Thermodynamic Characterization of the Binding of Tetrahydropterins to Phenylalanine Hydroxylase[§]

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Abstract: Phenylalanine hydroxylase (PAH) is the key enzyme in the catabolism of L-Phe. The natural cofactor of PAH, $6R$-tetrahydrobiopterin (BH_4), negatively regulates the enzyme activity in addition to being an essential cosubstrate for catalysis. The analogue 6-methyltetrahydropterin ($6M$-PH_4) is effective in catalysis but does not regulate PAH. Here, the thermodynamics of binding of BH_4 and $6M$-PH_4 to human PAH have been studied by isothermal titration calorimetry. At neutral pH and 25 °C, BH_4 binds to PAH with higher affinity ($K_d = 0.75 \pm 0.18$ μM) than $6M$-PH_4 ($K_d = 16.5 \pm 2.7$ μM). While BH_4 binding is a strongly exothermic process ($\Delta H = -11.8 \pm 0.4$ kcal/mol) accompanied by an entropic penalty ($-T\Delta S = 3.4 \pm 0.4$ kcal/mol), $6M$-PH_4 binding is both enthalpically ($\Delta H = -3.3 \pm 0.3$ kcal/mol) and entropically ($-T\Delta S = -3.2$ kcal/mol) driven. No significant changes in binding affinity were observed in the 5−35 °C temperature range for both pterins at neutral pH, but the enthalpic contribution increased with temperature rendering a heat capacity change (ΔC_p) of -357 ± 26 cal/mol/K for BH_4 and -63 ± 12 cal/mol/K for $6M$-PH_4. Protons do not seem to be taken up or released upon pterin binding. Structure-based energetics calculations applied on the molecular dynamics simulated structures of the complexes suggest that in the case of BH_4 binding, the conformational rearrangement of the N-terminal tail of PAH contribute with favorable enthalpic and unfavorable entropic contributions to the intrinsic thermodynamic parameters of binding. The entropic penalty is most probably associated to the reduction of conformational flexibility at the protein level and disappears for the L-Phe activated enzyme. The calculated energetic parameters aid to elucidate the molecular mechanism for cofactor recognition and the regulation of PAH by the dihydroxypropyl side chain of BH_4.

Introduction

Phenylalanine hydroxylase (phenylalanine 4-monooxygenase, PAH; EC 1.14.16.1) catalyzes the hydroxylation of L-Phe to L-Tyr, in the presence of $6R$-tetrahydrobiopterin (BH_4) and dioxygen as cosubstrates. This is the rate-limiting step in the catabolic pathway for L-Phe taking place mainly in the liver, and deficiency of human PAH activity causes phenylketonuria (PKU), which represents the most prevalent inborn error of amino acid metabolism.[1] The full-length human protein is tetrameric and each monomer (452 residues) has a three domain structure (for review, see refs 2,3): (1) an N-terminal regulatory domain (residues 1−110), (2) a catalytic domain (111−410) containing the active site iron and substrates binding sites, and (3) an oligomerization domain (411−452). PAH binds L-Phe

cooperatively, inducing conformational changes involving the tertiary and quaternary structure that increase specific activity[4] and thermal stability[5] and favors phosphorylation of the enzyme at Ser16.[6]

Enzymatic activity assays have earlier indicated that the natural cosubstrate $(6R,1'R,2'S)$-6-(1′,2′-dihydroxypropyl)-5,6,7,8-tetrahydrobiopterin (BH_4), referred traditionally to as cofactor, binds with higher affinity to PAH and to the homologous enzyme tyrosine hydroxylase than other functional synthetic cofactors such as $6S$-BH_4 and 6-methyl-tetrahydropterin ($6M$-PH_4).[7−9] In addition to its catalytic function, BH_4 specifically enforces regulatory properties on the enzymes; on PAH BH_4 induces a low-activity conformational state, blocks L-Phe activation and disfavors phosphorylation at Ser16,[10,11]

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[§] Abbreviations: BH4, (6R)-l-erythro-5,6,7,8-tetrahydrobiopterin; DTT, dithiothreitol; ITC, isothermal titration calorimetry; 6M-PH4, 6-methyl-tetrahydropterin; MD, molecular dynamics; PAH, phenylalanine hydroxylase; PKU, phenylketonuria.

(1) Scriver, C. R.; Eisensmith, R. C.; Woo, S. L. C.; Kaufman, S. *Annu. Rev. Genet.* **1995**, *28*, 141−165.

(2) Fitzpatrick, P. F. *Adv. Enzymol. Relat. Areas Mol. Biol.* **2000**, *74*, 235−294.

(3) Flatmark, T.; Stevens, R. C. *Chem. Rev.* **1999**, *99*, 2137−2160.

(4) Kaufman, S. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1993**, *67*, 77−264.

(5) Thórólfsson, M.; Ibarra-Molero, B.; Fojan, P.; Petersen, S. B.; Sanchez-Ruiz, J. M.; Martínez, A. *Biochemistry* **2002**, *41*, 7573−7585.

(6) Døskeland, A. P.; Flatmark, T. *Biochem J.* **1996**, *319*, 941−945.

(7) Bigham, E. C.; Smith, G. K.; Reinhard, J. F., Jr.; Mallory, W. R.; Nichol, C. A.; Morrison, R. W., Jr. *J. Med. Chem.* **1987**, *30*, 40−45.

(8) Bailey, S. W.; Dillard, S. B.; Ayling, J. E. *Biochemistry* **1991**, *30*, 10 226−10 235.

(9) Almås, B.; Toska, K.; Teigen, K.; Groehn, V.; Pfleiderer, W.; Martínez, A.; Flatmark, T.; Haavik, J. *Biochemistry* **2000**, *39*, 13 676−13 686.

(10) Xia, T.; Gray, D. W.; Shiman, R. *J. Biol. Chem.* **1994**, *269*, 24 657−24 665.

(11) Solstad, T.; Stokka, A. J.; Andersen, O. A.; Flatmark, T. *Eur. J. Biochem.* **2003**, *270*, 981−990.

whereas in tyrosine hydroxylase, it elicits negative binding cooperativity.[9,12] These specific effects of BH_4 seem to be associated to the dihydroxypropyl side chain at C6 and are not observed with 6M-PH_4.[4,13] Structural models for pterin binding based on crystal structures, NMR, and molecular modeling have been described in the past years and characterized pterin binding at a molecular level.[9,14–16] The aromatic amino acid hydroxylases show a high degree of sequence identity, and they share a common binding motif for the cofactor. The BH_4 binding pocket is located at the bottom of the hydrophobic active site opening. In PAH, the cofactor makes stacking interactions with an invariant phenylalanine (Phe254) and the N3 and the amino group at C2 hydrogen bonds with the carboxylic group of a conserved glutamate residue (Glu286). The side chain hydroxyl groups of BH_4 establish hydrogen bonding interactions with Ser251 at the catalytic domain and Ser23 of the N-terminal regulatory domain of PAH.[11,13] The structural information supports the role of the cofactor in the reduction of the iron in the activating prereduction step and in the formation of an iron-peroxo-pterin as the hydroxylating intermediate.[14,17,18]

Thermodynamic information on the interactions between the pterin cofactor and PAH is important for understanding the mechanism of recognition, function, and regulation at the molecular level, as well as to aid to elucidate the relationship between the structure of the enzyme-cofactor complexes and the thermodynamic parameters. Furthermore, a subtype of mild-PKU patients shows a normalization of plasma L-Phe concentration after oral administration of high doses of BH_4.[19] It has been hypothesized that the BH_4 responsive PAH mutations are K_m variants (decreased BH_4 binding affinity).[20] In this context, there is a need for an in-depth characterization of the cofactor interaction with PAH to develop the standards for later comparative binding studies with PKU mutants. Recently, we reported a preliminary attempt to characterize dihydrobiopterin (BH_2) and BH_4 binding to human PAH by isothermal titration calorimetry (ITC) using dithiothreitol (DTT) as reducing agent.[21] These experiments were however unsuccessful in conditions necessary to get a full thermodynamic characterization of reduced pterin binding to PAH. Here, we describe an experimental system under anoxic conditions using ITC that allows wide-range temperature binding measurements. This study provides novel information about the energetics of the binding of BH_4 and its analogue 6M-PH_4 to human wild-type PAH, including the enthalpy (ΔH), the dissociation constant (K_d), the stoichiometry (n), the free energy (ΔG), and the entropy (ΔS) of the binding. As in most ligand-protein binding processes, the interaction with both pterin cofactors results in a decrease in heat capacity (ΔC_p). To characterize the binding processes and to relate the overall BH_4 binding thermodynamics to specific physical contributions and conformational changes, we have compared the experimentally obtained ΔC_p and ΔH with the values estimated from the structural models of the complexes obtained by molecular dynamics (MD) simulations.[13] Finally, comparative ITC analyses of the binding of BH_4 to PAH in the presence of L-Phe were applied to further study the relation between the binding parameters and the conformational states of the enzyme, related to the substrate activation and the negative regulation by the cofactor.

