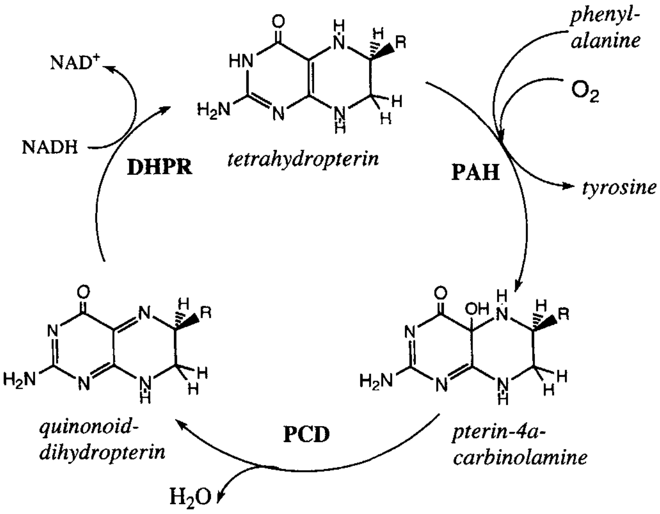
Pterin 	K_d $\{h\}$ for wt PCD	C81S PCD	C81R PCD
	µM		
(+)-Neopterin (oxidized) 	5 {0.6}	100 {0.6}	30 {1.2}
7,8-Dihydroneopterin 	20 {1.1}	60 {1.9}	40 {1.2}
(−)-Biopterin (oxidized) 	7 {0.6}	100 {1.4}	7 {0.7}
7,8-Dihydrobiopterin (H_2biopterin) 	20 {1.2}	80 {1.8}	20 {1.0}
Tetrahydrobiopterin (H_4biopterin) 	20 {1.2}	25 {0.8}	20 {1.1}
6-Aminomethylpterin R = $-CH_2-NH_3^+$	30 {0.9}	150 {1.5}	70 {1.0}
7,8-Dihydroneolumazine  , C(2)=O	15 {0.9}	100 {1.8}	20 {1.0}
Quinonoid 6,6-dimethyl-7,8-dihydro-pterin (q-6,6-Me_2H_2pterin) R = $(CH_3)_2$	0.9 {1.1}	20 {0.6}	1 {0.8}

Coomassie blue. Molecular mass standards were from BioRad. Sulfhydryl groups were modified with methyl-methanethiosulfonate (Me_2SO_2S) and 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2) according to Smith et al. [30] and Ellman [31]. Crosslinking was performed following the procedure of Baskin and Yang [32].

RESULTS

Expression and purification of human recombinant wild-type, C81S and C81R PCD. Recombinant PCD has been tagged at the COOH terminus with six histidine residues, which have a high binding affinity to immobilized Ni^{2+} ions [33], and expressed in *E. coli* BL21 (DE3) pLysS. This allowed isolation of as much as 20 mg (25% total soluble proteins) of pure enzyme from a 0.5-l culture in a one-step affinity purification procedure (see Fig. 1A). The ε_{280} of the pure protein, estimated as 12600 M^{-1} cm^{-1} for monomeric PCD based on the absorbancies of aromatic amino acids [34], was used for all concentration determinations of PCD. This value is comparable to that of ≈44000 M^{-1} cm^{-1} obtained by Huang et al. [35] for tetrameric PCD using the biuret method. Analysis by electrospray-ionisation MS of wild-type, C81S and C81R PCD produced spectra with major peaks corresponding to a protein molecular mass of 12690.56±1.65 Da, 12675.66±0.94 Da and 12744.37 ±1.9 Da, respectively. These mass values were approximately 131 Da less than the calculated masses (MacDNASIS 3.2. Hitachi software), implying that the recombinant proteins had the first N-terminal methionine cleaved off (residue mass of

Scheme 1. Reaction sequence, pterins, and enzymes involved in the hydroxylation of phenylalanine. PAH, phenylalanine hydroxylase; DHPR, dihydropteridine reductase.



