Structure of Phenylalanine Hydroxylase from Colwellia psychrerythraea 34H, a Monomeric Cold Active Enzyme with Local Flexibility around the Active Site and High Overall Stability*§

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The characteristic of cold-adapted enzymes, high catalytic efficiency at low temperatures, is often associated with low thermostability and high flexibility. In this context, we analyzed the catalytic properties and solved the crystal structure of phenylalanine hydroxylase from the psychrophilic bacterium Colwellia psychrerythraea 34H (CpPAH). CpPAH displays highest activity with tetrahydrobiopterin (BH4) as cofactor and at 25 °C (15 °C above the optimal growth temperature). Although the enzyme is monomeric with a single L-Phe-binding site, the substrate binds cooperatively. In comparison with PAH from mesophilic bacteria and mammalian organisms, CpPAH shows elevated [S0.5](L-Phe) (1.1 ± 0.1 mM) and Km(BH4) (0.3 ± 0.1 mM), as well as high catalytic efficiency at 10 °C. However, the half-inactivation and denaturation temperature is only slightly lowered (Tm ≈ 52 °C; where Tm is half-denaturation temperature), in contrast to other cold-adapted enzymes. The crystal structure shows regions of local flexibility close to the highly solvent accessible binding sites for BH4 (Gly87/Phe88/Gly89) and L-Phe (Tyr114–Pro118). Normal mode and COREX analysis also detect these and other areas with high flexibility. Greater mobility around the active site and disrupted hydrogen bonding abilities for the cofactor appear to represent cold-adaptive properties that do not markedly affect the thermostability of CpPAH.

The strictly psychrophilic Colwellia psychrerythraea strain 34H, found in near-freezing Arctic marine sediments (1), possesses a variety of adaptations to low temperatures, as recently shown by comparative genome analysis (2). To obtain insight into the molecular determinants for cold-active enzymes of this organism, Methe et al. (2) prepared 2026 structure models based on 176 templates from the Protein Data Bank. There appears to be an extensive increase in flexibility in certain regions of the structures investigated, accompanied by high activity at low temperatures and some increase in thermostability (2).

A few enzymes from C. psychrerythraea have been isolated and characterized in detail. These included an extracellular cold-active aminopeptidase, which shows characteristic cold-adapted activity and structural properties (3) and two isocitrate dehydrogenase isoforms, one found to be monomeric and cold-active, and one found to be dimeric with a mesophilic activity profile (4).

The genome of C. psychrerythraea notably includes a phenylalanine hydroxylase (PAH) ortholog (Fig. 1), an enzyme that had not achieved prominence in the previous comparative genomics study (2). PAH is a non-heme iron-dependent enzyme, which in mammals is found mainly in the liver catalyzing the hydroxylation of L-Phe using molecular oxygen and (6R)-1-erythro-5,6,7,8-tetrahydrobiopterin (BH4) as co-substrates. In mammals this is the first step in the catabolism of L-Phe to carbon dioxide and water. In humans, excessively high levels of L-Phe are toxic to the brain, and a dysfunction of human PAH is associated with phenylketonuria (for review see Ref. 5). In fact, other severe human diseases (e.g. alcaptonuria, tyrosinemia, Richner-Hanhart syndrome, and hawkinsinuria) are also associated with enzyme deficiencies in the catabolism of L-Phe and L-Tyr.

Over the past few years, the functional and structural studies of several forms of human and rat PAH, including free and ligand-bound states and disease-related mutants, have advanced the understanding of the main catalytic features of the enzyme and genotype-phenotype relationships in phenylalanine hydroxylase; BH4, 7,8-dihydrobiopterin; BH4, 6,7-dihydrobiopterin; BH4, 6,7,8-tetrahydrobiopterin; CpPAH, PAH from Colwellia psychrerythraea; CpPAH, PAH from Chromobacterium violaceum; DMPH4, 6,7-dimethyl-tetrahydropterin; hPAH, human PAH; MHi6, 6R)-5,6,7,8-tetrahydro-L-ornithinase; NLE, L-norleucine; NMA, normal mode analysis; r.m.s.d., root mean square deviation; THA, 3-(2-thienyl)-L-alanine; WAS, water-accessible surface; PDB, Protein Data Bank.

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ketonuria (6–9). In bacteria, PAH is relatively rare and is found predominantly within the phylum Proteobacteria. PAH from Chromobacterium violaceum (CvPAH) and Pseudomonas aeruginosa have been cloned, purified, biochemically characterized, and crystallized (10–15). Bacterial PAH crystal structure determination has been reported only for CvPAH, which shares the fold of the catalytic domain of human and rat PAHs (14).

The physiological role of PAH in bacteria is unclear, but studies of the PAH system in P. aeruginosa have provided some insights into the catabolic function of the enzyme. Interestingly, expression of PAH (PhhA) and of a bacterial ortholog of pterin 4a-carbinolamine dehydratase (PhhB), both included in the same operon, is required to enable the organism to utilize L-Phe as a sole carbon source (16, 17). However, the finding that activation of both PhhA and PhhB expression by L-Phe occurs also in the presence of better carbon sources such as glucose indicates that this operon might be dedicated to the provision of derivative compounds of L-Phe (17).

Functional studies of bacterial enzymes have improved our understanding of bacterial metabolism, adaptation, and evolution and are also relevant to the understanding of the mammalian orthologs. The evolution of mammalian PAH and of the other aromatic amino acid hydroxylases, i.e. tyrosine hydroxylase and tryptophan hydroxylases, 1 and 2, appears to have proceeded via juxtaposition of bacterial PAH with a precursor of the regulatory domain of chorismate mutase-prephenate dehydratase (P-protein) (18–21), corresponding to the N-terminal regulatory domain of the hydroxylases. Tetrameric eukaryotic PAH, which is highly regulated both by phosphorylation and positive cooperativity for L-Phe, could thus have evolved from a monomeric bacterial PAH.

Here we report the cloning, expression, purification, and the structural and functional characterization of the monomeric bacterial PAH from the psychrophilic bacterium C. psychrerythraea (CpPAH). The structure of the enzyme has been analyzed and compared with that of ortholog PAHs from the mesophilic prokaryote C. violaceum (CvPAH; optimum growth temperature, 25 °C) and human (hPAH). The structural features help to explain the kinetic properties and particular cold-adaptive characteristics of CpPAH.
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MATERIALS AND METHODS

Growth of C. psychrerythraea 34H and Detection of Gene Expression—C. psychrerythraea 34H was grown to stationary phase in Marine Broth (Difco 2216) at 9 °C. Pellets were resuspended in freshly prepared Tris-EDTA buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) with lysozyme (0.4 mg/ml) and incubated at room temperature for 5 min before the total RNA was isolated using the SV (“spin or vacuum”) total RNA isolation kit according to the manufacturer’s instructions (Promega). PAH mRNA was subsequently detected using the following primer and the reverse transcriptase SuperScript™ (Invitrogen): 5′-CCCTAAGCTCGAGACTGCTTTTTCACGGGAAATT-3′ and 5′-CCCTAAGCTCGAGACTGGCTTTTTCACGGGAAATT-3′. Amplification was performed using Phusion™ DNA polymerase (Finnzymes) in a PCR using the following primers: 5′-GGGAATTCCATATGGCAAAAGG-AACAAAGTATGTT-3′ and 5′-GGGAATTCCATATGGCAAAAGG-AACAAAGTATGTT-3′ (Ndel and XhoI restriction sites are underlined). C. psychrerythraea genomic DNA was used as positive control (not shown).