Experimental Section

Expression and Purification of Recombinant Human PAH Enzymes. Growth of *E. coli* transformed with the pMAL vectors for expression of human wild-type PAH and purification of the fusion protein with maltose binding protein (MBP–PAH) were performed as described.[22] Protein concentration was measured spectrophotometrically using $\epsilon_{280\ nm}$ (1 mg/mL) = 1.63.

BH_4 Oxidation Measurements and the Glucose Oxidase System. Fresh aliquots of BH_4 (72 mM in 10 mM HCl) were diluted to 20 mM NaHepes, 200 mM NaCl, pH 7.0 to a final concentration of 100 μM, in the absence or the presence of the components of the glucose oxidase system, i.e., 0.5 U/mL glucose oxidase (type II, Sigma), 750 U/mL Catalase (Sigma) and 20 mM D-Glucose[23] or 0.5 mM D-Glucose, 65 U/mL glucose oxidase and 1300 U/mL catalase,[24] at 25 °C. The effect of DTT (5 mM) on the oxidation rate of BH_4 was also investigated. In the glucose oxidase system, oxygen was removed from all buffers and solutions by incubation for 10 min. at room temperature before BH_4 dilution. The oxidation rate of BH_4 at different conditions was measured spectrophotometrically by monitoring the increase in $A_{330\ nm}$[25] with a Beckman UV/VIS DU 530 spectrophotometer.

Isothermal Titration Calorimetry. The experiments were performed using a VP-ITC titration calorimeter (MicroCal Inc.) at the indicated temperatures. The instrument was calibrated using the built-in electrical calibration check. The experiments were carried out in anoxic conditions using 20 mM NaHepes, 200 mM NaCl, pH 7.0 with the glucose oxidase system as described[23] (see above). In experiments aimed to investigate the influence of buffer ionization and of pH on the experimental ΔH, the buffers 20 mM MES, Phosphate, Hepes and Pipes, pH 6.0–8.0, containing 200 mM NaCl, were utilized, as indicated. The buffer was incubated at 25 °C for 15 min before the fresh aliquots of BH_4 (or 6M-PH_4) or enzyme were added to prepare the solutions used in the syringe and the sample cell, respectively. All of the solutions were filtered and degassed prior to titration. Tetrameric fusion protein (10–30 μM subunit, as indicated) with approximately 0.5 mol ferrous ammonium sulfate per mol PAH subunit was applied in the sample cell and 0.5–1.0 mM BH_4 or 6M-PH_4 in the syringe. In each run, 50–90 injections (3 μL) were performed into the sample cell, with a 240 s interval between injections, and mixed via the rotating stirrer syringe at 300 rpm. The final ratio [pterin]/[enzyme subunit] was typically ≥4, and the mean of the last 5–10 injections was used as experimental heat dilution, and routinely subtracted from the raw data. Under these anoxic conditions, the baseline was stable up to 10 h in the calorimetric cell. In some cases, the first injections presented defects in the baseline and these data were not included in the fitting process. Data were processed and fitted to the *Single set of identical*

(12) Flatmark, T.; Almås, B.; Knappskog, P. M.; Berge, S. V.; Svebak, R. M.; Chehin, R.; Muga, A.; Martínez, A. *Eur. J. Biochem.* **1999**, *262*, 840–849.
(13) Teigen, K.; Martínez, A. *J. Biomol. Struct. Dyn.* **2003**, *20*, 733–740.
(14) Teigen, K.; Frøystein, N. Å.; Martínez, A. *J. Mol. Biol.* **1999**, *294*, 807–823.
(15) Andersen, O. A.; Flatmark, T.; Hough, E. *J. Mol. Biol.* **2001**, *314*, 266–278.
(16) Wang, L.; Erlandsen, H.; Haavik, J.; Knappskog, P. M.; Stevens, R. C. *Biochemistry* **2002**, *41*, 12 569–12 574.
(17) Andersen, O. A.; Flatmark, T.; Hough, E. *J. Mol. Biol.* **2002**, *320*, 1095–1108.
(18) Fitzpatrick, P. F. *Biochemistry* **2003**, *42*, 14 083–14 091.
(19) Kure, S.; Hou, D. C.; Ohura, T.; Iwamoto, H.; Suzuki, S.; Sugiyama, N.; Sakamoto, O.; Fujii, K.; Matsubara, Y.; Narisawa, K. *J. Pediatr.* **1999**, *135*, 375–378.
(20) Erlandsen, H.; Stevens, R. C. *J. Inherit. Metab. Dis.* **2001**, *24*, 213–230.
(21) Pey, A. L.; Thórólfsson, M.; Erlandsen, H.; Stevens, R. C.; Ugarte, M.; Desviat, L. R.; Pérez, B.; Martínez, A. In *Pterins, Folates and Neurotransmitters in Molecular Medicine*; Blau, N., Thöny, B., Eds.; SPS Publications: Heilbronn, Germany, 2004, pp 155–160.

(22) Martínez, A.; Knappskog, P. M.; Olafsdottir, S.; Døskeland, A. P.; Eiken, H. G.; Svebak, R. M.; Bozzini, M.; Apold, J.; Flatmark, T. *Biochem. J.* **1995**, *306*, 589–597.
(23) Rajagopalan, P. T.; Pei, D. *J. Biol. Chem.* **1998**, *273*, 22 305–22 310.
(24) Bou-Abdallah, F.; Arosio, P.; Santambrogio, P.; Yang, X.; Janus-Chandler, C.; Chasteen, N. D. *Biochemistry* **2002**, *41*, 11 184–11 191.
(25) Davis, M. D.; Kaufman, S.; Milstien, S. *Eur. J. Biochem.* **1988**, *173*, 345–351.

sites model using Microcal Origin v.5.0 software. The total heat content Q of the solution (determined relative to zero for the unliganded species) contained in the active cell volume, V_o, was calculated according to the following equation, where K_a is the binding affinity constant, n is the number of sites, ΔH is the enthalpy of ligand binding, M_t and X_t is the bulk concentration of macromolecule and ligand, respectively, for the binding $X + M \leftrightarrow XM$

$$Q = \frac{nM_t \Delta H V_o}{2}\left[1 + \frac{X_t}{nM_t} + \frac{1}{nK_aM_t} - \sqrt{\left(1 + \frac{X_t}{nM_t} + \frac{1}{nK_aM_t}\right)^2 - \frac{4X_t}{nM_t}}\right] \quad (1)$$

The change in heat (ΔQ) measured between the completion of two consecutive injections is corrected for dilution of the protein and ligand in the cell between injections and is used for nonlinear least-squares fitting according to standard Marquardt methods. The ΔH, K_a, and n were thus obtained directly from the titration data (eq 1)[26,27] and the free energy (ΔG) and the entropy (ΔS) of the interaction were calculated by using the relationship

$$\Delta G = \Delta H - T\Delta S = -RT\ln K_a \quad (2)$$

where T is the absolute temperature and R is the gas constant.

