Inorg. Chem. **2008**, *47*, 4877−4883

Kinetics of Thermal Unfolding of Phenylalanine Hydroxylase Variants Containing Different Metal Cofactors (Fe^{II}, Co^{II}, and Zn^{II}) and Their Isokinetic Relationship

Aristobulo Loaiza,[†] Kathryn M. Armstrong,[‡] Brian M. Baker,[‡] and Mahdi M. Abu-Omar*[,†]

Brown Laboratory, Department of Chemistry, Purdue University, 560 Oval Drive, West Lafayette, Indiana 47907, and Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana 46556

Received January 29, 2008

The kinetics of thermal unfolding of apo- and holo-*Chromobacterium violaceum* phenylalanine hydroxylase (cPAH) was investigated using circular dichroism (CD) over the temperature range 44−76 °C. In addition to the native cofactor (Fe^{II}), the unfolding kinetics of holo-cPAH was characterized using Zn^{II} and Co^{II} as cofactors. Kinetic profiles for apo- and holo-cPAH showed a single-phase exponential rise in the CD signal at $\lambda = 222$ nm and a first-order dependence on protein concentration. The extrapolated unfolding rate constants (k_u) at ambient temperature followed the order apo > Fe > Zn ≫ Co. Transition-state analysis of the activation parameters revealed an isokinetic correlation, which suggests a common mechanism for the enzyme variants. The values of the entropy of activation ($\Delta S^{\ddagger}$) for apo- and Fe-cPAH were negative but small: -34 ± 24 and -32 ± 18 J mol^{-1} K^{-1}, respectively. On the other hand, $\Delta S^{\ddagger}$ values for Zn- and Co-cPAH were large and positive: 54 ± 9 and 175 ± 27 J mol^{-1} K^{-1}, respectively. Therefore, at higher temperatures the unfolding rates of Zn- and Co-cPAH are affected significantly by entropy, while the unfolding rates of apo- and Fe-cPAH are dominated by enthalpy even at higher temperatures. The rate of unfolding of holo-cPAH did not depend on excess metal concentrations and maintained single-phase kinetic profiles, refuting the occurrence of adventitious metal binding and the notion that unfolding occurs via apo-cPAH exclusively. Isothermal titration calorimetry (ITC) was employed to measure cPAH binding affinities for Fe, Zn, and Co as well as the enthalpy of metal coordination. Dissociation constants (K_d) decreased in the order Fe > Zn > Co. The non-native metals, Zn and Co, were bound more tightly than Fe. The activation enthalpy for unfolding ($\Delta H^{\ddagger}$) displayed a linear correlation with the enthalpy of metal binding obtained from ITC measurements (ΔH_{ITC}). On this basis, a common mechanism (transition state) is suggested for this family of metal cofactors, and the varying enthalpy of activation arises from the differing stabilities of enzyme variants having different metal cofactors.

Introduction

Phenyalanine hydroxylase (PAH) catalyzes the oxidation of L-phenylalanine to L-tyrosine using molecular oxygen as the oxidant and 6(R)-L-*erythro*-tetrahydrobiopterin (BH_4) as a cosubstrate (Scheme 1).[1] The active-site cofactor is an octahedral non-heme iron(II) ligated by two imidazoles (His), a bidentate carboxylate (Glu), and two water molecules.[2] A typical human diet is rich in L-phenylalanine, and therefore, we depend on PAH to metabolize more than 75% of our L-phenylalanine intake. Genetic deficiencies in the PAH gene adversely affect this metabolic pathway and lead to hyperphenylalaninemia (HPA), a hallmark of phenylketonuria (PKU).[3] In vitro studies have shown that PKU-associated mutations affect enzymatic activity, expression yields, stabil-

* Corresponding Author. E-mail: mabuomar@purdue.edu.
[†] Purdue University.
[‡] University of Notre Dame.