Cloning, Expression, and Purification—The PAH gene from C. psychrerythraea 34H (CpPAH) was amplified by PCR using the primers described above. The PCR products were purified using the Stratagene PCR purification kit, digested with Ndel and BamiHI, and ligated into Ndel-BamiHI-digested pET-30a using the rapid DNA ligation kit (Roche Applied Sciences). Recombinant CpPAH was overexpressed in Escherichia coli strain BL21 Star™(DE3) by growing transformed cells in LB broth containing kanamycin (30 μg/ml) at 37 °C and induction with 1 mM isopropyl β-D-galactopyranoside. Eight hours after induction at 22 °C (A600 nm = 0.7–0.8), the cells were harvested and disrupted using a French press, and the supernatant was applied to a TALON® Superflow metal affinity resin column (Clontech). C-terminal His6-tagged-CpPAH was purified at 4 °C by washing with 10 mM imidazole and eluting with 150 mM imidazole in 20 mM sodium phosphate, 300 mM NaCl, pH 7.5, followed by size-exclusion chromatography on a HiLoad Superdex 200 column, which also removed the imidazole. The enzyme was kept concentrated in 20 mM NaHepes, 200 mM NaCl, pH 7.0, in liquid nitrogen. The catalytic domain (Gly103–Gln428) of human PAH (hPAH) was purified as described previously (22).

Activity Measurements—Steady-state PAH activity was measured by high pressure liquid chromatography with fluorimetric detection of L-Tyr (23, 24), with reactions mixtures containing 100 mM Hepes, pH 7, catalase (0.04 mg/ml), and the indicated concentrations of L-Phe and BH4 (with 5 mM dithiothreitol) were used. Under the selected standard assay conditions, the amount of product was linear with time (up to 5 min) and amount of enzyme (up to 5 μg when L-Phe and BH4 were added simultaneously to initiate the assay). [S0.5] and Km values were determined at the specified temperature and concentrations of L-Phe and BH4 (with or without 5 min preincubation with L-Phe) and customarily with 1 μg of CpPAH and a 1-min assay. The data were fitted to a Hill equation (for L-Phe) or to a Michaelis-Menten equation (for BH4). Thermal inactivation experiments were performed by incubating the enzyme at the indicated temperatures in 20 mM NaHepes, 200 mM NaCl, pH 7, in the absence and the presence of 5 mM L-Phe for 10 min, followed by chilling of the samples on ice and immediate assay (1 min at 10 °C).

Tyrosine hydroxylase activity was measured at 10 and 20 °C as described (25), using reaction mixtures containing 0.2–1.0 μM of purified CpPAH, 0.05 mg/ml catalase, 20 μM ferrous ammonium sulfate, and 50 μM L-Tyr and initiating the reactions by adding BH4 (up to 1 mM) with 5 mM dithiothreitol, and up to a 10-min reaction time. The L-Tyr-dependent oxidation of the cofactor was measured using a similar reaction mixture but additionally including 200 μM NADH and 0.4 units of dihydropteridine reductase (from sheep liver; Sigma). The reaction was started by adding 75 μM BH4 and ΔA340 was recorded for 1 min. The rate of NADH oxidation (pterin oxidation) was calculated using ε340 nm = 6220 M−1·cm−1.

CD Spectroscopy—CD was performed as described (26), with 25 μM CpPAH in freshly made and degassed 20 mM NaHepes, 200 mM NaCl, pH 7.0. Stoichiometric amounts of ferrous ammonium sulfate were added when indicated. The samples were incubated for 10 min at the indicated temperature prior to acquisition of the CD spectra at the same temperature. CD-recorded thermal denaturation was also performed in the same samples as described (26) by following the changes in ellipticity at 222 nm, with a scan rate of 1 K/min in the range 4–75 °C. The amount of α-helical structure was estimated with the program CDNN, which employs a back-propagation neural network model for the quantitative analysis of protein UV CD spectra (27).

Differential Scanning Calorimetry (DSC)—DSC was performed as described (26), using 32 μM CpPAH in 20 mM NaHepes, 200 mM NaCl, pH 7.0, in the absence and the presence of L-Phe (5 mM) at 1 K/min scan rate, typically from 4 to 75 °C.

Crystallization, Data Collection, and Structure Determination—Crystallization was performed using the hanging drop method. Crystals of native CpPAH were grown by mixing 1-μl drops of 10 mg/ml protein (in 20 mM NaHepes, 200 mM NaCl, pH 7.0) with a 1-μl reservoir solution containing 1.6–1.8 mM ammonium sulfate, 100 mM NaCl, and 20 mM NaHepes, pH 7.5. The drops were equilibrated at 4 °C, and crystals suitable for data collection purposes appeared after about 3 weeks. The crystals had overall dimensions of ~120 × 100 × 80 μm³, were cryo-protected with 25% glycerol and the reservoir additives, and flash-cooled in a nitrogen stream (Oxford Instruments) operating at 100 K. One crystal was soaked in 1 mM 7,8-dihyd罗pterin (BH2), 1.9 mM ammonium sulfate, 80 mM NaCl, 20 mM Heps, pH 7.5, and 25% glycerol for 15 min at 4 °C and then flash-cooled. This surprisingly resulted in the apo form of CpPAH with the iron atom missing in the crystal structure.

All diffraction data were collected at the European Synchrotron Radiation Facility (ESRF), Grenoble, France, at ID14-EH1 (for the native, holo-CpPAH) and at the Swiss-Norwegian Beamline (CpPAH-apo). The crystals belong to the space group P21 with unit cell dimensions for the native structure of a = 40.28 Å, b = 86.02 Å, c = 87.57 Å, and β = 97.01°. The two molecules in the asymmetric unit (A/B) give a water content of 49% and a Matthews coefficient of 2.5 Å³/Da. The native structure was solved by the molecular replacement method using
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TABLE 1
X-ray data collection statistics for CpPAH and CpPAH-apo

| X-ray parameters | CpPAH | CpPAH-apo |
|------------------|-------|-----------|
| Beamline         | ID14-EH1 | SNBL     |
| Space group      | P2    | P2        |
| Unit cell        | a = 40.28 Å | a = 40.43 Å |
|                  | b = 86.02 Å | b = 86.29 Å |
|                  | c = 87.57 Å | c = 87.67 Å |
|                  | β = 97.01° | β = 96.64° |
| Resolution (Å)   | 45-1.5 | 45-1.95   |
| (Highest bin)    | (1.58-1.50) | (2.06-1.95) |
| Wavelength (Å)   | 0.934 | 0.873     |
| No. of unique reflections | 94,151 | 42,547 |
| Multiplicity     | 4.2 (4.1) | 2.4 (2.4) |
| Completeness (%) | 99.6 (97.5) | 97.6 (96.8) |
| Mean ⟨I/(σI)⟩   | 12.7 (2.7) | 7.0 (2.3) |
| R<sub>free</sub> (%) | 7.2 (46.2) | 11.0 (40.6) |
| Wilson B-factor (Å<sup>2</sup>) | 12.65 | 17.06 |

MOLREP (28) and the crystal structure of PAH from C. violaceum (CvPAH, PDB 1LTV) as search model. The refinement and incorporation of solvent molecules were done using REFMACS (29) interspersed with rounds of manual rebuilding in O (30). The final native model includes 536 residues, 2 iron atoms, and 847 water molecules, resulting in an R-factor of 19.52%. All ion pairs were included in the refinement statistics, see Tables 1 and 2, respectively.