Structural Energetics Calculations. The structure-based calculations of the thermodynamic parameters were based on previously published formalisms.[28–30] The structural models of dimeric full-length human PAH bound with either BH_4 (PAH·BH_4) or 6M-PH_4 (PAH·6M-PH_4) were obtained by MD simulations as described.[13] Briefly, the models with the respective cofactor were prepared based on the structure of the rat enzyme[31] and aligned with the structure of the binary complex between the catalytic domain of human PAH and BH_4[14,15] in order to use the pterin ring as a template for appropriate attachment of the corresponding side chain at C6. Then, MD simulations using an implicit treatment of water, i.e., a dielectric constant of 4 and a distance dependent dielectric function, were performed for 0.5 ns using the Discover module of InsightII (Accelrys) with the Amber force field.[32] The cutoff radius for nonbonded interactions was 10 Å, with a secondary cutoff radius of 15 Å. Additional details for the simulations are described in.[13] The minimized average structures from the last 50 ps of the simulations were used to calculate the changes in polar and apolar solvent accessible surface area, ΔASA_{polar} and ΔASA_{apolar}, respectively, using GETAREA.[33] The use of MD simulated structures did not allow the explicit consideration of changes in solvent accessibility related to putative buried water molecules within the bound complexes, which should be included in the calculations only when high-resolution crystal structures are available.[30]

Results

Tetrahydrobiopterin Oxidation and the Glucose Oxidase System. BH_4 rapidly oxidizes at pH 7 at aerobic conditions in the absence of reductants such as DTT.[21,25] We tested two systems based on the glucose/glucose oxidase/catalase coupled reaction as described by Rajagopalan and Pei[23] and by Bou-Abdallah et al.[24] to create an anoxic environment and prevent

(26) Bundle, D. R.; Sigurskjold, B. W. *Methods Enzymol.* **1994**, *247*, 288–305.
(27) Wiseman, T.; Williston, S.; Brandts, J. F.; Lin, L. N. *Anal. Biochem.* **1989**, *179*, 131–137.
(28) Murphy, K. P.; Freire, E. *Adv. Protein Chem.* **1992**, *43*, 313–361.
(29) Baker, B. M.; Murphy, K. P. *J. Mol. Biol.* **1997**, *268*, 557–569.
(30) Luque, I.; Freire, E. *Proteins* **2002**, *49*, 181–190.
(31) Kobe, B.; Jennings, I. G.; House, C. M.; Michell, B. J.; Goodwill, K. E.; Santarsiero, B. D.; Stevens, R. C.; Cotton, R. G.; Kemp, B. E. *Nat. Struct. Biol.* **1999**, *6*, 442–448.
(32) Weiner, S. J.; Kollman, P. A.; Nguyen, D. T.; Case, D. A. *J. Comput. Chem.* **1986**, *7*, 230–252.
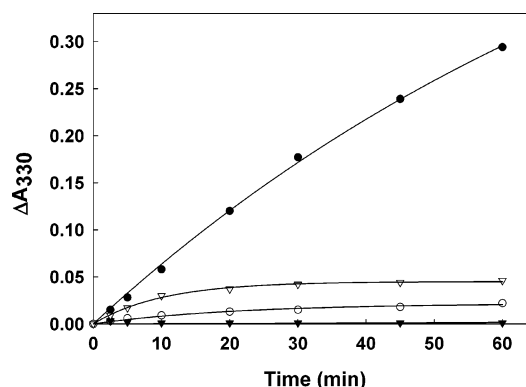(33) Fraczkiewicz, R.; Braun, W. *J. Comput. Chem.* **1998**, *19*, 319–333.

Figure 1. Effect of different protective systems on the autoxidation of BH_4 in 20 mM NaHepes, 0.2 M NaCl, pH 7.0 at 20 °C. No protective system (●), 5 mM DTT (○), glucose/glucose oxidase/catalase systems described by Rajagopalan and Pei[23] (▼) and by Bou-Abdallah et al. [24] (▽).

BH_4 oxidation. As seen in Figure 1, best results were obtained with the system of Rajagopalan & Pei[23] which prevented oxidation of the cofactor for up to 60 min and was selected for ITC studies. Further control calorimetric experiments demonstrated the stability of BH_4 in this anoxic environment for several hours (data not shown). In addition, the use of anaerobic conditions allows for the investigation of the binding of the reduced cofactors to the enzyme without interfering changes in the protein due to reduction of ferric forms of the active site metal, as reduction has been reported not to take place at these conditions.[34,35]

Binding of BH_4 and 6M-PH_4. The binding of reduced pterin cofactors to the tetrameric wild-type PAH in anoxic NaHepes, 0.2 M NaCl, pH 7.0 at different temperatures (5–35 °C) was studied by ITC. Good signal-to-noise ratios were obtained at a protein concentration of 12 μM subunit for the binding of BH_4, while 30 μM subunit was necessary for experiments with 6M-PH_4. The anoxic environment created by the glucose oxidase system was calorimetrically well-behaved in all the cases, and stable in the time and temperature ranges used. In all experiments at pH 7.0, the pterin cofactors bind to PAH stoichiometrically. A representative thermogram and isotherm for BH_4 binding at 25 °C is shown in Figure 2. At this temperature, BH_4 binding is strongly exothermic ($\Delta H = -11.8 \pm 0.4$ kcal/mol), and is accompanied by a remarkable entropic penalty (3.4 kcal/mol), rendering a $\Delta G = -8.4 \pm 0.2$ kcal/mol and $K_a = (1.4 \pm 0.4)\cdot10^6$ M^{-1}. In the temperature range under study (5–35 °C) the affinity just decreased slightly with increasing temperature, but the enthalpy/entropy contributions to ΔG varies significantly, being entropically favored at low temperature and strongly penalized at higher temperatures. The entropic contribution/penalization to binding became zero at 13 °C (Figure 3A). The thermodynamic parameters obtained in these experiments are compiled in Table 1. From the linear fitting of the temperature dependency of ΔH (Figure 3A), a $\Delta C_p = -357 \pm 26$ cal/mol/K was calculated. Such a relatively large negative capacity change is indicative of specific and apolar interactions.[36,37]

(34) Bloom, L. M.; Benkovic, S. J.; Gaffney, B. J. *Biochemistry* **1986**, *25*, 4204–4210.
(35) Marota, J. J.; Shiman, R. *Biochemistry* **1984**, *23*, 1303–1311.
(36) Gomez, J.; Freire, E. *J. Mol. Biol.* **1995**, *252*, 337–350.
(37) Ye, H.; Wu, H. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 8961–8966.
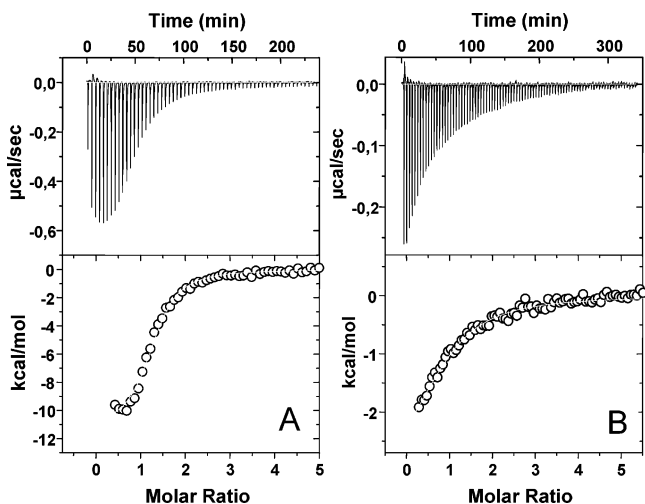
Figure 2. Thermograms (upper panel) and binding isotherms (lower panel) obtained for the binding of BH_4 (A) and 6M-PH_4 (B) to tetrameric human PAH (12 μM and 30 μM subunit in A and B, respectively) at 25 °C in 20 mM NaHepes, 0.2 M NaCl, pH 7.0. The enzyme was titrated with 60 injections, 3 μL per injection, 0.5 mM BH_4 (A) and 80 injections, 3 μL per injection, 1 mM 6M-PH_4 (B).