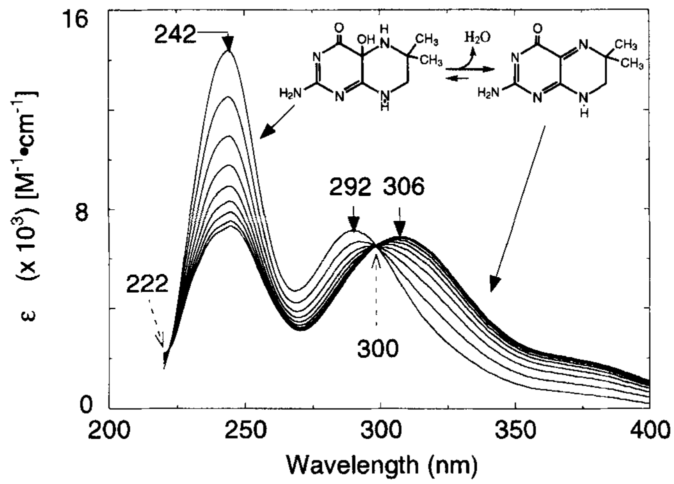


Fig. 4. Spectral characterization of the 6,6-Me_2-4a-OH-H_2pterin and changes occurring during dehydration to its quinonoid form. The spontaneous rate of dehydration is 1.2 min^{-1} in 10 mM Tris/HCl pH 8.5 at 4 °C.

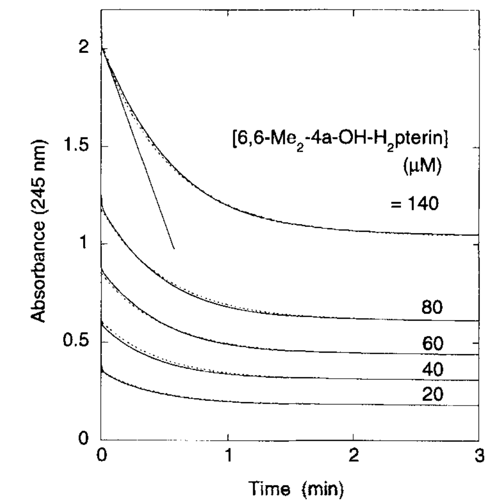


Fig. 5. Analysis of time and concentration dependence of dehydration of 6,6-Me_2-4a-OH-H_2pterin catalyzed by wild-type PCD. Conditions: [PCD] = 1 μM, in 10 mM Tris/HCl pH 8.5 and variable concentrations of 6,6-Me_2-4a-OH-H_2pterin at 4 °C. Concentrations of 6,6-Me_2-4a-OH-H_2pterin were determined from the final absorbance at 245 nm based on the ε_{245} derived from Fig. 4. (——) Experimental data. Note that a somewhat higher absorbance at the beginning of the traces is due to an experimental artefact connected with closure of the photometer chamber and beginning of recording. Monophasic curve fits (exponential decay) are superimposable to the experimental curve in all cases. (- - -) These traces represent the lines obtained by simulation using the reactions of Eqns (1) and (2), the kinetic parameters listed in Table 2, ε = 14500 M^{-1} cm^{-1} for 6,6-Me_2-4a-OH-H_2pterin and 7500 for q-6,6-Me_2-H_2pterin (the product), and the 6,6-Me_2-4a-OH-H_2pterin concentrations shown. The initial rate was determined numerically from the initial slope of the fit (example: straight line to the experimental curve at [6,6-Me_2-4a-OH-H_2pterin] = 140 μM).

131 Da). This was confirmed by amino acid analysis of wild-type PCD (not shown) and N-terminal amino acid sequence determination which yielded the sequence AGKAH. Therefore we conclude that all three recombinant PCD proteins start with Ala as the first residue similarly to what was found for the mature form of the human and rat enzymes which have N-acetylalanine as the N-terminal residue [9].

Fluorescence properties of PCD and binding of pterins and pterin analogs. As reported by Rebrin et al. [36], PCD exhibits a fluorescence with a $\lambda_{max} \approx 340$ nm when excited at 280 nm, the absorbance maximum of tryptophan (Fig. 2). PCD contains two Trp residues at positions 24 and 65 [9] which are located in the hydrophobic core of the monomer [17]. Time-dependent irradiation of PCD with ultraviolet light centered around 280 nm leads to a progressive decrease of the fluorescence emission (Fig. 2). First the emission maximum is shifted slightly to the red (Fig. 2, curves 2–4) and subsequently the tryptophan emission is essentially abolished; the residual emission (curve 8) is probably due to low-fluorescence decay product(s). The time dependence of this process (Fig. 2, inset) is strongly biphasic with each phase contributing about half of the total observed fluorescence decrease. Since the two Trp residues mentioned above are located in different environments [17], Trp65 not being accessible to solvent, it appears that the two fluorophores are affected differently by light irradiation. The present data do not, however, allow an assignment of the two phases of fluorescence decrease to photodestruction of either Trp24 or Trp65.