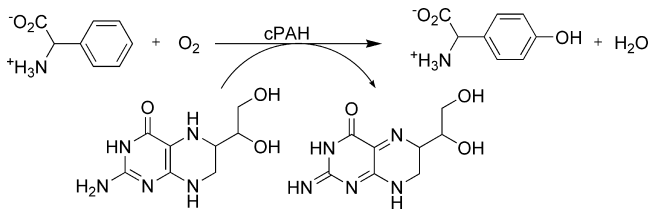
(1) (a) Abu-Omar, M. M.; Loaiza, A.; Hontzeas, N. *Chem. Rev.* **2005**, *105*, 2227–2252. (b) Fitzpatrick, P. F. *Biochemistry* **2003**, *42*, 14083–14091. (c) Flatmark, T.; Stevens, R. C. *Chem. Rev.* **1999**, *99*, 2137–2160. (d) Kappock, T. J.; Caradonna, J. P. *Chem. Rev.* **1996**, *96*, 2659–2756.

(2) Erlandsen, H.; Kim, J. Y.; Patch, M.; Han, A.; Volner, A.; Abu-Omar, M. M.; Stevens, R. C. *J. Mol. Biol.* **2002**, *320*, 645–661.

(3) Erlandsen, H.; Stevens, R. C. *Mol. Genet. Metab.* **1999**, *68*, 103–125.

10.1021/ic800181q CCC: $40.75
Published on Web 04/23/2008

Scheme 1



ity, and folding of the recombinant products, suggesting that these may play a role in vivo.[4] Despite the implication of misfolded structures in PKU recombinant mutants, no studies have been reported on the (un)folding kinetics and mechanisms of PAH.

Investigations of (un)folding of metalloproteins represent only a small fraction of all protein (un)folding research. However, that percentage is likely to increase given the critical role metals play in misfolding diseases such as Alzheimer's, Parkinson's, and ALS. Metal dependence of protein folding has been investigated in blue copper proteins, cytochromes, polypeptides containing Fe−S clusters, and synthetic metallopeptides.[5]

We have investigated the kinetics of unfolding in *Chromobacterium violaceum* phenylalanine hydroxylase (cPAH) (Figure 1). In comparison to the human enzyme (hPAH), cPAH lends itself well to protein folding studies because of its structural simplicity. It is a monomeric, single-domain protein, while hPAH consists of three domains (tetramerization, catalytic, and regulatory). Despite sharing only 35% amino acid sequence identity, cPAH and hPAH have nearly indistinguishable folding motifs.[2] Furthermore, cPAH is slightly less compact than hPAH, with molecular dimensions that render its active site more solvent-exposed, yet cPAH is more resistant to thermal denaturing.[6] This structural feature enables metal reconstitution in cPAH, which is not possible in the human enzyme. In addition to the native holo-cPAH (Fe-cPAH), which contains iron, we have prepared cobalt- and zinc-substituted cPAH variants.[6]

Herein we report the kinetics and activation parameters for the unfolding of apo- and holo-PAH using biologically

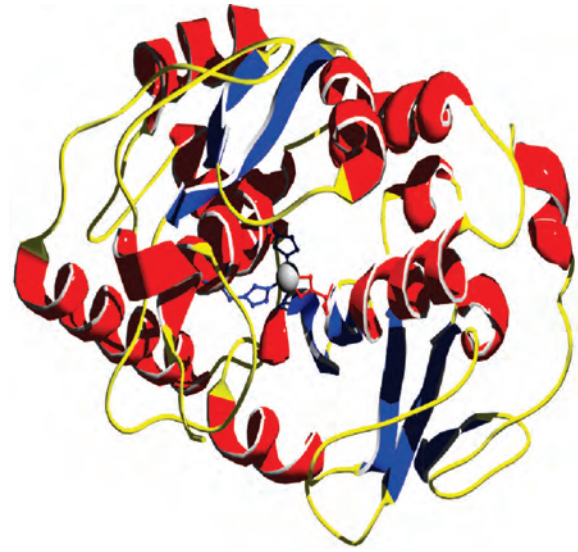


Figure 1. Structure of bacterial phenylalanine hydroxylase from *C. violaceum* (PDB entry 1LTZ). The active-site iron is in gray, His ligands are in blue, and Glu is in red.