Structural Analysis—Root mean square deviations (r.m.s.d.) were calculated with the Secondary Structure Matching (SSM) data base. The program HBPLUS Version 3.15 (31) was used to calculate hydrogen bonds satisfying the following criteria for the parameters Donor (D), Acceptor (A), Acceptor Antecedents (AA), and (calculated) hydrogen (H): maximum distances for D-A 3.5 Å, H-A 2.5 Å; minimum angle for D-H-A, D-A-AA, and H-A-AA of 90°. All ion pairs were included in the calculations.

The WHAT IF Web Interface (32) was used to identify ion pairs with interatomic distances <6 or <4 Å between the side chains of Asp or Glu to Arg, Lys, or His residues. Electrostatic surfaces were calculated with DelPhi (33) and displayed with PyMOL (DelLano Scientific, San Carlos, CA).

Models of CpPAH with only BH<sub>4</sub> and with BH<sub>4</sub> and the substrate analog (2-thienyl)-L-alanine (THA) were prepared based on the hPAH·BH<sub>4</sub> (PDB 18528) and hPAH·Fe(II)·BH<sub>4</sub>·THA (PDB 1MMK) crystal structures.

Computational Structural Analysis—The low frequency normal modes of CpPAH (molecules A and B), CvPAH (PDB 1LTV) and hPAH (PDB 1PAH), which provide information on regions with slow motions, were computed and analyzed by normal mode analysis (NMA) using the web server WebNM (34). The regional folding/unfolding conformational fluctuations and residue-specific stability constants were calculated using the COREX/BEST algorithm (35) on the COREX/BEST web server (36). A more detailed description of the algorithms and experimental details for NMA and COREX calculations is provided at the servers (34, 36). Calculations of the energies of charge-charge interactions were performed using a Tanford-Kirkwood model with the solvent accessibility correction of Gurd (at pH 7); other methodological details are given in Refs. 37, 38. Dielectric constants of 4.0 and 78.5 were used for the protein and the aqueous solvent, respectively. In this study, we calculated the energy of charge-charge interactions of group i with the rest of the ionizable groups in the protein (W<sub>i</sub>), which can be used to estimate the total charge-charge interaction energy in the proteins (⟨W<sub>q,q</sub>⟩), as shown in Equation 1.

\[
⟨W_{q,q}⟩ = \frac{1}{2} \sum_{i=1}^{n} ⟨W_i⟩
\]  

RESULTS

Expression of PAH in C. psychrerythraea 34H; Cloning, Expression, and Purification of CpPAH—C. psychrerythraea displayed a growth optimum around 9 °C when cultivated in Marine Broth at temperatures from 0 to 15 °C. Reverse transcriptase PCR confirmed that PAH was expressed in C. psychrerythraea cultivated under these conditions (Fig. 2A). Addition of L-Phe at concentrations of up to 30 mM added to the Marine Broth medium stimulated the growth. This is opposite to what is found for E. coli and other bacteria that do not possess PAH (39) where L-Phe in high amounts appears to be toxic. Nevertheless, C. psychrerythraea 34H did not grow in minimal medium (M9), and the addition of L-Phe (up to 50 mM) to this medium did not stimulate growth.

After cloning the CpPAH gene into the vector pET-30a, the enzyme was successfully expressed in E. coli, and purified by chromatography on TALON<sup>®</sup> resin and consequent size-exclusion chromatography. CpPAH showed apparent molecular masses of 31 and 28 kDa by SDS-PAGE and analytic gel filtration chromatography, respectively (Fig. 2B and data not shown). The enzyme, with estimated molecular weight from the amino acid sequence of 30.71 kDa (31.77 kDa when the His tag is included), thus appears to be monomeric.

Catalytic Properties of CpPAH—The enzyme activity is stimulated (40–70% depending on assay conditions and temperature) by the addition of 100 μM Fe(II) added as ferrous ammonium sulfate. This stimulation by iron is also found in other non-heme iron-dependent aromatic amino acid hydroxylases.
expression and purification of cloned CpPAH in E. coli. A. expression of PAH in C. psychrerythraea grown in Marine Broth at 9 °C. Lane 1, 1-kb DNA ladder (Invitrogen); lanes 2 and 3, amplification of first strand synthesis reactions (reaction in lane 2 had half the starting RNA amount in first strand synthesis than that in lane 3); lane 4, amplification of first strand synthesis reaction without C. psychrerythraea RNA (negative control). B. purification of CpPAH. SDS-PAGE gel showing low molecular weight standards (lane 1), supernatant of cell extract (E. coli) after French press (lane 2), and purified CpPAH (lane 3).

FIGURE 2. Characterization of the CpPAH activity. A, effect of temperature on the enzyme activity measured with 0.5 mM BH₄ and 5 mM L-Phe (1 min assay). Inset, Arrhenius plot for activity in the 0–25 °C range giving Ea = 4.9 ± 0.3 kcal/mol. B, effect of L-Phe concentration on CpPAH activity at 10 °C, 0.5 mM BH₄ and 1 min assay, with (○) 5-min preincubation at the indicated concentrations of L-Phe. Similar concentrations of L-Phe were used in the preincubation and in the assay. C, time course of L-Phe hydroxylation by CpPAH without (●) (simultaneous addition of L-Phe and BH₄) and with (□) 5-min preincubation of the enzyme with 5 mM L-Phe. Note the lag phase in the time course for the L-Phe-preincubated enzyme. D, time course for the recovery of CpPAH activity after inhibition of the enzyme by preincubation for 10 min with 10 mM L-Phe. At time 0, the preincubation was stopped by 100-fold dilution of the enzyme in 100 mM Hepes, pH 7, and catalase (0.04 mg/ml), and the activity was assayed at the indicated times after dilution by simultaneous addition of L-Phe (5 mM) and BH₄ (0.5 mM). Inset, inhibition of CpPAH as a function of the concentration of L-Phe used in preincubation for 5 min.

The nature of the cofactor for the bacterial PAHs is unclear. For eukaryote forms of the aromatic amino acid hydroxylases, it has been shown that 6(R)-BH₄ (BH₄) is the natural cofactor (42). C. psychrerythraea and other bacteria that contain the PAH gene also include enzymes supporting BH₄ synthesis and recycling, but it was interesting to investigate the capacity of other cofactors to sustain CpPAH activity. We selected the following: (i) synthetic 6,7-dimethyl-tetrahydropterin (DMPH₄), which elicits high activity in CvPAH (41); (ii) (6R)-5,6,7,8-tetrahydro-L-monapterin (MH₄), which is the most abundant pterin in E. coli (43), although E. coli does not contain PAH; and (iii) tetrahydrofolate, which has been found to support nitric-oxide synthase activity in Bacillus subtilis (44, 45). We thus compared the BH₄-supported activity of CpPAH (1680 nmol of L-Tyr/min/mg at 0.5 mM BH₄ and 5 mM L-Phe at 10 °C) with that obtained with these other cofactors. The activities relative to BH₄ were 20, 17, and 7% for tetrahydrofolate, DMPH₄, and MH₄, respectively, also assayed at 0.5 mM. This indicates that BH₄ is the probable natural cofactor for CpPAH. Further characterization of the enzyme was thus performed with BH₄.