The tryptophan fluorescence is also affected by the addition of pterins and pterin analogs, as demonstrated in Fig. 3. For example, titration of PCD with H_2biopterin leads to a nearly complete fluorescence quenching. This has been used to determine the binding constants of a set of pterin analogs listed in Table 1. For these pterins binding to PCD leads to a monophasic decay of the fluorescence emission such as depicted in Fig. 3, inset. In a Hill plot using the same data, a linear dependence with a coefficient h = 1.2 is obtained.

Spontaneous dehydration of 6,6-Me_2-4a-OH-H_2pterin. Enzyme activity of PCD can be measured in an enzyme-coupled assay based on the reactions shown in Scheme 1. This method, however, is rather tedious in that it requires PCD preparations free of the accessory enzymes phenylalanine hydroxylase and dihydropteridine reductase, and suffers from the intrinsic problems of enzyme-coupled measurements. Therefore it appears highly desirable to develop a more direct assay for the activity of PCD, in particular to obtain a biochemical characterization of the catalyzed reaction. The question of whether the catalytic activity of PCD is affected by the presence of other enzymes such

Table 2. Velocities and kinetic parameters of PCD catalysis. V_{max} and K_m were calculated from the increase in velocity observed upon addition of PCD to the phenylalanine hydroxylase (PAH) assay (see legend to Fig. 8 for details) or estimated directly as shown in Fig. 6. Simulation was obtained as detailed in the text.

Parameter	Value for			
	wt PCD	C81S PCD	C81R PCD	simulation
V_{max} by PAH assay (acceleration factor)	9.9	9.5	11.9	
V_{max} by direct assay (nmol/min)	76	52	<20	
K_m by PAH assay (μM)	0.039	0.052	0.62	
K_m by direct assay (μM)	83	80		77
k_1 (M · min^{-1})				$3 \cdot 10^6$
k_{-1} (min^{-1})				150
k_2 (min^{-1})				80
K_3 (M)				$5 \cdot 10^{-5}$
k_4 (min^{-1})				1.5

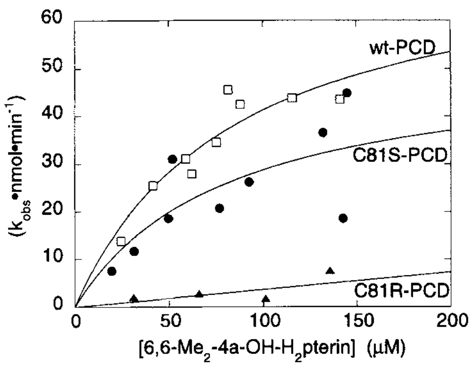


Fig. 6. Dependence of the rate of dehydration from the concentration of 6,6-Me$_2$-4a-OH-H$_2$pterin catalyzed by wild-type (wt), C81S and C81R PCD. Conditions: [PCD] = 1 μM, in 10 mM Tris/HCl pH 8.5 at 4°C. Freshly prepared 6,6-Me$_2$-4a-OH-H$_2$pterin (see Materials and Methods) was rapidly added to the sample cuvette containing PCD. The ensuing reaction was followed and analyzed as described in Fig. 5 (see also text). The rates observed are corrected for the spontaneous decay of the 6,6-Me$_2$-4a-OH-H$_2$pterin. The lines are the best fits to the data points obtained using the Michaelis-Menten equation. (R^2 = 0.86 and 0.55 for the wt and C81S PCD, respectively). See Table 2 for rate constants.