relevant metals: iron, zinc, and cobalt. We examine the isokinetic relationship ($\Delta H^{\ddagger}$ vs $\Delta S^{\ddagger}$, where $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are the enthalpy and entropy of activation, respectively) for the series and discuss its implications for the mechanism of cPAH unfolding induced by a temperature jump. We also report on the thermodynamics of metal binding to cPAH for Fe^{II}, Zn^{II}, and Co^{II}. Isothermal titration calorimetry (ITC) measurements were used to obtain dissociation constants (K_d) and enthalpies (ΔH) for metal coordination. The relationship between the activation enthalpies for unfolding of cPAH and metal-binding enthalpies (ΔH) is discussed in light of the isokinetic relationship.

Experimental Methods

Enzyme Expression and Purification. *C. violaceum* phenylalanine hydroxylase (cPAH) was expressed in *Escherichia coli* BL21 (DE3) pLysS bacterial host according to a previously published procedure.[7] Recombinant cPAH was purified via chromatography (DEAE cellulose followed by gel filtration on Sephadex G-75), and collected fractions of cPAH were concentrated to 750 μM and stored at −80 °C; protein concentration was determined spectrophotometrically, and enzyme activity measurements were performed by following tyrosine production at 275 nm, as described previously.[7] Holo-cPAH samples were prepared from apo-cPAH and the metal salts $Fe(SO_4)$, $ZnCl_2$, and $CoCl_2$.[7,8]

Unfolding Kinetics. The unfolding reactions of apo- and holo-cPAH were monitored using circular dichroism (CD) (on a Jasco J-715 spectropolarimeter equipped with a Peltier temperature control unit) at $\lambda = 222$ nm, which is characteristic of α-helical secondary structure. Unfolding was initiated by adding apo- or holo-cPAH from a 735 μM stock solution to a buffer solution (5.0 mM Hepes, pH 7.40) equilibrated at the desired temperature (44−76 °C) to give a final protein concentration of 20 μM. Alternatively, to investigate the metal dependence of the unfolding kinetics, an

(4) (a) Blau, N.; Erlandsen, H. *Mol. Genet. Metab.* **2004**, *82*, 101–111. (b) Erlandsen, H.; Pey, A. L.; Gamez, A.; Perez, B.; Desviat, L. R.; Aguado, C.; Koch, R.; Surendran, S.; Tyring, S.; Matalon, R.; Scriver, C. R.; Ugarte, M.; Martinez, A.; Stevens, R. C. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 16903–16908. (c) Gjetting, T.; Petersen, M.; Guldberg, P.; Guttler, F. *Mol. Genet. Metab.* **2001**, *72*, 132–143. (d) Gamez, A.; Perez, B.; Ugarte, M.; Desviat, L. R. *J. Biol. Chem.* **2000**, *275*, 29737–29742. (e) Pey, A. L.; Desviat, L. R.; Gamez, A.; Ugarte, M.; Perez, B. *Hum. Mutat.* **2003**, *21*, 370–378. (f) Pey, A. L.; Perez, B.; Desviat, L. R.; Martinez, M. A.; Aguado, C.; Erlandsen, H.; Gamez, A.; Stevens, R. C.; Thorolfsson, M.; Ugarte, M.; Martinez, A. *Hum. Mutat.* **2004**, *24*, 388–399.

(5) (a) Bushmarina, N. A.; Blanchet, C. E.; Vernier, G.; Forge, V. *Protein Sci.* **2006**, *15*, 659–671. (b) Bren, K. L.; Kellogg, J. A.; Kaur, R.; Wen, X. *Inorg. Chem.* **2004**, *43*, 7934–7944. (c) Ghosh, D.; Pecoraro, V. L. *Inorg. Chem.* **2004**, *43*, 7902–7915. (d) Wittung-Stafshede, P. *Acc. Chem. Res.* **2002**, *35*, 201–208. (e) Apiyo, D.; Jones, K.; Guidry, J.; Wittung-Stafshede, P. *Biochemistry* **2001**, *40*, 4940–4948. (f) Wittung-Stafshede, P.; Malmstrom, B. G.; Sanders, D.; Fee, J. A.; Winkler, J. R.; Gray, H. B. *Biochemistry* **1998**, *37*, 3172–3177. (g) Leckner, J.; Bonander, N.; Wittung-Stafshede, P.; Malmstrom, B. G.; Karlsson, B. G. *Biochim. Biophys. Acta* **1997**, *1342*, 19–27. (h) Winkler, J. R.; Wittung-Stafshede, P.; Leckner, J.; Malmstrom, B. G.; Gray, H. B. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 4246–4249.