With respect to substrate specificity, CpPAH did not catalyze the hydroxylation of L-Tyr measured as l-DOPA production when analyzed at concentrations of both cofactor and L-Tyr up to 1 mM. With L-Tyr, CpPAH only catalyzed cofactor oxidation (as seen by the NADH-dependent dihydropteridine reductase activity [data not shown]), which is similar to the PAHs investigated so far (46, 47).

Preliminary activity assays indicated that maximal activity of CpPAH was obtained at higher concentrations of substrate and cofactor than for the human enzyme (hPAH) (48) and CvPAH (41). We therefore adjusted the concentrations used in the customary standard PAH assays from 1 mM L-Phe and 75 μM BH₄ to 5 mM L-Phe and 0.5 mM BH₄ in order to investigate the catalytic properties of CpPAH. At these conditions, CpPAH had an optimum temperature for activity of about 25 °C (Fig. 3A). The activation energy of the reaction catalyzed by CpPAH (Ea = 4.9 ± 0.3 kcal/mol) is lower than for mesophilic CvPAH (13), indicating that CpPAH possesses cold-adapted features. Studies of psychrophilic enzymes show that they have lowered their Ea in order to reduce the temperature dependence of kcat, making catalysis less affected by a decrease in temperature (49, 50).

The enzyme activity was about eight times higher when simultane-ously adding BH₄ and L-Phe (or
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TABLE 3
Catalytic properties of CpPAH at 10 and 25 °C

Parameters were measured without or with (values in parentheses) 5 min of preincubation with l-Phe prior to the start of the assay by addition of BH₄. Steady-state conditions are not met when the enzyme is preincubated with l-Phe (Fig. 3C).

| Temperature (°C) | Preincubation with l-Phe | Vₘₐₓ (µmol l-Tyr/min·mg) | [S₀.₅](l-Phe) (mM) | Hill coefficient (h) | Kₘ(BH₄) (mM) |
|------------------|--------------------------|--------------------------|-------------------|-------------------|---------------|
| 10               | No                       | 1800 ± 101               | 1.1 ± 0.1         | 1.9               | 0.3 ± 0.1     |
| 10               | Yes                      | (300 ± 38)              | (0.6 ± 0.1)       | (2.5)             |               |
| 25               | No                       | 2800 ± 240              | 1.3 ± 0.2         | 2.0               | 0.5 ± 0.2     |
| 25               | Yes                      | (451.5 ± 26.4)          | (0.8 ± 0.1)       | (2.2)             |               |

*Measured at 0.5 mM BH₄ in l-Phe saturation curves. Fitting was performed in the concentration range 0–5 mM l-Phe due to substrate inhibition.

*Measured at 5 mM l-Phe in the BH₄ saturation curves.

by 5 min-preincubation with the BH₄ cofactor) compared with the activity obtained after preincubation with l-Phe (Fig. 3B). This contrasts what is found for the mammalian forms of PAH, which are activated only by preincubation with l-Phe (22, 51). To study this inhibitory effect of preincubation with l-Phe, we investigated the time course of l-Tyr formation by CpPAH. For the preincubated enzyme a lag phase was observed (Fig. 3C), although the time course on simultaneous addition of substrate and cofactor was linear, satisfying initial velocity and steady-state conditions. Moreover, the inhibition upon incubation with l-Phe (5 mM) is time-dependent with a calculated pseudo-first-order rate constant (kₐₙₐₜ) of 0.22 ± 0.03 min⁻¹ (data not shown). The inhibition was also dependent upon the concentration of l-Phe (Fig. 3D, inset) and appeared to be reversible (Fig. 3D). Elucidation of the kinetic mechanism of this inhibition would require the determination of the catalytic mechanism for the enzyme, including the order of binding for the reactants. However, the sigmoidal saturation curves for l-Phe obtained at all concentrations of cofactor tested (see below), in addition to the substrate inhibition, prevented detailed steady-state analyses of CpPAH. A previous study with noncooperative CvPAH with synthetic DMPH₄ as cofactor concluded with an ordered mechanism where l-Phe binds as the second substrate, after the pterin cofactor (41). Thus, assuming a similar mechanism for CpPAH, our results on the inhibition by l-Phe preincubation would be best explained by formation of long-lived dead-end complexes with the substrate, leading to different activities depending on the preincubation scheme (Fig. 3B).

The activity of CpPAH as a function of l-Phe and cofactor concentration was measured both at 10 °C, close to the optimum growth temperature for C. psychrerythraea, and at 25 °C. Considering that CpPAH is a monomeric enzyme, it was remarkable that its activity as a function of substrate concentration resulted in sigmoidal curves both with and without preincubation with l-Phe (Hill coefficient (h) ~ 2; see Fig. 3B and Table 3). The concentrations of l-Phe for half-maximal activity ([S₀.₅](l-Phe)) depended on the l-Phe preincubation scheme (Table 3). The BH₄-dependent activity showed hyperbolic kinetics, and the Kₘ(BH₄) values are also included in Table 3. The catalytic efficiency (Vₘₐₓ/Kₘ) and Vₘₐₓ/[S₀.₅] values) appears to be maximal at 25 °C and also high at 10 °C. Both the Kₘ(BH₄) and [S₀.₅](l-Phe) values were about 10 times higher than other PAHs characterized so far (at 25 °C), which show [S₀.₅](l-Phe) in the range 90–300 µM and Kₘ(BH₄) about 20–30 µM for mammalian enzymes (48, 51, 52), and even lower for CvPAH (41).

Many enzymes from psychrophilic organisms show elevated Kₘ levels for substrates and cofactors compared with mesophilic homologs, indicative of reduced affinity (49, 50). Moreover, the Kₘ values often increase further with increasing temperature (53). We observed a slight increase in both the Kₘ(BH₄) and [S₀.₅](l-Phe) of CpPAH when the assay temperature was raised from 10 to 25 °C (Table 3). This is the opposite to what is observed for the temperature effect of Kₘ(l-Phe) in the mesophilic CvPAH (41). PAH catalyzes a complex reaction with three reactants, and the Kₘ values are usually higher than the equilibrium dissociation constants (Kₐ) (54). We therefore also measured the thermodynamic parameters for the equilibrium binding of l-Phe at both temperatures by ITC, and these results more clearly support a decrease in affinity with increasing temperature for l-Phe (supplemental Fig. S1 and Table S1). The almost 3-fold increase in Kₐ from 10 to 25 °C arises mainly from an increased entropic penalty to binding (supplemental Table S1). In addition, the binding assays confirmed the kinetic origin of the positive cooperativity because the binding isothersms were hyperbolic (supplemental Fig. S1).

Thermal Effect on the Conformational Stability of CpPAH—

As earlier found for PAHs from other sources (55, 56), the far-UV CD spectrum of CpPAH (200–260 nm) recorded at 4 °C shows two minima at 208 and 222 nm (Fig. 4A), characteristic for proteins with high α-helical content. Processing the spectra with the program CDNN (27) provided a 40% α-helix content for CpPAH, a content that is not affected by addition of Fe(II) or l-Phe, up to 5 mM (data not shown). However, the CD spectrum in the presence of the substrate showed a high level of noise at <210 nm, and an accurate estimation of any change in secondary structure could therefore not be performed. On the other hand, higher temperatures induced a reduction in α-helical structure (8% decrease from 4 to 37 °C; Fig. 4A). This conformational change was found to be almost reversible up to 37 °C because a helical content of ~40% was also estimated by CDNN after cooling the sample back from 37 to 4 °C.