as phenylalanine hydroxylase [1, 2] also can be studied appropriately using a direct assay. Bailey et al. [37] and Lazarus et al. [2] have shown that carbinolamines of pteridines carrying simple substituents at position 6 are substrates of PCD. We have thus selected 6,6-Me$_2$-4a-OH-H$_2$pterin as substrate because its product, quinonoid 6,6-dimethyl-7,8-dihydropterin (q-6,6-Me$_2$-H$_2$pterin), does not undergo rearrangement to the 5,6-dihydro-tautomer but yields a stable end product, in contrast to 6-mono-substituted quinonoid 7,8-dihydropterins which rearrange readily. By virtue of its stability, q-6,6-Me$_2$-H$_2$pterin can in turn be used to study the effect of product inhibition on catalysis. The ultraviolet/visible spectrum of 6,6-Me$_2$-4a-OH-H$_2$pterin and its decay to the quinonoid form is depicted in Fig. 4, which also shows that the reaction can be conveniently monitored at 245 or 310 nm. The rate of spontaneous dehydration measured this way at 4°C and at pH 8.5 is 1.1−1.5 min^{-1}. It should be pointed out that this dehydration has been, reported by Bailey et al. [37] to be subject to general acid-base catalysis. We have not addressed this point in the present context.

Kinetic analysis of the spontaneous and PCD-catalyzed dehydration. The spontaneous decay of 6,6-Me$_2$-4a-OH-H$_2$pterin is a simple monophasic process (see Fig. 5). The reaction observed in the presence of PCD is more complex since it consists of the sum of the spontaneous decay (Eqn 1, below) and of the catalyzed reaction (Eqn 2), the two rates being of similar magnitude and having half-times of the order of 20−40 s at 4°C. For these reasons 4a-carbinolamine was produced *in situ* immediately before use (see Materials and Methods) and the occurring spectral changes were recorded until they had virtually ceased (3−5 min). A curve fit was then generated to the experimental data and the initial rate was determined numerically from the initial slope of the curve fit. The velocity obtained is the sum of the spontaneous (Eqn 1) and of the enzyme-catalyzed decay (Eqn 2) of the 6,6-Me$_2$-4a-OH-H$_2$pterin. The same analysis was done in parallel with traces obtained from the spontaneous decay, and subtraction of the latter from the former yields the velocity of the PCD-catalyzed reaction. The validity of the method and of the assumed scheme was assessed by simulation of the experimental data using the set of reactions (1 and 2), and as described in Materials and Methods.

$$\text{6,6-Me}_2\text{-4a-OH-H}_2\text{pterin} \xrightarrow{k_4} \text{q-6,6-Me}_2\text{-H}_2\text{pterin} \quad (1)$$

$$\begin{aligned}&\text{6,6-Me}_2\text{-4a-OH-H}_2\text{pterin} + \text{PCD}\\ &\underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{6,6-Me}_2\text{-4a-OH-H}_2\text{pterin} \approx \text{PCD}\\ &\xrightarrow{k_2} \text{q-6,6-Me}_2\text{-H}_2\text{pterin} \approx \text{PCD}\\ &\overset{K_3}{\rightleftharpoons} \text{PCD} + \text{q-6,6-Me}_2\text{-H}_2\text{pterin} \quad (2)\end{aligned}$$

where 6,6-Me$_2$-4a-OH-H$_2$pterin ≈PCD = Michaelis complex with substrate; q-6,6-Me$_2$-H$_2$pterin ≈PCD = Michaelis complex with product; k_1, k_{-1} = rates of substrate binding/dissociation; k_2 = rate of dehydration; K_3 = K_d of product binding to PCD and k_4 = rate of spontaneous decay of 6,6-Me$_2$-4a-OH-H$_2$pterin.

Thus using the rates listed in Table 2 and the absorption coefficients given in the legend to Fig. 5, simulations yielded the traces shown in Fig. 5, which are in fair agreement with the experimental ones. One exception is K_3, the binding of product (leading to inhibition) which is ≈50-fold higher than the K_d estimated by fluorescence quenching for the binding of the quinonoid pterin (q-6,6-Me$_2$-H$_2$pterin; Table 1). In agreement with the results of the simulation, initial rates of the PCD-catalyzed reaction were not influenced by the addition of product q-6,6-Me$_2$-H$_2$pterin to the assay immediately before that of PCD, ([q-6,6-Me$_2$-H$_2$pterin] ≤0.25 · [6,6-Me$_2$-4a-OH-H$_2$pterin] and ≥[PCD]; higher concentrations can not be used due to the high absorption of q-6,6-Me$_2$-H$_2$pterin). Product inhibition does thus not appear to play a role, at least in the case of q-6,6-Me$_2$-H$_2$pterin. As shown in Fig. 6, extrapolation to saturating 6,6-