(6) Zoidakis, J.; Loaiza, A.; Vu, K.; Abu-Omar, M. M. *J. Inorg. Biochem.* **2005**, *99*, 771–775.

(7) (a) Volner, A.; Zoidakis, J.; Abu-Omar, M. M. *J. Biol. Inorg. Chem.* **2003**, *8*, 121–128. (b) Zoidakis, J.; Sam, M.; Volner, A.; Han, A.; Vu, K.; Abu-Omar, M. M. *J. Biol. Inorg. Chem.* **2004**, *9*, 289–296.

(8) Han, A. Y.; Lee, A. Q.; Abu-Omar, M. M. *Inorg. Chem.* **2006**, *45*, 4277–4283.

aliquot of apo-cPAH (735 μM stock solution) was added to a buffer solution (5.0 mM Hepes, pH 7.40) containing 20−100 μM $CoCl_2$, $ZnCl_2$, or $Fe(SO_4)$ and equilibrated at the desired temperature (44−76 °C) to give a final protein concentration of 20 μM. Thermal unfolding of Fe-cPAH was conducted as stated above, except that the buffer containing Fe^{II} was prepared in a wet box pre-equilibrated with N_2(g) in order to prevent oxidation of Fe^{II}. Unfolding reactions were followed for at least five half-lives, and KaleidaGraph (version 3.6) was used to fit the time profiles (ellipticity vs time) to the first-order equation $\theta_t = \theta_\infty + (\theta_0 - \theta_\infty)\exp(-kt)$, where θ is the CD signal (mdeg), k is the first-order rate constant, and t is the time. Each reaction at a given temperature was performed at least in duplicate.

Activation energies (E_a) for each cPAH variant were obtained directly from the Arrhenius equation: $k = A\exp(-E_a/RT)$, where T is the absolute temperature and R is the gas constant. The activation parameters $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$ were computed in accordance with results from transition-state theory (TST) using eqs 1–3:[9]

$$\Delta G^{\ddagger} = RT\left(\ln\frac{k_B}{h} + \ln T - \ln k\right) \quad (1)$$

$$\Delta H^{\ddagger} = E_a - RT \quad (2)$$

$$\Delta S^{\ddagger} = \frac{(\Delta H^{\ddagger} - \Delta G^{\ddagger})}{T} \quad (3)$$

where k_B is Boltzmann's constant and h is Planck's constant.

Isothermal Titration Calorimetry. ITC experiments were carried out at 37.0 ± 0.2 °C (unless indicated otherwise) on a MicroCal VP-ITC calorimeter. Enzyme and metal solutions were buffered in 50 mM Hepes (pH 7.44). In the case of the Fe^{II} titration, the buffer and metal solutions contained 2.5 mM tris(carboxyethyl)phosphine (TCEP). Addition of TCEP was necessary to keep iron reduced in the 2+ oxidation state. The binding constant for the Fe-cPAH titration was acquired by one-site-model fitting of the difference data obtained after subtraction of a control ΔH without enzyme. A typical experiment consisted of titrating a 120−400 μM metal solution into an identically buffered 20 μM apo protein solution. ITC data are presented as the baseline-adjusted calorimetric trace (top panel) and the concentration-normalized, peak-integrated heat of reaction (bottom panel) versus the molar ratio of metal ion to protein monomer (see Figure 3 in the Results). ITC data were fitted to a one-site equilibrium binding model using Origin software. The one-site equilibrium model yields three parameters: stoichiometry (N_{ITC}), change in enthalpy (ΔH_{ITC}), and binding constant (K_{ITC}).