Thermal denaturation experiments monitored by either CD (ellipticity changes at 222 nm) (Fig. 4B) or DSC (Fig. 4C) resulted in one thermal transition with a Tₘ ~ 52 °C. CpPAH unfolds reversibly, but it appears that the unfolding transition obtained by DSC can be fitted to equilibrium thermodynamic models, because the Tₘ is not significantly affected by the scan rate from 0.25 to 1.5 K/min (data not shown) (57). The unfolding parameters obtained by DSC were Tₘ = 52.1 ± 0.1 °C and calorimetric enthalpy change (ΔH) = 94.6 ± 1.3 kcal/mol. These values were similar in the presence of stoichiometric
amounts of Fe(II), but when the experiments were performed with 5 mM l-Phe the $T_m$ increased to 56.4 ± 0.1 °C ($\Delta H = 96.6 \pm 0.6$ kcal/mol) (Fig. 4, B and C). Inactivation experiments, where CpPAH was preincubated for 10 min at temperatures from 0 to 70 °C prior to assay of remaining activity at 10 °C, showed half-maximal inactivation at 47 and 52 °C when samples were preincubated in the absence and presence of 5 mM l-Phe, respectively (data not shown). These results, together with the $T_m$ values obtained by CD and DSC indicate a relatively high thermostability of the enzyme. A comparison of the $T_m$ values for CpPAH with those obtained for CvPAH and the catalytic domain (Gly$^{103}$–Glu$^{429}$) of human PAH (hPAH) are shown in Table 4.

**TABLE 4**

$T_m$ values obtained by CD-monitored thermal unfolding and DSC

| PAH | l-Phe | $T_m$ by CD °C | $T_m$ by DSC °C | Reference |
|-----|-------|---------------|---------------|-----------|
| CpPAH | 0 | 52 | 52 | This work |
| CpPAH | 5 | 55 | 56 | This work |
| CvPAH | 0 | 62 | 64 | 13 |
| hPAH | 0 | 55 | 53 | This work |
| hPAH | 1 | 59 | 57 | This work |

The Crystal Structure of CpPAH—The final native model of CpPAH included 536 amino acids from the two molecules (A/B) in the asymmetric unit. Both molecules lack the first three residues, and molecule B has eight additional residues comprising the C-terminal His tag. The active site residues of each monomer contain one iron atom coordinated by His$^{122}$, His$^{127}$, and Glu$^{167}$ and two water molecules (W1 and W2). The final R-factor is 16.4%; R-free is 19.5%, with a satisfactory geometry of the model (Table 2).

Fig. 5 shows the superposition of molecules A and B of CpPAH, which have an r.m.s.d. of 0.42 Å for 258 C-α atoms (residues 10–267). The main differences (>0.7 Å) are found for...
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TABLE 5
Structure and sequence comparison of CpPAH, CvPAH, and hPAH

|                   | CpPAH   | CpPAH-apo | CvPAH   | CvPAH-apo | hPAH    | hPAH-BH4   |
|-------------------|---------|-----------|---------|-----------|---------|------------|
| No. of residues in gene sequence/in CC | 267/226 | 297/227   | 267/226 | 297/227   | 452/229 | 1J8U       |
| (Ile + Leu)/(Ile + Leu + Val)/in CC | 0.75/0.78 | 0.70/0.73 | 0.75/0.78 | 0.70/0.73 | 0.80/0.80 | 1J8U       |
| Arg/(Arg + Lys)/in CC | 0.39/0.48 | 0.61/0.57 | 0.48/0.48 | 0.61/0.57 | 0.44/0.32 | 1J8U       |
| No. of Met/Gly/Pro in CC | 6/13/12 | 6/14/14   | 6/13/12 | 6/14/14   | 2/15/12 | 1J8U       |
| Sequence identity/similarity | 34/57% | 31/53%    | 34/57% | 31/53%    | 17/31%  | 14/34%     |
| to CpPAH          |         |           |         |           |         |            |
| to CvPAH          |         |           |         |           |         |            |
| CC sequence identity/similarity | 41/67% | 32/52%    | 41/67% | 32/52%    |         |            |
| to CpPAH          |         |           |         |           |         |            |
| to CvPAH          |         |           |         |           |         |            |
| PDB entry         | 2V27    | 2V28      | 1LTV    | 1LTU      | 1PAH    | 1J8U       |
| Resolution (Å)    | 1.50    | 1.90      | 2.0     | 1.73      | 1.68    | 1.50       |
| No. of protein residuesa | 264/64 | 264/64    | 275/64 | 275/64    | 284/64 | 308/64     |
| First-last residue | Gly6–Ser267 | Gly6–Ser267 | Val6–Leu163 | Gly6–Ser267 | Asn6–Ala265 | Thr16–Leu424 |
| r.m.s.d. (Å) (no. of residues)b | 0.22 (264) | 1.42 (242) | 1.43 (242) | 1.65 (227) | 1.68 (227) | 1.68 (227) |
| No. of H-bonds per residue | 0.981 | 0.951      | 0.891   | 0.887     | 0.919   | 0.941      |
| Ion pairs         | 27/76   | 26/71     | 14/60   | 18/64     | 38/91   | 34/83      |
| No. <4/6 Å | 0.102/0.288 | 0.098/0.269 | 0.051/0.218 | 0.063/0.225 | 0.123/0.295 | 0.111/0.270 |

a Data are from crystal structure.
b Data compared with CpPAH-molecule A.
A Abbreviations used are as follows: MM, main chain-main chain; MS, main chain-side chain; SS, side chain-side chain.

The calculations were made for the complete sequence, the catalytic core (CC), or for the residues in the crystal structures, as indicated. Two PDB files for each protein have been used for hydrogen binding and ion pair calculations to draw more confident conclusions and obtain better statistics.

The N-terminal residues 4–9 that adopt different conformations in the two molecules (Fig. 5). The His tag in CpPAH-molecule B is folded into the enzyme, a feature most likely reflecting a crystallization artifact with the tag probably being very mobile in solution. The tag does not appear to be tightly reflecting a crystallization artifact with the tag probably being very mobile in solution. The tag does not appear to be tightly

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The iron-bound and iron-free CpPAH structures have an overall r.m.s.d. of 0.22–0.44 Å for the C-α atoms (Table 5), indicative of no substantial overall structural differences between the two. On the other hand, in the active sites there are some rearrangements of residues His122, His127, and Glu167 in CpPAH-apo to compensate for the lack of the charged iron atom. Furthermore, the water molecules W1 and W2 are missing, and W3 is displaced relative to the CpPAH-holo as shown in Fig. 6, A and B.