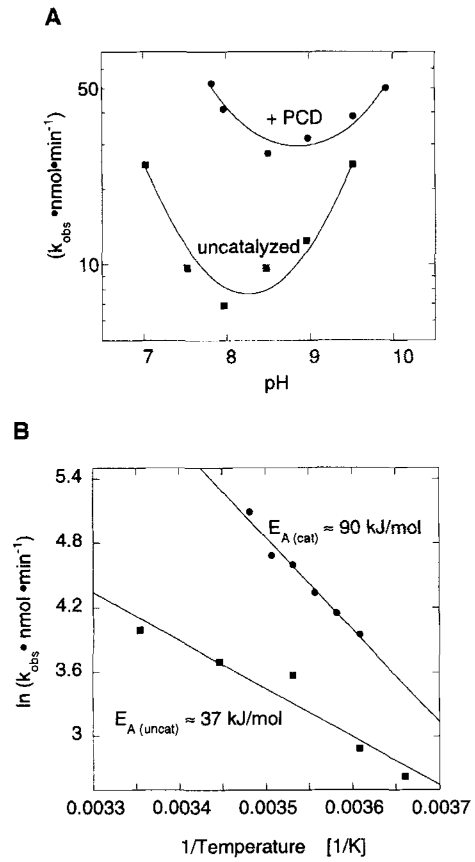


Fig. 7. Dependence of the observed rate of dehydration of 6,6-Me_2-4a-OH-H_2pterin on pH (A) and temperature (B). Conditions: [PCD] = 1 μM, [6,6-Me_2-4a-OH-H_2pterin] = 25 μM in 10 mM Tris/HCl, pH as shown. For the pH dependence (A) the solution contained [KCl] = 0.1 M, and the pH was adjusted with HCl. The curves are the best fit obtained using the appropriate H^+-dependence equation of k_{obs} [41]. The temperature dependence (B) was obtained at pH 8.5.

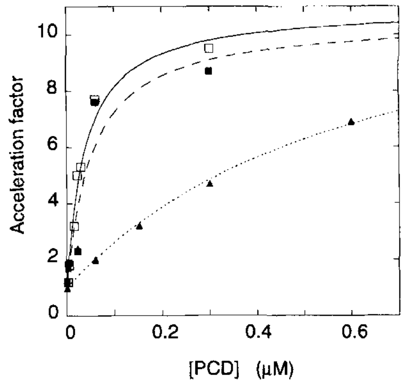


Fig. 8. Activity of wild-type (□), C81S (■) and C81R (▲) PCD in the phenylalanine-hydroxylase-coupled assay. Activities of PCD were measured in presence of 50 mM Tris/HCl pH 8.3, 20 μg catalase and 0.5 unit dihydropteridine reductase, 2.9 nmol H_4biopterin, 100 nmol NADH, 1 μmol phenylalanine at 25 °C. The activity is expressed as the rate acceleration of NADH consumption ($\Delta A_{340\,nm}$) compared to the same rate measured in the absence of enzyme. The lines are the best fits obtained using the Michaelis-Menten Eqn +1 (1 = no acceleration); the values obtained are listed in Table 2.

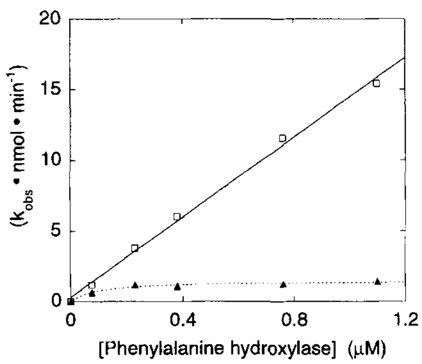


Fig. 9. Rate enhancement of phenylalanine hydroxylation by PCD in the phenylalanine-hydroxylase-coupled assay as a function of phenylalanine hydroxylase concentration. The rates in the absence (▲) or presence (□) of saturating concentration of PCD (> 1 μM) were determined at the phenylalanine hydroxylase concentration shown and under the conditions detailed in the legend to Fig. 8.