6M-PH_4 binds also stoichiometrically to PAH, but significantly weaker ($K_a = (6.1 \pm 0.7) \cdot 10^4$ M^{-1}) than BH_4 under our experimental conditions (Figure 2B and Table 1). These results are as expected from previous studies.[4,38] The enthalpic contribution to the interaction of PAH with 6M-PH_4 is smaller than for BH_4 binding in the temperature range under study and, interestingly, the binding is also entropically favorable at all temperatures (Table 1 and Figure 3B). Also, the ΔC_p ($= -63 \pm 12$ cal/mol/K) calculated for the binding of 6M-PH_4 was markedly smaller than for BH_4 (Figure 3B).

Effect of Buffer Ionization and pH on the Binding Enthalpy. Potential protonation/deprotonation effects coupled to tetrahydropterin binding to PAH were investigated by testing the possible contribution from buffer ionization to the experimental ΔH (ΔH_{exp}). ITC measurements were carried out at similar buffer concentrations at pH 7.0, with three reaction buffers of different ionization enthalpies at 25 °C.[39] The ΔH_{exp} obtained was plotted as a function of the ionization enthalpy of the buffer ($\Delta H_{ionization}$) (Figure 4) and fitted to the equation

$$\Delta H_{exp} = \Delta H_{indep} + nH^+ \cdot \Delta H_{ionization} \qquad (3)$$

where ΔH_{indep} is the buffer independent enthalpy and nH^+ is the number of protons taken up or released by the enzyme upon binding. The slope of the linear regression in Figure 4 indicated that the number of protons exchanged was close to zero ($nH^+ = -0.01 \pm 0.09$) and that the ΔH_{indep} ($= -11.75 \pm 0.35$ kcal/mol) is identical to the experimental values obtained in Hepes buffer ($\Delta H_{exp} = -11.78 \pm 0.43$ kcal/mol).

The absence of protonation−deprotonation events of the enzyme or the cofactor upon binding was also confirmed by the relatively similar thermodynamic properties at pH values ranging from 6.0 to 8.0 (Table 2). These limits of the allowed pH range for the calorimetric assays are determined by the pH range at which PAH is stable.[5] Moreover, at pH > 8.0 the enzyme undergoes an activating conformational change that affects its tertiary and quaternary structure, resulting in its dissociation to dimers.[40,41] At pH 6.0 a marked decrease (10-fold) in the affinity of PAH for BH_4 is observed, and a small decrease in binding stoichiometry was also detected. Experiments performed using 6M-PH_4 at pH $\leq$ 7.0 were unsuccessful under our experimental conditions most probably due to low affinity (10^4 $M^{-1} \geq K_a$), beyond the limits of the calorimetric determination.

Binding of Pterins to PAH in the Presence of L-Phe. Incubation of PAH with L-Phe leads to cooperative conformational changes that increase the specific activity with BH_4 more than 20-fold for the rat and 3.5-fold for the human enzyme.[3,4,42] The prior activation of the enzyme has very little effect on the PAH activity with 6M-PH_4.[43] The activation affects the subunit secondary and tertiary structures, as seen for instance by circular dichroism and fluorescence spectroscopy.[42,44] The activation also results in an increase of the hydrodynamic radius of the tetrameric form [45,46] and a more accessible active site.[42] To characterize the effect of activation on the affinity and the thermodynamics of BH_4 and 6M-PH_4 binding, and exploiting the fact that anoxic conditions abolished PAH activity (by removing one of the substrates, dioxygen) we performed calorimetric analysis with activated PAH in the presence of 1 mM L-Phe. Binding thermograms in the presence of L-Phe did not show any calorimetric evidence of catalysis or distortions in baseline. Analysis of the samples obtained after the titration using HPLC showed small but detectable L-Tyr levels (<9% L-Phe had been hydroxylated to L-Tyr after 7−8 h incubation in the calorimetric cell). The thermodynamic parameters for the binding are shown in Table 3. Notably, preincubation of the enzyme with 1 mM L-Phe and the presence of the substrate at the same concentration during the titrations resulted in a decreased binding affinity for BH_4, but not for 6M-PH_4. Also, the presence of the substrate was accompanied by a decreased enthalpy change for the binding of both BH_4 and 6M-PH_4, and a concomitant rise in the entropic contribution to binding.

Comparison of Measured and Calculated Thermodynamic Binding Parameters. Tetrahydropterin cofactors bind to PAH by interactions of the pterin ring with an invariant phenylalanine residue (Phe254 in human PAH) and a hydrogen bonding network involving positions N1, N2, N3, N5, and N8 of the pterin ring.[14,15,17] The available crystal structures of the complexes correspond to N-terminal truncated forms of PAH and in order to prepare the structure of full-length PAH bound to BH_4 and analogues and to study the structural changes that accompany the binding of the pterins, we have recently performed molecular modeling and MD simulations (Figure 5).[13] The MD-converged structures of the PAH·BH_4 (Figure 5B) and PAH·6M-PH_4 (Figure 5C) complexes were used to calculate the buried polar and apolar solvent accessible surface area in the complexes (ΔASA_{polar} and ΔASA_{apolar}) (Table 4). As expected

(38) Haavik, J.; Døskeland, A. P.; Flatmark, T. *Eur. J. Biochem.* **1986**, *160*, 1−8.
(39) Baker, B. M.; Murphy, K. P. *Biophys. J.* **1996**, *71*, 2049−2055.
(40) Parniak, M. A.; Davis, M. D.; Kaufman, S. *J. Biol. Chem.* **1988**, *263*, 1223−1230.
(41) Knappskog, P. M.; Martinez, A. *FEBS Lett.* **1997**, *409*, 7−11.
(42) Thórólfsson, M.; Teigen, K.; Martínez, A. *Biochemistry* **2003**, *42*, 3419−3428.
(43) Kappock, T. J.; Caradonna, J. P. *Chem. Rev.* **1996**, *96*, 2659−2756.
(44) Knappskog, P. M.; Haavik, J. *Biochemistry* **1995**, *34*, 11 790−11 799.
(45) Kappock, T. J.; Harkins, P. C.; Friedenberg, S.; Caradonna, J. P. *J. Biol. Chem.* **1995**, *270*, 30 532−30 544.
(46) Kleppe, R.; Uhlemann, K.; Knappskog, P. M.; Haavik, J. *J. Biol. Chem.* **1999**, *274*, 33 251−33 258.
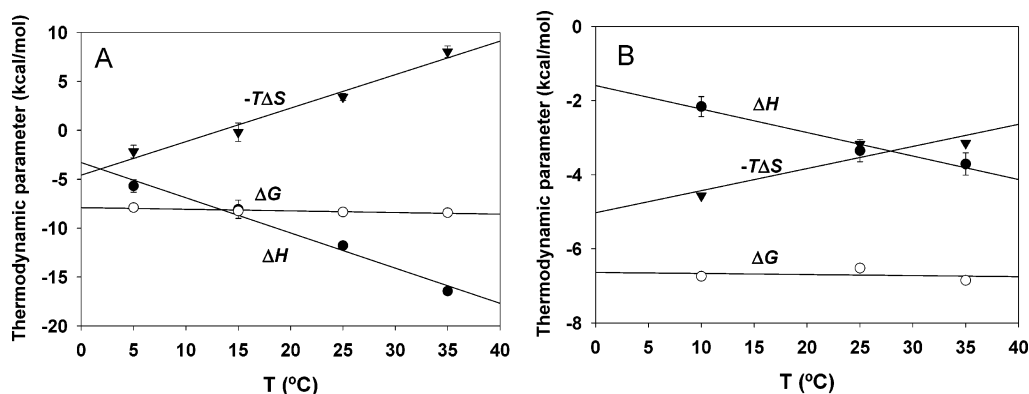
Figure 3. Temperature dependence of the thermodynamic parameters (ΔG, ΔH and $-T\Delta S$) determined for the binding of BH$_4$ (A) and of 6M-PH$_4$ (B) to tetrameric human PAH in 20 mM NaHepes, 0.2 M NaCl, pH 7.0 by ITC. The results are shown as mean $\pm$ S.D from three independent experiments at each temperature for BH$_4$ and from fitting of single experiments for 6M-PH$_4$. The slope of the linear regression of ΔH vs temperature yields a $\Delta C_p = -357 \pm 26$ cal/mol/K for BH$_4$ and -63 ± 12 cal/mol/K for 6M-PH$_4$.