Structural Comparison with Other PAHs—To understand the structural basis for some of the functional and adaptive properties of PAH enzymes, a structural comparison of CpPAH, CvPAH, and hPAH (catalytic domain) was performed, and from the two latter different complexes were used and are referred to throughout this paper. CpPAH is most similar to CvPAH in terms of sequence identity (34%) and overall structure (r.m.s.d. of 1.42 Å for 242 residues; see Table 5). The N terminus in CvPAH is longer and forms a short α-helix; the C terminus is also longer and folds up on the same side of the protein molecule as in CpPAH (Fig. 7). The catalytic domain of hPAH has low sequence identity (17%) and structurally high r.m.s.d. (1.65 Å for 227 residues; see Table 5) compared with CpPAH. hPAH also has an extended N terminus with two non-conserved helices (colored green in Fig. 7G) and a slightly longer C terminus folded into two β-strands (Figs. 1 and 7G), adjacent to the α-helix carrying Phe254 (Phe91 in CpPAH), that stacks with BH4 (9, 58). For the catalytic core residues (Trp20–Met245 in CpPAH), the sequence identities are higher (Table 5), and the fold of the three proteins is more conserved (Fig. 7).

The 6-coordinated iron in CpPAH is similar to CvPAH with two histidines, two water molecules, and a skewed bidentate coordination of the glutamate. On the other hand, in hPAH-Fe(III) (PDB 1PAH) and hPAH-Fe(II)-BH4 (PDB 1J8U), the iron has the acidic residue (Glu330) binding with only one carboxyl oxygen atom, three water molecules (W1, W2, and W3), and two histidine residues in the coordination sphere (Fig. 6D). Other comparative values of the three PAHs are presented in Table 5.

Cofactor-binding Site—Crystal structures of binary CpPAH complexes of either BH4 (or its oxidized and stable analog BH2) or l-Phe could not be obtained, but models of CpPAH with only BH4 and with both BH4 and THA (a substrate analog, see below) were prepared. Structurally, the loop Leu85–Gly89 (in CpPAH) includes highly different residues in the three enzymes (Figs. 1 and 6), and both residues Leu85 and Phe88 in CpPAH (molecule A) are poorly defined in the final electron density, with high B-factors. Furthermore, Phe88 in CpPAH is incapable of making the same type of ionic interactions with BH4 as are found in CvPAH-BH4, where the corresponding residue Asp104 forms two hydrogen bonds to O-1’ of BH4 (14) (Fig. 6C). Likewise, in the hPAH-BH4 complex there is one important hydrogen bond from the corresponding residue Ser251 O-γ (Phe88 in CpPAH) to the cofactor (Fig. 6D) (59). Furthermore, Ser251 in hPAH is more stabilized than Phe88 in CpPAH, because the preceding and subsequent residues (Ser250 and Arg252) are involved in hydrogen-binding and ion pair interac-
CpPAH has two glycine residues at these positions (Gly87 and Gly89) making the loop more flexible and Phe88 a poor stabilizer for BH$_4$ binding, in agreement with the high $K_{m}$ value of CpPAH compared with the other PAHs. CpPAH should still be able to form two hydrogen bonds from Ile86 O and N to BH$_4$, as observed in CvPAH-BH$_2$ and hPAH-BH$_4$ (14, 58) (Fig. 6, C and D).

**Substrate-binding Site**—With respect to the substrate-binding site, there is no crystal structure available with the natural substrate (L-Phe), but for hPAH there are two ternary crystal structure complexes with the substrate analogs L-norleucine (NLE; PDB 1MMT; hPAH-Fe(II)-BH$_2$-NLE) and 3-(2-thienyl)-L-alanine (THA; PDB 1MMK; hPAH-Fe(II)-BH$_2$-THA) (9). These structures essentially confirm the L-Phe-binding mode as deduced by NMR and molecular docking (60). Both substrate analogs (NLE and THA) have similar hydrogen-binding patterns for the carboxyl and amino groups, with the NH1 and NH2 atoms of Arg270 (Arg107 in CpPAH) almost symmetrically bound to the carboxyl group (Fig. 6F). Ser349 O=O (Ser186 in CpPAH), Thr278 O and N atoms (Leu115 in CpPAH), and two water molecules also participate in the hydrogen-binding network of the carboxyl and amino groups, which are believed to be the same in an hPAH-L-Phe complex (9, 60). The model of THA bound to CpPAH showed that the hydrogen-binding network for the carboxyl and amino groups of the substrate present in hPAH (9, 60) can be maintained by Ser186, Arg107, and Leu115 N and O (Fig. 6E). Furthermore, CpPAH can provide a hydrophobic binding pocket for the aromatic ring of L-Phe with the conserved residues Pro118, His122, Trp163, and Phe168 (Fig. 1). However, CpPAH also has Gly181, Gly182, and Gly183 (Gly, Ala, and Gly in CvPAH and hPAH) in a loop opposite to Pro118 (Fig. 6E), resulting in a less rigid substrate-binding pocket. Additional structural explanations for the low affinity for L-Phe binding to CpPAH relative to the other enzymes (41, 48, 51, 52) might be attributed to Gln116 and Glu117 located in the “water-exposed loop” (residues 112–118) making up one side of the L-Phe-binding site (Fig. 6). Gln116 is poorly defined in

![FIGURE 6. Active site comparison. Structural comparison of the BH$_4$-binding sites in CpPAH (A), CpPAH-apo (B), CvPAH-BH$_2$ (1LT2) (C), and hPAH-BH$_4$ (PDB 1J8U) (D). The L-Phe-binding sites of CpPAH (E) and the hPAH-BH$_4$-THA (PDB 1MMK) (F) crystal structure with the substrate analog THA. For A and E, BH$_4$ and THA have been modeled by docking the ligands according to their position in the hPAH-BH$_4$ and hPAH-BH$_4$-THA structures, respectively. See the text for further details.](image-url)
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FIGURE 7. Structural comparison. Ribbon diagrams (left panels), calculated electrostatic surface potentials (middle panels), and mobility analysis (right panels). A–C are CpPAH (residues 4–267); D and E are CvPAH-BH$_2$ (PDB 1LTZ, residues 9–283); F is CvPAH (PDB 1LTV, residues 9–283), G and H are hPAH-BH$_4$ (PDB 1J8U), and I is hPAH (PDB 1PAH), all in similar orientations. In CvPAH-BH$_2$, Tyr$_{130}$ was poorly defined in electron density and its orientation very uncertain (14). The surface potentials are color coded in red, white, and blue for the charge $-10$, $0$, and $+10 K/bT/e$, respectively. The normalized squared atomic displacements were calculated for mode 8 (C, F, and I) and are color-coded from small (dark blue) to large (red) mobility on the ribbon diagrams. All figures were made with PyMOL (DeLano Scientific, San Carlos, CA).
electron density (high B-factors) in both molecules A and B, whereas Glu\textsuperscript{117} makes one hydrogen bond and two water-mediated hydrogen bonds in molecule A and is not defined in molecule B, implying some flexibility for these residues in CpPAH. Both residues also have high water-accessible surfaces (WAS) than the equivalent residues Pro\textsuperscript{279} and Glu\textsuperscript{280} in hPAH structures (Table 6).