Me_2-4a-OH-H_2pterin concentrations yields a maximal (experimental) rate of ≈76 nmol 6,6-Me_2-4a-OH-H_2pterin consumption/min (sample volume = 1 ml, [PCD] = 1 μM), which is assumed to be the limiting rate at the given enzyme concentration. This value is reasonable keeping in mind the value of k_2 (= 80 min^{-1}) used to simulate the experimental data with the set of reactions (1) and (2) shown above. There is also a good agreement between the experimentally determined K_m and that resulting from the simulation ($(k_1 + k_2)/k_1$ (= K_m), Table 2; the comparatively low absolute values which were required for simulation indicate that k_1 can become partially rate-limiting, in particular at low 6,6-Me_2-4a-OH-H_2pterin concentration. These results demonstrate that PCD is competent in catalyzing dehydration of 4a-carbinolamines in the absence of phenylalanine hydroxylase.

Temperature and pH dependence of spontaneous dehydration of 6,6-Me_2-4a-OH-H_2pterin and of catalysis by PCD. The dehydration of 6,6-Me_2-4a-OH-H_2pterin is markedly dependent on pH and temperature as shown in Fig. 7. In addition, general acid-base catalysis was found in Tris/HCl (data not shown), as also reported by Bailey et al. for the 6-methyl-4a-carbinolamine [37]. The rate of dehydration for the uncatalyzed reaction is lowest around pH 8.5 and, for the enzyme-catalyzed reaction, the pH minimum shifts to a somewhat higher pH as shown in Fig. 7A. The Arrhenius plots of the temperature dependence of the uncatalyzed and catalyzed rates (activation energies ≈37 versus 90 kJ/mol, Fig. 7B) indicate that the rate of enzyme-catalyzed dehydration will be accelerated by a factor of ≈200 and ≈450 at 25 °C and 37 °C, respectively, where the reactions cannot be measured directly (see above). This indicates a moderate catalytic effectiveness of PCD for 6,6-Me_2-4a-OH-H_2pterin as substrate. This demonstrates the higher sensitivity of this assay method compared to the phenylalanine-hydroxylase-coupled assay, where acceleration factors of 9–10 are observed at 25 °C.

Effect of wild-type, C81S and C81R PCD on the phenylalanine-hydroxylase-catalyzed hydroxylation of phenylalanine, and on the formation of 7-pterins. The increase of the rate of the phenylalanine-hydroxylase-catalyzed reaction was noted earlier by Kaufman's group and an enhancement of ≈9 was reported for the enzyme isolated from rat liver [24]. The assay is based on the reactions depicted in Scheme 1, and the parameters are chosen such that dehydration is rate-limiting [24]. This assay was performed using phenylalanine hydroxylase purified according to the method of Shiman [20], which yields enzyme essentially devoid of PCD activity. The results are depicted in

Fig. 8. It is apparent from this that wild-type PCD, as well as the C81S PCD, have a similar stimulatory effect, corresponding to a factor of 9–10 at saturating enzyme concentration, while the C81R PCD shows a 20% lower factor at saturation. However the latter has an approx. tenfold higher K_m compared to that of the wild-type and C81S PCD with the natural substrate H_4biopterin (Table 2). We have performed the same assays using PCD freshly isolated from rat liver according to the method of Rebrin [38] and have found, within experimental error, the same acceleration factor and K_m as with wild-type PCD (not shown), which also correspond to the values reported by Kaufman [1, 24].

In this assay, the concentration of phenylalanine hydroxylase is critical for the extent of the rate acceleration obtained with PCD as demonstrated in Fig. 9. The saturating concentration of PCD was found to be ≈ 1 μM and, under these conditions, the observed NADH consumption increases linearly with the phenylalanine hydroxylase concentration up to a value of 80 nmol NADH/min. In the absence of PCD, as expected, the rate of NADH consumption saturates at [phenylalanine hydroxylase] ≈ 0.2 μM, where the spontaneous dehydration of biopterin-4a-carbinolamine probably becomes limiting. PCD partially purified from rat liver showed a similar rate acceleration in the coupled assay both in terms of the maximum velocity of NADH oxidation as well as the dependency on the PCD concentration (data not shown), indicating that recombinant PCD exhibits similar properties in terms of catalysis. The effect of PCD on the formation of 7-H_4biopterin was studied by HPLC as detailed earlier [8, 37]. Thus, in the absence of PCD, and under the conditions of Fig. 8 (incubation time = 15 min) $\approx 7\%$ of 7-pterin were found, while in the presence of 0.1 μM of both wild-type and C81R PCD, 7-pterins were not detectable (see also Materials and Methods).