Table 1. Thermodynamic Parameters Obtained for BH$_4$ and 6M-PH$_4$ Binding to Tetrameric Human PAH at Different Temperatures in 20 mM NaHepes, 0.2 M NaCl, pH 7.0 Using ITC[a]

T (°C)	n	K_a (M^{-1})	K_d (μM)	ΔG (kcal/mol)	ΔH (kcal/mol)	ΔS (cal/mol·K)	$-T\Delta S$ (kcal/mol)
			BH$_4$				
5	1.07 ± 0.18	$(1.59 \pm 0.10) \times 10^6$	0.63 ± 0.04	-7.90 ± 0.04	-5.69 ± 0.64	$+7.93 \pm 2.20$	-2.18 ± 0.65
15	1.12 ± 0.05	$(1.82 \pm 0.13) \times 10^6$	0.55 ± 0.04	-8.25 ± 0.03	-8.07 ± 0.93	$+0.67 \pm 3.36$	-0.19 ± 0.95
25	1.15 ± 0.11	$(1.40 \pm 0.38) \times 10^6$	0.75 ± 0.18	-8.36 ± 0.16	-11.78 ± 0.43	-11.41 ± 1.16	$+3.41 \pm 0.35$
35	0.98 ± 0.11	$(0.95 \pm 0.31) \times 10^6$	1.11 ± 0.37	-8.42 ± 0.20	-16.44 ± 0.40	-26.05 ± 1.95	$+8.03 \pm 0.60$
			6M-PH$_4$				
10	0.72 ± 0.07	$(15.9 \pm 3.90) \times 10^4$	6.3	-6.74	-2.16 ± 0.27	$+16.16$	-4.57
25	0.92 ± 0.07	$(6.05 \pm 0.68) \times 10^4$	16.5	-6.52	-3.35 ± 0.30	$+10.66$	-3.18
35	0.88 ± 0.07	$(7.23 \pm 1.08) \times 10^4$	13.8	-6.85	-3.71 ± 0.38	$+10.21$	-3.15

[a] The data are the mean $\pm$ S. D. from three independent experiments for BH$_4$ and mean $\pm$ S. E. of the fitting obtained from single experiments for 6M-PH$_4$. Protein concentration was 12 μM subunit for BH$_4$ binding and 30 μM for 6M-PH$_4$ binding.
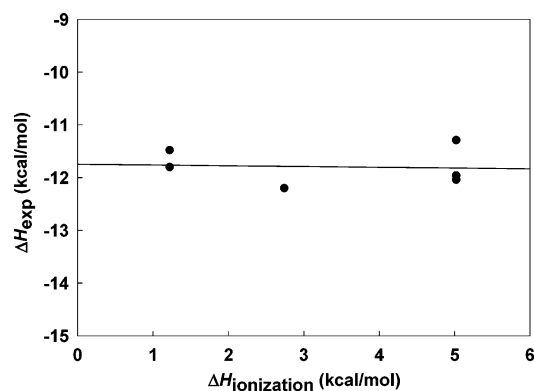

Figure 4. Dependence of the ΔH experimental (ΔH_{exp}) for the binding of BH$_4$ to human PAH on the ionization enthalpy of the reaction buffer ($\Delta H_{ionization}$). Experiments were performed at 25 °C at pH 7.0 in 20 mM of each buffer and 0.2 M NaCl. The $\Delta H_{ionization}$ for the buffers used were: Phosphate (1.22 kcal/mol), Pipes (2.74 kcal/mol) and Hepes (5.02 kcal/mol).[58]

from the size of the ligand, the total interface area is larger in PAH·BH$_4$ than in PAH·6M-PH$_4$. In addition, the apolar areas are larger than the polar in both complexes (Table 4).

The large and slow conformational changes induced by L-Phe involve the tetrameric forms of the protein and cannot be properly simulated in achievable computational times. Preliminary insights on the structural changes related to L-Phe binding to the full-length PAH have been undertaken by restricted MD simulations,[42] but the binary PAH·L-Phe and ternary PAH·L-Phe·BH$_4$ (or PAH·L-Phe·6M-PH$_4$) complexes obtained could not be used for an accurate calculation of the ΔASA. These

calculations in the ternary complexes must thus await high-resolution structures.

One of the most important thermodynamic parameter to characterize the energetics of protein folding and molecular recognition is ΔC_p,[47] which is negative for most binding processes. In general, the largest contribution to the ΔC_p is due to changes in dehydration of ligand and protein surfaces.[36,47] The ΔC_p thus becomes proportional to the change in water exposed surface area according to the following empirical relationship[28]

$$\Delta C_p = -0.26 \text{ cal/(mol·K·Å}^2)\Delta ASA_{polar} + 0.45 \text{ cal/(mol·K·Å}^2)\Delta ASA_{apolar} \quad (4)$$

While the ΔC_p of polar hydration is negative, the hydration ΔC_p of apolar groups is positive. Thus, the opposite contributions are expected from the dehydration accompanying binding. The calculated ΔC_p ($\Delta C_{p,calc}$) on the final modeled structures of the PAH·BH$_4$ and PAH·6M-PH$_4$ complexes (Figure 5B,C), agrees well with the experimental results for the binding of 6M-PH$_4$ at pH 7.0, but not for BH$_4$ binding (Table 4). Discrepancies between the $\Delta C_{p,exp}$ and $\Delta C_{p,calc}$ for the binding of the natural cofactor are most probably related to the conformational rearrangement of the enzyme, notably at the N-terminal[13] (see also below).

The current structural energetics formulations also assume a relation between other thermodynamic parameters as ΔH and ΔS to the surface area burial of polar and apolar groups upon

(47) Gómez, J.; Hilser, V. J.; Xie, D.; Freire, E. *Proteins* **1995**, *22*, 404−412.

Table 2. Thermodynamic Parameters Obtained for BH$_4$ and 6M-PH$_4$ Binding to Tetrameric Human PAH at Different pH at 25 °C Using ITC[a]

buffers, pH	N	K$_a$ (M^{-1})	K$_d$ (μM)	ΔG (kcal/mol)	ΔH (kcal/mol)	ΔS (cal/mol·K)	−TΔS (kcal/mol)
			BH$_4$				
MES, 6.0	0.70 ± 0.03	(0.14 ± 0.02) × 10^6	7.1	−7.02	−9.75 ± 0.57	−9.47	+2.82
phosphate, 6.5	0.92 ± 0.04	(0.86 ± 0.18) × 10^6	1.2	−8.08	−10.22 ± 0.61	−7.14	+2.13
hepes, 7.0[c]	1.15 ± 0.11	(1.40 ± 0.38) × 10^6	0.75 ± 0.18	−8.36 ± 0.16	−11.78 ± 0.43	−11.41 ± 1.16	+3.41 ± 0.35
hepes, 7.5	1.31 ± 0.01	(0.87 ± 0.05) × 10^6	1.1	−8.10	−9.86 ± 0.12	−5.90	+1.76
hepes, 8.0	1.28 ± 0.02	(1.82 ± 0.24) × 10^6	0.55	−8.53	−10.49 ± 0.23	−6.51	+1.94
			6M-PH$_4$[b]				
hepes, 7.0	0.92 ± 0.07	(6.05 ± 0.68) × 10^4	16.5	−6.52	−3.35 ± 0.30	+10.66	−3.18
hepes, 8.0	1.19 ± 0.09	(7.40 ± 1.30) × 10^4	13.5	−6.64	−3.04 ± 0.32	+12.08	−3.60

[a] Data are given as mean ± S. E. of the fitting from single experiments, except for BH$_4$ at pH 7.0 (mean ± S. D. from three independent experiments). Protein concentration was 12 μM subunit for BH$_4$ binding (except at pH 6.0 that was 33 μM) and 30 μM for 6M-PH$_4$ binding. [b] Not titratable with MES buffer at pH 6.0 due to too low affinity. [c] Similar values were measured with Pipes, pH 7.0 and Phosphate, pH 7.0 (Figure 4).