**Regional Flexibility in CpPAH**—The crystal structures of CpPAH point to regions of expected increased flexibility at both the BH\textsubscript{4} and the \textsuperscript{\textalpha}-Phe-binding sites (see above). At the same time, the \( T_m \) of 52 °C for this enzyme compared with 62 and 55 °C for the mesophilic counterparts CvPAH and hPAH, respectively (Table 4), indicates that overall stability is not seriously compromised. To further substantiate the predicted local flexibility, we employed normal mode analysis (NMA) (34). The first six vibrational modes correspond to global rotation and translation of the system and are ignored in the analyses. The lowest frequency mode of interest are modes 7 and 8, regarded as having functional significance describing large movements in regions of the protein. The two modes exhibited similar regional fluctuations in PAHs analyzed here (see supplemental Fig. S2, A–C, for mode 8; see also Fig. 7, C, F, and I, for a representation of the NMA in the structures). For CpPAH, no significant differences between molecule A (not shown) and molecule B (supplemental Fig. S2A) were found. Highly mobile regions include the N-terminal residues 1–34, the C-terminal residues 239–267, residues 81–87 at the cofactor-binding site (Fig. 6A), 105–119 at the substrate-binding site (including the water-exposed loop) (Fig. 6E), and residues 196–203, a surface-exposed loop at the entrance to the substrate-binding site. All these regions show elevated B-factors and poorer electron density in the crystal structure. For CvPAH and hPAH similar regions with mobility are encountered, but the fluctuations are estimated to be smaller, except for the region around residue 166–174 (CvPAH), corresponding to a loop between two important helices that define the architecture of the active site (Fig. 7D) and the loop 143–154 in hPAH, which is not present in the other enzymes.

Regional variations in local folding/unfolding equilibria were also calculated by the COREX/BEST algorithm (36, 61). The residue stability constant is the ratio of the summed probabilities of the states in the ensemble in which a particular residue is in a folded conformation, to the summed probability of the states in which a residue is in an unfolded conformation. We observed that the three PAHs regions with high mobility according to NMA are associated with low stability constants (supplemental Fig. S2). Comparative studies of the role of electrotropics in temperature adaptation have found several cold-adapted enzymes with increased negative surface charge and uncompensated (destabilizing) charge-charge interactions (see Refs. 50, 62, 63 and references therein). It has been suggested that the acidic surface in psychrophilic enzymes would enhance protein solvation, whereas charge compensation would ensure active site flexibility (63). CpPAH has a more negative surface potential, notably around the active site, which also appears to be wide and open (Fig. 7B). However, when compared with its mesophilic homologs, CpPAH does not include a greater proportion of destabilizing charged residues. In fact, as inferred from electrostatic analysis by implementation of the Tanford-Kirkwood model on the structures (37, 38), CvPAH presents more regions with destabilizing charge-charge interactions (i.e. positive energy of interaction of the charged groups with the rest of the ionizable groups in the protein (\( \langle W_{i} \rangle > 0 \)) (supplemental Fig. S2, G–I), and these regions are located around the active site and present high mobility (supplemental Fig. S2, B and E). Accordingly, the total charge-charge of \( \langle W_{q} \rangle \) (see Equation 1) of −25.3 kJ/mol for CvPAH was compared with −42.5 and −67 kJ/mol for CpPAH and hPAH, respectively. This is also consistent with the number of ion pairs found in the crystal structures with CvPAH having least ionic interactions followed by CpPAH and hPAH (Table 5). \( W_{q} \) is regarded as an approximation of the charge-charge contributions to the unfolding Gibbs energies (38), and our results indicate that CpPAH has generally stabilizing interactions, in agreement with the relatively high \( T_m \). This also suggests that regional mobility in this enzyme is not primarily a result of unfavorable electrostatics. Other factors, such as a more open water-accessible active site and the higher proportion of Gly residues in some high mobility regions, appear to play an important role. In fact, important specific stabilizing charge-charge interactions were observed for CpPAH involving ion-pair networks with interactions <5 Å and very negative \( W_{i} \) values (supplemental Fig. S2G) indicative of strong links like: (i) Glu\textsuperscript{23}–Arg\textsuperscript{109}–Glu\textsuperscript{30}–Arg\textsuperscript{4}–Asp\textsuperscript{113}, and (ii) Glu\textsuperscript{90}–Arg\textsuperscript{93}–Glu\textsuperscript{97}–Lys\textsuperscript{9}–Glu\textsuperscript{78}, which are not present in CvPAH nor in hPAH. These networks might also play a role in the refolding observed after cooling samples that were heated to 37 °C (Fig. 4A)

Despite the fact that detailed analysis of the hydrogen bonding and ion pair patterns are dependent on the resolution of the crystal structure, both CpPAH structures have more hydrogen

### Table 6

| CpPAH           | CpPAH Mol. A/B | CpPAH-apo Mol. A/B | hPAH  | hPAH (1PAH) | hPAH-BH\textsubscript{4} (1BU) | hPAH-BH\textsubscript{4}-THA (1MMK) |
|-----------------|----------------|---------------------|-------|-------------|-----------------------------|-----------------------------------|
| \( \delta^2 \)  | \( \delta^2 \)  | \( \delta^2 \)  | \( \delta^2 \)  |
| Arg\textsuperscript{117} | 12/12 | 11/16 | Arg\textsuperscript{270} | 9 | 11 | 8 |
| Tyr\textsuperscript{14} | 121/123 | 125/129 | Tyr\textsuperscript{277} | 98 | 117 | 19 |
| Leu\textsuperscript{19} | 21/28 | 17/26 | Thr\textsuperscript{278} | 40 | 41 | 31 |
| Glu\textsuperscript{16} | 118/106 | 135/125 | Pro\textsuperscript{279} | 56 | 65 | 5 |
| Glu\textsuperscript{17} | 56/53 | 45/51 | Glu\textsuperscript{280} | 25 | 24 | 15 |
| Pro\textsuperscript{16} | 52/45 | 52/57 | Pro\textsuperscript{261} | 53 | 62 | 16 |
| Trp\textsuperscript{16} | 35/16 | 32/27 | Trp\textsuperscript{266} | 51 | 30 | 24 |
| Phe\textsuperscript{16} | 15/14 | 12/12 | Phe\textsuperscript{31} | 18 | 19 | 16 |
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bonds than the other PAH structures, and fewer ion pairs (<4 Å) per residue than hPAH but more than CvPAH (Table 5). This also fits with the fact that CpPAH is not cold-adapted in terms of lower global thermal stability.

**DISCUSSION**

The Function of CpPAH—In some prokaryotic organisms containing PAH the initial steps in a catabolic pathway to mediate Phe and Tyr carbon recycling involve the function of the phh operon and include the reactions L-Phe → L-Tyr (catalyzed by PAH) and L-Tyr → 4-hydroxyphenylpyruvate (catalyzed by tyrosine aminotransferase) (64), similar to the steps for L-Phe catabolism in eukaryotes. 4-Hydroxyphenylpyruvate is then further oxidized to homogentisic acid (65). The fact that carbon recycling involve the function of the phe operon and include the reactions L-Phe → L-Tyr (catalyzed by PAH) and L-Tyr → 4-hydroxyphenylpyruvate (catalyzed by tyrosine aminotransferase) (64), similar to the steps for L-Phe catabolism in eukaryotes. 4-Hydroxyphenylpyruvate is then further oxidized to homogentisic acid (65). The fact that P. aeruginosa as red-brown pigmented, and in several of them, such as Hyphomonas strain sp., Shewanella colwelliana, and P. aeruginosa (66, 67), it has been shown that the pigment provides this color, usually referred to as pyomelanin or alkapton, derives from the oxidation and oligomerization of homogentisic acid. Homogentisic acid is a derivative of L-Tyr (the product of PAH), and it seems very likely that PAH is the first enzyme in the biosynthesis of pyomelanin. Studies on the synthesis of this pigment have usually focused on enzyme activities downstream in the pathway, e.g. hydroxyphenylpyruvate dehydrogenase (67). Pyomelanin is associated with several functions, such as biofilm formation, redox activity, and protection against oxidants and radiation (67–69). To the best of our knowledge, the function of the pigment in C. psychrerythraea has not been described previously or clarified, although we have observed that pigment production increases when the bacteria grow forming biofilms.