Role of Cys81 in protein association and catalysis. We have studied the role of Cys81, the only sulfhydryl present in human PCD in oligomerization and catalysis. Reaction with the sulfhydryl reagents Nbs_2 or Me_2SO_2S did not inactivate wild-type PCD, which is in good agreement with the observation of activity of the C81S PCD described above. Reaction with Nbs_2 showed that $\approx 80\%$ of the Cys81 SH groups are present as free thiols. Under denaturing conditions (SDS and heat) and in the absence of a reducing agent, however, two bands are observed on SDS/PAGE (Fig. 1 B; for details see Materials and Methods); one of them is at ≈ 12 kDa, the position reported for monomeric PCD [19] and a second one at ≈ 25 kDa. In the presence of a reducing agent, such as mercaptoethanol, the 25-kDa band disappears completely. C81S PCD shows a single band on SDS/PAGE corresponding to ≈ 12 kDa, regardless of the presence of a reducing agent.

Crosslinking of wild-type PCD with $[MeOC(NH)C_2H_4S]_2$ leads to the appearance of four major bands on SDS/PAGE corresponding approximately to those expected for the mono-, di-, tri- and tetrameric forms of PCD. With wild-type PCD, due to the presence of Cys81, formation of a dimer can arise also from disulfide bridging. We have thus repeated the experiment using C81S PCD, where the same results were obtained (Fig. 1 B). In addition, we have confirmed the results of Hauer et al. [9] that the molecular mass of PCD is ≈ 45 kDa by gel filtration (Superose 12 HR, Pharmacia); this value, and thus the degree of oligomerization, is not modified when the filtration is performed in the presence of 2.2 μM q-6,6-Me_2-H_2pterin in the elution buffer. The same results were obtained using C81S PCD, which demonstrates that Cys81 does not play a significant role in oligomer formation.

DISCUSSION

Structural aspects. A major question, which has been the subject of some debate, is the state of oligomerization of PCD both in its function as a dehydratase, and also regarding its mode of interaction with HNF-1α. The dimerization cofactor of HNF-1α has been reported to be a dimer [39]; PCD, on the other hand, crystallizes as a tetramer, or, better, as a dimer of dimers [17]. A tetramer is consistent with the results of our gel filtration experiments performed in the presence and absence of q-6,6-Me_2-H_2pterin, which is one of the best ligands studied, and of the PAGE experiments under non-denaturing conditions [38] (and this work). Ligand binding thus does not appear to affect the state of oligomerization of PCD. Also, Cys81 does not appear to play any role in oligomer formation since essentially the same results were obtained in all pertinent experiments when C81S PCD was used. This is in full agreement with the three-dimensional structure which shows that Cys81 is at the protein surface and fully exposed to solvent [17]. The observed formation of polymers ($n \geq 2$) on gels in the absence of reducing agents is thus attributed to adventitious intermolecular formation of S-S bonds.

The photodecomposition experiments detailed in Fig. 2 are consistent with a nonequivalence of Trp24 and Trp65. This apparent inequivalency contrasts with the course of fluorescence emission quenching occurring upon binding of pterins and analogs, which is apparently monophasic (cf. Fig. 3) for all derivatives listed in Table 1, when measured under the conditions detailed in the legend of Fig. 3.

Binding of pterins. All pterins tested bind to wild-type PCD and quench the tryptophan fluorescence essentially $>95\%$. The K_d for binding of H_4biopterin to wild-type PCD (Table 1) is similar to that reported earlier by Rebrin et al. [36] for binding to PCD from rat liver. However, in the previous work, the photolability of the fluorescence was not recognized. There is no apparent correlation between specific structural features, such as the state of oxidation, substituents in the pyrimidine subnucleus or the nature of the C6 side chain and the binding constants K_d listed in Table 1. This suggests that the binding pocket(s) has a relatively high degree of flexibility and might not be designed to recognize only the 4a-carbinolamine of biopterin. The low interaction constant observed in the phenylalanine hydroxylase assay suggests that PCD has a much better affinity for the 4a-carbinolamine of biopterin compared to that of 6,6-dimethylpterin. Noteworthy is the discrepancy between the K_d determined by fluorescence quenching for q-6,6-Me_2-H_2pterin, which should reflect simple binding, and the absence of relevant inhibition during the assays; the latter being in agreement with the high binding constant (K_d) of q-6,6-Me_2-H_2pterin required for the simulations. From their data Ficner et al. suggest that the putative active site might be located in the vicinity of residues Asp60, His61, His62, Glu64, His79 and Glu80. From this Trp65 would be the one which could be located in close proximity of bound ligands and its fluorescence could be quenched directly or indirectly as a consequence of a conformational change induced by binding [17]. Nevertheless, from the three-dimensional data, the existence of multiple binding or recognition sites cannot be excluded [17]. This would be in line with the puzzling observation that the slope (h) of Hill plots (cf. Fig. 3 and Table 1) which reflect the stoichiometry of interaction of pterins with the different forms of PCD vary considerably. Specifically, finding of stoichiometries >1 suggests that binding can occur at different loci. On the other hand, values of $n > 1$ were found in general only in those cases when the apparent K_d is high (i.e. >20 μM). Conversely, when binding of the ligand is tight (K_d