Table 3. Thermodynamic Parameters Obtained for BH$_4$ and 6M-PH$_4$ Binding to Tetrameric Human PAH in the Absence or Presence of L-Phe 1 mM at 25 °C in 20 mM Hepes, 200 mM NaCl, pH 7.0 using ITC[a]

L-Phe	N	K$_a$ (M^{-1})	K$_d$ (μM)	ΔG (kcal/mol)	ΔH (kcal/mol)	ΔS (cal/mol·K)	−TΔS (kcal/mol)
			BH$_4$				
−	1.15 ± 0.11	(140 ± 38) × 10^4	0.75 ± 0.18	−8.36 ± 0.16	−11.78 ± 0.43	−11.41 ± 1.16	+3.41 ± 0.35
+	0.89 ± 0.05	(37 ± 6) × 10^4	2.70	−7.60	−6.61 ± 0.49	+3.33	−0.99
			6M-PH$_4$				
−	0.92 ± 0.07	(6.0 ± 0.7) × 10^4	16.5	−6.52	−3.35 ± 0.30	+10.66	−3.18
+	0.88 ± 0.08	(6.8 ± 1.1) × 10^4	14.7	−6.59	−1.85 ± 0.21	+15.91	−4.74

[a] The protein concentration used was 12 μM for the binding of BH$_4$ and 30 μM for the binding of 6M-PH$_4$. Data are given as mean ± S. E. of the fitting from single experiments, except for BH$_4$ in the absence of L-Phe (mean ± S. D. from three independent experiments)

binding.[28,29] Moreover, a recent work has successfully proven the empirical parametrization of the binding enthalpy for small ligands (MW < 800) in terms of structural information using structure/enthalpy correlations derived from protein stability data.[30] The total binding enthalpy of small ligands can be considered as the combination of at least three terms

$$\Delta H = \Delta H_{\text{intrinsic}} + \Delta H_{\text{conformation}} + \Delta H_{\text{protonation}} \quad (5)$$

where $\Delta H_{\text{intrinsic}} + \Delta H_{\text{conformation}}$ corresponds to ΔH_{indep} in eq 3. $\Delta H_{\text{intrinsic}}$ represents the noncovalent interactions between the ligand and the protein and the changes in solvation upon binding; $\Delta H_{\text{conformation}}$ is the enthalpy associated with any possible conformational change in the protein or ligand upon binding, and $\Delta H_{\text{protonation}}$ is the enthalpy associated with protonation/deprotonation events. $\Delta H_{\text{intrinsic}}$ corresponds to the enthalpy that would be observed if protein and ligand had the same conformation in the free and bound states and is expected to scale-up with changes in accessible surface areas. These changes are calculated on the structures of the final complex. On the other hand, $\Delta H_{\text{conformation}}$ is not easily parametrized in terms of $\Delta\Delta ASA$, specially for large proteins, as is the case for PAH ($ASA_{\text{total}} = 18\,000$ Å^2/subunit), and its theoretical quantification would require high-resolution structures of diverse enzyme-ligand complexes with pterins inducing similar conformational changes.[30]

In the absence of protonation/deprotonation events, which is the case for the binding of reduced pterins to PAH (see above and Figure 4), and at 25 °C

$$\Delta H = \Delta H_{\text{conformation}} - a \cdot \Delta ASA_{\text{apolar}} + b \cdot \Delta ASA_{\text{polar}} \quad (6)$$

where the enthalpy values are in cal/mol and the a (7.35(± 2.55)) and b (31.06(± 6.32)) coefficients in cal·(mol·Å^2)$^{-1}$. The second and third terms in eq 6 thus constitute the calculated ΔH (ΔH_{calc}) (Table 4). ΔH_{calc} would correspond to the $\Delta H_{\text{intrinsic}}$ (see eqs 5 and 6), and is calculated without considering

conformational changes using the structure of the BH$_4$-bound or 6M-PH$_4$-bound PAH (Figure 5B,C) both in the presence and absence of the corresponding cofactor.[30] As seen in Table 4 the calculated enthalpies at 25 °C compare very well with the experimental values for the binding of 6M-PH$_4$, but there was no agreement for BH$_4$ binding. This seems to indicate a large negative enthalpic contribution from conformational changes ($\Delta H_{\text{conformation}}$ in eq 6) upon binding of BH$_4$, again in agreement with the results from MD simulations[13] (Figure 5A,B).

Finally, the total binding entropy change (ΔS) can be considered to be the sum of at least three terms[29]

$$\Delta S = \Delta S_{\text{solv}} + \Delta S_{\text{conformation}} + \Delta S_{\text{mixing}} \quad (7)$$

where ΔS_{solv} is the solvation term at 25 °C. Baker and Murphy have proposed that the entropy of both polar and apolar solvation is close to zero near 385 K,[29] whereas Freire and co-workers[48,49] consider that while this temperature value fits for the apolar solvation, the entropy of polar solvation is zero near 335.15 K. ΔS_{solv} was thus calculated according to both approximations (Table 4). $\Delta S_{\text{conformation}}$ is the change in side-chain and backbone conformational entropy associated to binding and ΔS_{mixing} accounts for entropy changes due to variations in translational/rotational degrees of freedom. For bimolecular interactions ΔS_{mixing} is equal to the "cratic" term −7.96 cal/mol/K.[29] As seen in Table 4, it is again for the interaction of PAH with 6M-PH$_4$ that the calculated and experimental entropy contributions show a good agreement at 25 °C, notably when ΔS_{solv} was calculated as indicated by Baker and Murphy,[29] indicating the absence of a significant $\Delta S_{\text{conformation}}$. On the other hand the PAH·BH$_4$ structure was calculated to have a favorable solvation (plus mixing) entropy change, whereas the opposite is measured experimentally, indicative of a large penalization by conforma-

(48) Luque, I.; Gómez, J.; Freire, E. *Proteins* **1998**, *30*, 74−85.
(49) D'Aquino, J. A.; Gómez, J.; Hilser, V. J.; Lee, K. H.; Amzel, L. M.; Freire, E. *Proteins* **1996**, *25*, 143−156.
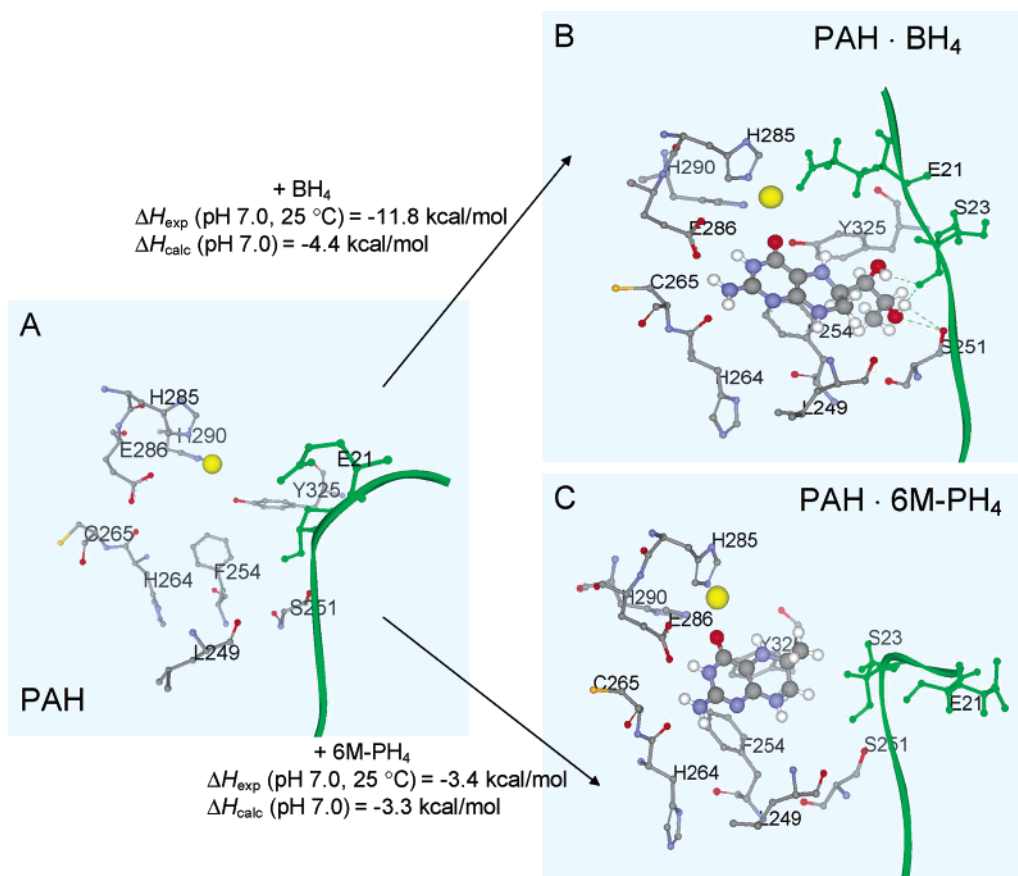
Figure 5. Detailed views of the unbound resting PAH (A), and of the PAH·BH₄ (B) and PAH·6M-PH₄ (C) complexes obtained by MD simulations. The structures in (B) and (C) represent the average energy minimized conformer from the last 50 ps of the 500 ps-simulations.[13] The N-terminal tail backbone is shown as a green ribbon and the active site iron as a yellow sphere. The cofactor, the residues at the cofactor binding site and relevant residues from the N-terminal tail are shown in ball-and-stick representation. Note the interaction between the dihydroxypropyl side chain in BH₄ and both Ser23 and Ser251 (B).