Results

Unfolding Kinetics and Activation Parameters. Protein unfolding reactions were initiated via a temperature jump by taking a concentrated protein sample directly from a 5 °C ice bath and pipetting the desired amount into a quartz cuvette containing a buffered solution pre-equilibrated at the desired temperature (44−76 °C). The volume of added protein amounted to only 2−3% of the volume of buffer in the cuvette and hence did not disturb the temperature in the reaction solution. Subsequent loss of α-helical content was followed by CD at 222 nm (Figure 2a). The corresponding unfolding time profiles (Figure 2b) followed a clean first-order dependence on protein concentration and afforded first-order unfolding rate constants (k_u).

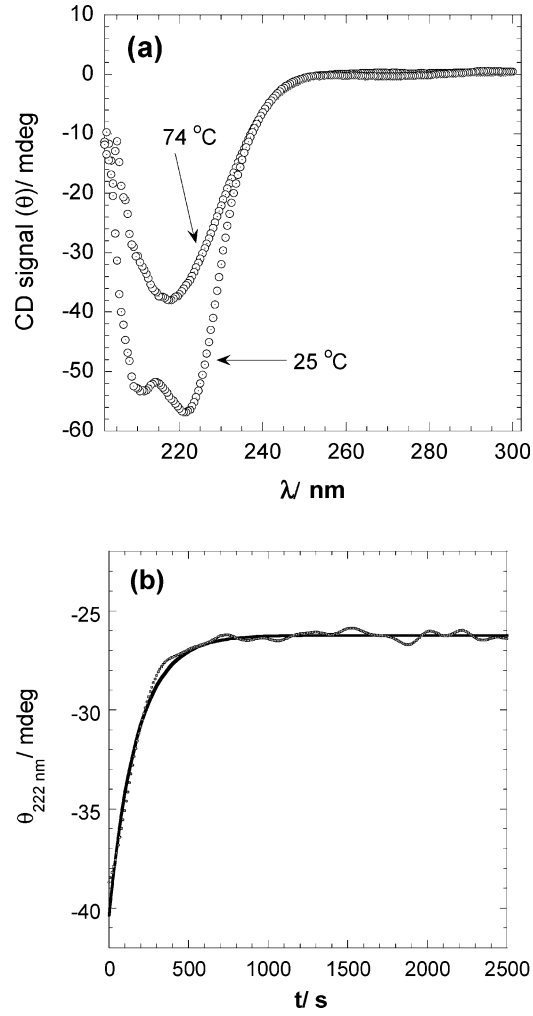


Figure 2. (a) CD spectra of native Co-cPAH at 25 °C and denatured Co-cPAH at 74 °C. (b) Typical kinetic profile for thermal unfolding of Co-cPAH (12 μM) at 66 °C in 5.0 mM Hepes buffer monitored at 222 nm (the solid line is a fit of the data to a first-order equation).

Unfolding rate constants for apo-cPAH (k_u^{apo}) were obtained at various temperatures ranging from 319 to 339 K by fitting the time profiles to a single-exponential equation. The first-order dependence on protein concentration, single-phase exponential time profiles, and absence of a significant accumulation of an intermediate suggest that unfolding of cPAH follows a two-state model/mechanism.

Similarly, unfolding rate constants for holo-cPAH (k_u^{M}) were obtained over a temperature range of 317−349 K. The kinetics followed a first-order dependence on protein concentration and displayed a single-phase exponential rise in the CD signal (Figure 2). The corresponding rate constants and activation parameters for unfolding of each protein variant are gathered in Table 1. The rate of cPAH unfolding showed a discernible dependence on the cofactor. For example, the rate constant for apo-cPAH at 335 K was 1.6, 2, and 8 times larger than those for Fe-cPAH, Zn-cPAH, and Co-cPAH, respectively. These rate constants indicate that apo-cPAH loses its secondary structure more readily than

(9) Lonhienne, T.; Gerday, C.; Feller, G. *Biochim. Biophys. Acta* **2000**, *1543*, 1–10.