The Effect of Substrate Preincubation—The specific activity of CpPAH under standard conditions is highly affected by the order of addition of substrate and cofactor in the assay, where preincubation with L-Phe resulted in very low activity (Fig. 3B; Table 3). Our results support formation of slow-dissociating dead-end enzyme complexes with L-Phe if the substrate binds before the cofactor (Fig. 3, C and D). In combination with a possible ordered kinetic mechanism, with BH₄ binding first in the active site, L-Phe second, and dioxygen last, as earlier determined for CvPAH (41), these results may provide an explanation for the low specific activity following preincubation with L-Phe. The orientation of L-Phe when bound to free CpPAH in the absence of BH₄ is probably not optimal, and only when the substrate binds to the binary CpPAH-BH₄ complex do the reactants adopt a correct orientation relative to each other and to the iron. A structural effect of BH₄ binding might be that the loop Leu⁸⁵–Phe⁹¹ (in CpPAH) becomes more ordered when Phe⁹¹ stacks against the cofactor and hydrogen bonds to the main chain of Ile⁸⁶ are formed, as found for the corresponding residues in hPAH (58). Ordering or different folding of the N terminus of CpPAH, which is highly mobile and adjacent to the L-Phe-binding site (Fig. 7C and Fig. S2A), might also facilitate correct L-Phe binding.

Positive Cooperativity for L-Phe—It was interesting to find positive cooperativity for the activity of CpPAH as a function of L-Phe concentration (Fig. 3B and Table 3). This regulatory mechanism is characteristic of tetrameric mammalian PAHs (48, 51, 52). Positive cooperativity occurs in a few monomeric enzymes, of which the most studied is glucokinase (72). The equilibrium binding of glucose to glucokinase displays hyperbolic saturation curves, in contrast to the sigmoidal dependence on glucose in turnover (73), indicating that the positive cooperativity with the substrate originates from a kinetic property. The kinetic mechanism is, however, not completely understood, and several models have been proposed to explain the cooperative kinetics, such as a mnemonical model (74) and a slow transition model (75). Both models are based on the existence of different conformational forms of glucokinase that interconvert by substrate binding, and a slow transition leading to the catalytically competent enzyme-glucose complex relative to the catalytic step (73, 76). Similarly to what is found for glucokinase, hyperbolic binding curves were found for the binding of the substrate L-Phe to CpPAH (supplemental Fig. S1), also indicating a kinetic nature for the cooperativity. Our results are thus compatible with a slow conformational transition in CpPAH induced by L-Phe binding and leading to a catalytically competent enzyme when L-Phe binds after BH₄ (see also previous paragraph). On the other hand, sigmoidal binding curves are found for the binding of L-Phe to tetrameric mammalian PAH (26, 77).

The existence of positive cooperativity in CpPAH is remarkable. This mechanism is present at different evolutionary stages from bacteria to man, most probably as a means for the enzyme to sensitize the L-Phe concentrations and maintaining homeostatic levels of this essential amino acid. However, the elucidation of the molecular basis for the positive cooperativity as well as the nature of the regulatory and kinetic states of the enzyme, must await the structural determination of binary and ternary complexes with substrate and cofactor.

Cold Adaptation of CpPAH—Under the conditions used in this study, CpPAH shows maximal activity and catalytic efficiency at 25 °C. This temperature is about 15 °C above the optimal growth temperature for C. psychrerythraea. Other enzymes from the same bacterium and other cold-adapted organisms also show maximal activity 10–20 °C above the optimal growth
temperature (2–4). The drop in activity at temperatures above 25 °C is not associated with low overall stability (Fig. 4). The $K_m$(BH$_4$) and $[S]_{0.5}$([L-Phe]) values might further increase at temperatures >25 °C, although definitive measurement of these values at high temperatures was hampered by a more marked substrate inhibition at these conditions. A very likely explanation of the temperature-induced inactivation might be associated with failure in the formation of a catalytically competent CpPAH-Fe([II])BH$_4$-[L-Phe]O$_2$ complex. The formation of this complex is absolutely dependent on the correct arrangement of all substrates, with the right distance and orientation with respect to each other (9, 60, 78). The temperature-dependent increased mobility around the active site may not directly affect the binding of the individual reactants but rather their spatial organization and the coupling between substrate hydroxylation and cofactor oxidation.

The increasing availability of crystal structures from other psychrophilic organisms, notably bacteria, has further revealed that cold-adapted enzymes often show high flexibility and thermostability compared with their mesophilic and thermophilic counterparts (50, 53, 79). Most cold-adapted enzymes show a global rather uniform distribution of their flexibility throughout the three-dimensional structure (50). However, there are also examples in which the flexibility is locally increased, notably in regions around the active site, resulting in a weaker binding of the substrate (high $K_m$ values), and high $k_{cat}$ values at low temperatures (see Refs. 50, 53 and references therein), as is the case of CpPAH.

Overall conformational flexibility in cold-adapted enzymes appears to be obtained by the following: (i) a lower number of prolines and arginines; (ii) more glycines; (iii) fewer hydrogen bonds and fewer ion pair interactions; (iv) higher numbers of serine and methionine; and (v) reduced hydrophobicity (particularly for internal residues) expressed as lower (Ile + Leu)/(Ile + Leu + Val) ratio; or (vi) density of charged surface residues (50, 53, 79). As can be seen in Table 5, these structural features are not distinctive for CpPAH, except for the Gly clusters Gly$^{87}$–Phe$^{88}$–Gly$^{89}$, located in the highly mobile loop 81–87 in the BH$_4$-binding site, and Gly$^{181}$–Gly$^{182}$–Gly$^{183}$, at the top of the α-Phe active site (Figs. 6E and 7C). An additional cold-adaptive feature encountered in CpPAH is a more open active site (53) (Table 6), mostly due to a shorter N terminus (Fig. 7). This open, solvent-accessible active site could also contribute to local flexibility, notably in the region 105–119 (supplemental Fig. S2A), which includes the water-exposed loop. Moreover, CpPAH and hPAH have several Pro residues at the beginning or endings of α-helices and loops not present in CpPAH (Fig. 1).

The distribution of charge on the surface of proteins is important for their stability, and as we move from psychrophiles to mesophiles to thermophiles, a clear trend can be observed that shows an increase in the number of ionic attractions on the protein surface (38, 80). CpPAH presents a slightly higher negative charge at neutral pH and negative surface potential around the active site (Fig. 7B), but there are no regions with very dense charge, as is the case in the other PAHs (Fig. 7). CpPAH does not have a typical cold-adapted surface with a greater negative charge or uncompensated charge-charge interactions (Fig. 7B and Fig. S2G) that can lead to structural destabilization and thermostability.

The present biochemical and structural characterization of PAH from a cold-adapted organism shows an enzyme with relatively high thermostability and low affinity for substrate and cofactor, most probably associated with high local flexibility around the active site and disrupted hydrogen-bonding capacity of the cofactor.

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