Table 4. Structural-Derived Energetic Parameters Calculated (calc) on the MD Simulated Structures of the PAH−Tetrahydropterin Complexes[13] (Figure 5B,C)[a]

parameter	PAH·BH₄	PAH·6M-PH₄
ΔASA_{polar} (Å²)	−216	−180
ΔASA_{apolar} (Å²)	−313	−307
$\Delta C_{p,exp}$ (cal/mol/K)	−357	−63
$\Delta C_{p,calc}$ (cal/mol/K), from ΔASA	−85	−89
ΔH_{exp} (kcal/mol), 25 °C	−11.8	−3.35
ΔH_{calc} (kcal/mol), from ΔASA	−4.4	−3.32
ΔS_{exp} (cal/mol/K), 25 °C	−11.4	10.6
$\Delta S_{solv,calc}{}^{b}$ + ΔS_{mixing} (cal/mol/K)	13.7	14.1
$\Delta S_{solv,calc}{}^{c}$ + ΔS_{mixing} (cal/mol/K)	21.5	22.0

ᵃ Comparison with experimental (exp) parameters from ITC at pH 7.0. *ᵇ* $\Delta S_{solv,calc}$ of polar and apolar solvation at 25 °C calculated from the $\Delta C_{p,calc}$ according to ref 29 ($\Delta S_{solv,calc} = \Delta C_p \cdot \ln(298.15/385.15)$). *ᶜ* $\Delta S_{solv,calc}$ of polar and apolar solvation at 25 °C calculated from the $\Delta C_{p,calc,polar}$ and $\Delta C_{p,calc,apolar}$ according to refs 48 and 49 ($\Delta S_{solv} = \Delta C_{p,apolar} \cdot \ln(298,15/385,15) + \Delta C_{p,polar} \cdot \ln(298,15/335,15)$).

tional entropy at 25 °C. For the binding of BH₄ in the presence of L-Phe (activated enzyme) the negative contribution of this conformational rearrangement to the total entropy change appears to disappear and it is even positive at neutral pH and 25 °C (Table 3).

Discussion

As the key enzyme in the catabolism of L-Phe, PAH is highly regulated by its substrate and natural cofactor BH₄.[4] PAH has been believed to include regulatory sites for both L-Phe and

BH₄, in addition to the catalytic sites.[4,31,43] Recent work from our group has nevertheless proven that the full-length tetrameric enzyme only contains one L-Phe binding site per subunit.[5] The binding of the amino acid substrate at the active site of one subunit induces the displacement of the N-terminal autoregulatory sequence (residues 1−33), and the activating conformational changes are transmitted to the other subunits in the tetramer by homotropic positive cooperative events.[42] Here, we also show that full-length PAH contains only one BH₄ binding site per subunit (Table 1), where BH₄ would elicit both its catalytic and regulatory functions. Thus, our results do not support the presence of an additional site different to the catalytic site and that has been putatively located at the N-terminal domain.[31] It has been recognized for a long time that the specific regulatory properties of the 6R isomer of BH₄ are related to interactions between its dihydroxypropyl side chain and the N-terminal regulatory domain of PAH.[4,8,43] A likely molecular mechanism for the negative regulation elicited by BH₄ upon binding to the active site has been put forward by MD simulations.[13] The synthetic cofactor 6M-PH₄ does not negatively modulate PAH activity and is frequently used in activity assays with PAH due to its high catalytic efficiency.[43,50]

Our novel calorimetric experiments reveal the high affinity of the enzyme for BH₄ ($K_d = 0.75 \pm 0.18$ μM) as compared to that for the analogue 6M-PH₄ ($K_d = 16.5 \pm 2.7$ μM) at neutral

(50) Kaufman, S.; Citron, B. A.; Davis, M.; Milstien, S. *Adv. Exp. Med. Biol.* **1993**, *338*, 97−102.

pH and 25 °C. The K_d values are 40- and 6-fold smaller than the K_m values obtained from steady-state kinetic measurements with the human enzyme, for BH$_4$ and 6M-PH$_4$, respectively.[22] A decreased binding affinity at turnover conditions is not so unexpected for a multisubstrate enzyme reaction. Hence, the introduction of ground-state destabilization of the reactive portion of the bound substrate at turnover conditions is expected to decrease the affinity (i.e., $K_m > K_d$) with respect to simple enzyme–substrate complex formation.[51] Also, for BH$_4$, a fraction of this reduced apparent affinity encountered at turnover conditions may be partly explained by the observed 3.6-fold increase in K_d in the presence of L-Phe (Table 3 and see below), whereas binding assays performed at aerobic conditions show that dioxygen does not affect the affinity for BH$_4$.[21] The high affinity value measured for the natural cofactor corroborates earlier binding affinities measured by fluorescence quenching[38] and by the inhibitory effect of BH$_4$ on the rate of phosphorylation of the enzyme at Ser16 (half-maximum inhibition [I]$_{0.5}$ = 1.4 μM).[11] In addition to providing the stoichiometry and the equilibrium binding affinity constant of human PAH for its cofactors, ITC represents an important advantage compared with other methods in that it provides a complete thermodynamic description of the associating system. The comparative analysis of the binding of BH$_4$ and 6M-PH$_4$ to PAH also allows to characterize specific energetic contributions from either the pterin ring or the dihydroxypropyl side chain. 6M-PH$_4$ binding is both moderately enthalpically and entropically driven from 10 to 35 °C. On the other hand, BH$_4$ binding is strongly enthalpically driven and moderately entropic penalized at 25 °C, and the unfavorable entropic contribution increases at higher temperatures. These differences in energetic contribution (ΔH and ΔS) to the binding can be explained, at least partially, using structure-energetics relationships. 6M-PH$_4$ binds by π-stacking interaction with Phe254 and by an extensive hydrogen-bonding network between the pterin ring and residues from the PAH catalytic domain.[14,17] In this case, and due to the absence of ionization effects upon binding, the thermodynamic parameters can be evaluated by intrinsic binding considerations (noncovalent bond interactions and solvation changes upon binding) considering that the protein would have the same conformation in the free and bounded states.[30] Notably, ΔH and ΔC_p scale-up with the changes in apolar and polar accessible surface area upon binding (Table 4). This agreement between the experimental and the calculated intrinsic contribution to ΔH and ΔC_p, as well as the favorable ΔS, correspond well with results from MD simulation of the binding of 6M-PH$_4$ to PAH showing that the unbound and bound enzyme structures are very similar (Figure 5A,C).[13] The favorable entropy change can also be mainly explained by desolvation of both the tetrahydropterin and the active site of the PAH upon binding and by the highly nonpolar protein–ligand interface.[29,36] The presence of the dihydroxypropyl side chain at C6 in BH$_4$ is expected to provide a noticeably input to the binding energetics of PAH·BH$_4$ complex formation besides the contribution of the pterin ring. The aliphatic side chain interacts via O2′ with Ser251 at the catalytic domain, and both O1′ and O2′ interact with residues of the autoregulatory N-terminal sequence (residues 1–33), and notably with Ser23 (Figure 5B). These specific interactions and the resulting dehydration of higher binding interface should be