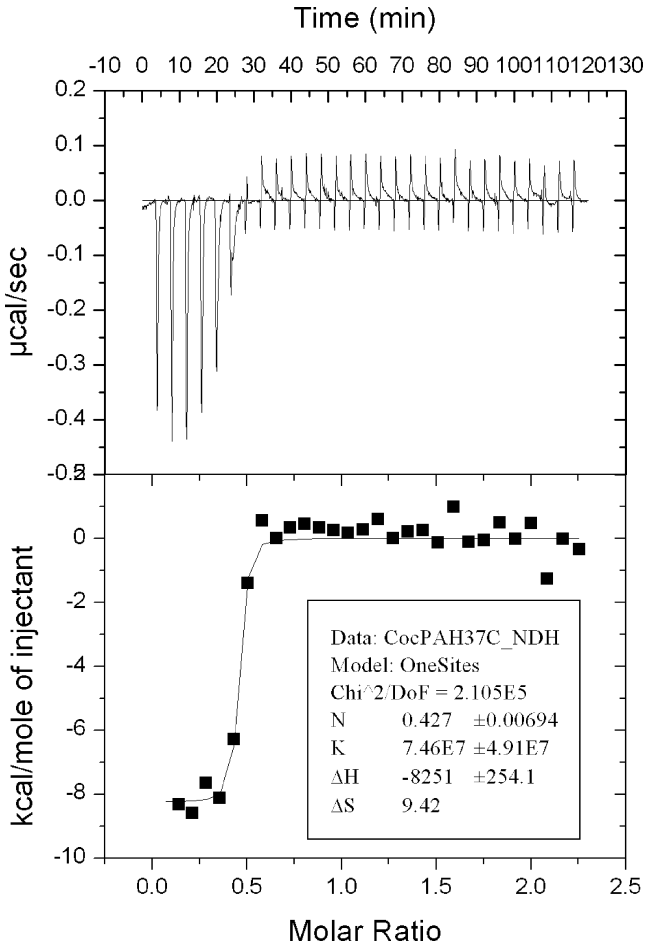


Figure 3. (top) Calorimetric trace for titration of 200 μM $CoCl_2$ into 20 μM apo-cPAH in 50 mM Hepes (pH 7.44) at 37 °C. (bottom) Plot of the net heat released as a function of the molar ratio of $CoCl_2$ to apo-cPAH. Inset: binding constant [K_a (M^{-1})], thermodynamic parameters [ΔH_{ITC} (cal mol^{-1}), ΔS_{ITC} (cal mol^{-1} K^{-1})], and stoichiometry (N_{ITC}).

its metalated (holo) counterparts at physiological temperatures and suggest that the minimum energy needed to reach the activated state must be lowest for apo-cPAH and highest for Co-cPAH. It has been noted that metalloproteins may redistribute their metal during thermal unfolding.[10] Such redistribution can be detected using CD because it gives rise to biphasic unfolding kinetics, which was not observed here for cPAH. Adventitious metal binding has also been encountered during metalloprotein unfolding.[11] However, the kinetics of holo-cPAH unfolding was found to be independent of excess metal concentrations up to 10-fold that of the protein (see below).

Arrhenius analysis of the unfolding rate constants afforded linear plots of ln k_u versus $1/T$ having excellent regression coefficient values ($R \geq 0.98$). The pre-exponential term A represents the maximum unfolding rate; its value is related to the rate at which proteins can sample different conformations in the energy landscape and is thought to be temperature-dependent.[12] As expected, the energies of activation for all four protein variants were temperature-independent over the temperature range investigated. The E_a values for apo-cPAH, Fe-cPAH, Zn-cPAH, and Co-cPAH were 85 ± 8, 87 ± 6, 116 ± 3, and 160 ± 9 kJ mol^{-1}, respectively. While the activation energies for apo- and Fe-cPAH are comparable, Co-cPAH requires twice as much energy to reach the unfolding transition state (TS). One probable explanation for this result is an increase in metal-dependent stabilization in the series Fe < Zn < Co, which is interesting in view of the fact that evolutionary pressures have made cPAH an Fe-containing enzyme.