< 20 μM), binding of one ligand per dimer (or ≈0.5 ligand/monomer) appears to be prevalent. In summary, the presence of different recognition sites, might be related to the bifunctional character of PCD. The clarification of the peculiar binding modes of pterins will have to await the results of ongoing experiments designed to study the mode and kinetics of binding of ligands to PCD and the results of crystallization studies of PCD with bound ligand(s).

While single structure elements of the pteridine do not appear to play specific roles in binding (Table 1), both mutations of Cys81 affected the binding strength of the ligands investigated. whereas the C81R mutation increases only the K_d of some ligands, C81S PCD exhibits a significantly weaker binding of all ligands (see Table 1). Conversely the K_m for 6,6-Me_2-4a-OH-H_2pterin is highest for C81R PCD while the difference between wild-type and C81S PCD is not significant. This is in agreement with the three-dimensional structure which suggests that position 81, while not being part of the putative active site, is located in its immediate vicinity [17].

Catalysis by PCD and mutants. The estimation of the catalytic effect of human wild-type PCD using the phenylalanine-hydroxylase-linked assay, originally introduced by Kaufman's group, yields values which are similar to those reported by Kaufman [1, 24] for rat liver enzyme and for the native enzymes. The finding of the same values for rat liver PCD, for wild-type PCD containing the His-tag motif at the C-terminal (this work) and for wild-type PCD devoid of the latter (Rhee, K.-H., Ficner, R. and Suck, D., personal communication) confirms that recombinant PCD folds similarly to native protein and yields enzyme with the same catalytic parameters. It should be noted that the estimated activity parameters are based on concentrations of PCD calculated assuming that the catalytic species is the monomer. The simulations of the primary kinetic traces of the dehydration of 6,6-Me_2-4a-OH-H_2pterin catalyzed by wild-type PCD allow a fair estimation of the rate-limiting step in catalysis as ≈80 min^{-1}. This corresponds to an enhancement of the catalyzed reaction by a factor 50–70 over the rate of the spontaneous decay (1.1–1.5 min^{-1}) under the conditions detailed in the legends to Figs 5 and 6. While this is a rather low value for an enzyme-catalyzed reaction, it should be noted that it reflects the rate increase measured at 4°C. Due to the different Arrhenius activation energies of catalyzed versus uncatalyzed reactions (Fig. 7B), the enhancement can be estimated as ≥450-fold at 37°C and pH 8.5 and will be higher at lower pH values. Based on this, and as suggested by Bailey et al. [37], the rate increase of the PCD-catalyzed dehydration of the 4a-carbinolamine of H_2biopterin at 37°C can be assumed by extrapolation to be much higher and sufficient to sustain efficient hydroxylation of aromatic amino acids. In this context it should be pointed out that the catalytic parameters of the two mutants are significantly different from those of wild-type PCD. In particular, both V_{max} and K_m of C81R PCD, i.e. the mutation encountered in a patient with a mild hyperphenylalaninaemia, are sufficiently different as to be responsible for the symptoms observed [16]. The finding that C81R PCD is as effective as the wild-type in suppressing the formation of 7-pterins during hydroxylation of phenylalanine catalyzed by phenylalanine hydroxylase suggests that the C81R mutation as such might not be sufficient for rationalizing the high level of 7-pterins found in some hyperphenylalaninaemic patients [8, 25]. An intriguing mechanism for the induction of a hyperphenylalaninaemia in patients with a defective PCD arises from the recent suggestion of Zhao et al. [40] that PCD might regulate the expression of phenylalanine hydroxylase in *Pseudomonas aeruginosa.*

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