manifested in a higher ΔH than that obtained upon binding of 6M-PH$_4$, as reflected in the theoretical estimates of these parameters for PAH·BH$_4$ (-4.4 vs. -3.3 kcal/mol for PAH· 6M-PH$_4$) (Table 4). Nevertheless, opposite to the good correlation obtained for the PAH·6M-PH$_4$ complex, the structure-based estimate of ΔH are significantly less negative than the experimental values for PAH·BH$_4$ (Table 4). In the resting unbound enzyme a hydrogen bonding network connects Tyr377 and both Ser23 and Glu21,[31] but the binding of BH$_4$ disturbs this network. MD simulations show that the interaction of the natural cofactor with Ser23 facilitates the switch of Glu21 toward the iron further pulling out the N-terminal into the active site of PAH and blocking the L-Phe binding site.[13] 6M-PH$_4$ cannot interact favorably with Ser23, and does not induce the inhibitory conformational change on PAH (Figure 5C). The specific contacts between the side-chain of BH$_4$ and PAH thus would trigger a regulatory conformational change that would contribute to the energetics of the binding process, and notably to an increase of enthalpic contribution, due to the concomitant changes in hydrogen bonding, van der Waals interactions and the increase in packing density. Conformational changes in the protein as a major contributor to ΔH have been demonstrated in many binding systems.[30,52] Upon BH$_4$ binding the enzyme adopts a more closed conformation with a less flexible N-terminal autoregulatory sequence[13] and, most probably, a drastic reduction of conformational entropy, in agreement with the entropic penalty at temperatures > 13 °C.

The BH$_4$ dependent PAH activity has been described to be pH dependent, presenting higher activity at mild alkaline pH (8.5–9.0) and lower activity at mild acidic pH compared to pH 7.0.[40,41,53] The higher activity at alkaline pH with BH$_4$ appears to be related to a conformational activation of the enzyme at this pH and in fact the activity for the L-Phe activated enzyme is highest at neutral pH.[40,41] The pH optimum for maximal activity is ~7.0 with 6M-PH$_4$ as cofactor both for activated and nonactivated PAH.[22] As seen by the pH dependent ITC measurements, the binding affinities are also highest for both cofactors at pH 7.0–8.0 (Table 2). The affinity for BH$_4$ decreased 10-fold at pH 6.0 and the titration with 6M-PH$_4$ at that pH was unsuccessful due to a very low affinity. The similar pH effect on the binding of both cofactors suggests that the pH dependence of tetrahydropterin binding affinity is most probably related to the interactions of the pterin ring with little contribution from the dihydroxypropyl side chain. The reduced specific activity of PAH under mild acidic conditions may thus well be explained by the large decrease in affinity at pH < 7.0 for the cofactor. In addition, the activation rate with L-Phe is also reduced at mild acidic pH.[53] At physiologic pH, biopterin primarily exists as an uncharged species, and the N5 and N1 amide groups have pKa values of 5.6 and 1.3, respectively.[54,55] A protonation of the N5 at mild acidic conditions would be electrostatically unfavorable for the interaction of the pterin with PAH due to the proximity of the protonated amide to the charged metal ion (Figure 5) and would explain the pH dependence of tetrahydropterin binding.

(51) Miller, B. G.; Wolfenden, R. *Annu. Rev. Biochem.* **2002**, *71*, 847–885.

(52) Spolar, R. S.; Record, M. T., Jr. *Science* **1994**, *263*, 777–784.
(53) Shiman, R.; Gray, D. W. *J. Biol. Chem.* **1980**, *255*, 4793–4800.
(54) Eberlein, G.; Bruice, T. C.; Lazarus, R. A.; Henrie, R.; Benkovic, S. J. *J. Am. Chem. Soc.* **1984**, *106*, 7916–7924.
(55) Wei, C. C.; Crane, B. R.; Stuehr, D. J. *Chem. Rev.* **2003**, *103*, 2365–2383.

Finally, the use of anaerobic conditions allowed us to investigate the effect of the substrate L-Phe on tetrahydropterin binding. PAH exhibits positive cooperative for the binding of L-Phe, reflecting a conformational change from a low-activity and low-affinity for the substrate to a high-activity and high-affinity state.[4] L-Phe and BH$_4$ elicit opposite effects on the activation state of the enzyme, the rate of phosphorylation at Ser16 by cAMP-dependent protein kinase and the susceptibility to proteolysis, presumably by inducing opposed and specific effects on the regulatory domain conformation and the quaternary structure of PAH.[4,11,56] Activation of human PAH by L-Phe causes a 3.6-fold decrease of binding affinity for BH$_4$, mainly by a large reduction in favorable enthalpic contribution, whereas the affinity and the thermodynamic parameters for 6M-PH$_4$ binding are only slightly different under activation conditions. The results for BH$_4$ binding indicate that the enthalpically favorable conformational change induced by specific interactions of the dihydroxypropyl side chain and associated to a reduction of conformational entropy at the protein level (see above), are hindered in the presence of L-Phe. The L-Phe activated enzyme thus would have a more open conformation of the N-terminal autoregulatory sequence which is not closed upon binding of BH$_4$ in the presence of L-Phe. This is in agreement with the favorable entropy change upon binding of BH$_4$ to the L-Phe activated enzyme (Table 3), indicating that at 25 °C there is no penalization by conformational entropy at the protein level.

Recent studies have highlighted the applicability of structure-energetics relationships to provide a quantitative and rational description of the different thermodynamic contributions to small ligand binding to proteins based on comparison between the calorimetric data and energetics parameters obtained in high-resolution structures[30] or by combining ITC and MD simula-tions.[57] No high-resolution structures for full-length PAH, with or without amino acid substrate and cofactor, have so far been solved. In this work, we have thus applied these calculations to structures of the enzyme-pterin complexes generated by MD simulations. The structure-energetics relationships appear to satisfactorily interpret at the molecular level the intrinsic binding contributions to the thermodynamic parameters. The quantification of the contributions due to conformational rearrangements, notably induced by the natural cofactor BH$_4$ both in the absence and the presence of substrate, would require the high resolution structures of PAH associated to pterins inducing similar conformational changes and the use of site-directed and truncated mutant forms of the enzyme. Meanwhile, the calorimetric analyses presented in this work represent a powerful approach to understand the thermodynamics of tetrahydropterin binding to PAH, and the conformational implications of important functional and regulatory events elicited by the substrate and the natural cofactor. In addition, this study provides a reference frame to investigate the functional effects of PKU mutations, notably on the affinity for BH$_4$, to help on the selection of genotypes predictably associated with a positive response to cofactor treatment.

Note Added after ASAP Posting. After this paper was posted ASAP on October 2, 2004, corrections were made to the exponents in column 3 of Tables 1-3. The corrected version was posted October 5, 2004.

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(56) Døskeland, A. P.; Døskeland, S. O.; Øgreid, D.; Flatmark, T. *J. Biol. Chem.* **1984**, *259*, 11 242−11 248.
(57) Talhout, R.; Villa, A.; Mark, A. E.; Engberts, J. B. *J. Am. Chem. Soc.* **2003**, *125*, 10 570−10 579.
(58) Fukada, H.; Takahashi, K. *Proteins* **1998**, *33*, 159−166.