The $\Delta S^{\ddagger}$ values for apo- and Fe-cPAH (the native form) were similar within the experimental uncertainty and close to zero. Their negative values may indicate that while the molten form of the protein loses secondary structure, it remains in a globular form. In other words, unfolding of apo- and Fe-cPAH ensues through an associative (compact) TS. Nevertheless, the enthalpic contributions to the Gibbs free energy of activation ($\Delta G^{\ddagger}$) were dominant for apo- and Fe-cPAH. On the other hand, the $\Delta S^{\ddagger}$ values were large and positive for Zn- and Co-cPAH (54 and 175 J mol^{-1} K^{-1}, respectively).

Isothermal Titration Calorimetry. We performed ITC experiments in order to obtain binding constants for the holo-cPAH variants investigated in this study. We found that cPAH binds Fe^{II}, Zn^{II}, and Co^{II} in 1:1 stoichiometry with K_d values decreasing in the order Fe-cPAH > Zn-cPAH > Co-cPAH. This binding trend is in agreement with the observed unfolding kinetics data: the strongest binder, Co, afforded the most slowly unfolding protein (Co-cPAH) in the series. Values of the dissociation constants and thermodynamic parameters for binding of the three metals are summarized in Table 2.

Titration of apo-cPAH in Hepes (pH 7.44) with a 10-fold excess of $CoCl_2$ at 37 °C yielded a K_d^{Co} value of 13 nM and an N_{ITC} value of 0.41 when the ITC data were fitted to a one-site model. Figure 3 shows the baseline-corrected raw data and the concentration-normalized, peak-integrated heat of reaction as a function of the molar ratio of Co^{II} to cPAH. A ΔG_{ITC} value of –46 kJ mol^{-1} was calculated from $\Delta H_{ITC} = -34$ kJ mol^{-1} and $\Delta S_{ITC} = 39$ J mol^{-1} K^{-1}. Titration of apo-cPAH with a 10-fold excess of $ZnCl_2$ at 37 °C yielded a K_d^{Zn} value of 46.5 nM and an N_{ITC} value of 0.71 when the data were fitted to a one-site model. The ΔG_{ITC} for binding of Zn^{II} to apo-cPAH was −40 kJ mol^{-1}. Zn^{II} coordination featured an enthalpy change ΔH_{ITC} of −28 kJ mol^{-1} and entropy change ΔS_{ITC} of 40 J mol^{-1} K^{-1}. Titration of apo-cPAH in 50 mM Hepes (pH 7.44) with a 20-fold excess of $Fe(NH_3)SO_4$ at 37 °C yielded a K_d^{Fe} of 0.860 μM and an N_{ITC} value of 3.02. The ΔG_{ITC} value of –32 kJ mol^{-1} for Fe^{II} binding was also enthalpically driven, with a ΔH_{ITC} value of −20 kJ mol^{-1} and a ΔS_{ITC} value of 48 J mol^{-1} K^{-1}. This is the first reported K_d^{Fe} value for an aromatic amino acid hydroxylase determined using ITC, and it is comparable to the value of K_d^{Fe} reported for ACC oxidase.[15b] The entropy

(10) Huus, K.; Havelund, S.; Olsen, H. B.; van de Weert, M.; Frokjaer, S. *Biochemistry* **2005**, *44*, 11171–11177.

(11) (a) Shrake, A.; Ross, P. D. *J. Biol. Chem.* **1990**, *265*, 5055–5059. (b) Shrake, A.; Ross, P. D. *J. Biol. Chem.* **1988**, *263*, 15392–15399.

(12) Kuhlman, B.; Luisi, D. L.; Evans, P. A.; Raleigh, D. P. *J. Mol. Biol.* **1998**, *284*, 1661–1670.

Table 1. Rate Constants and Activation Parameters for Thermal Unfolding of Apo- and Holo-cPAH with Different Metal Cofactors

apo-cPAH		Fe-cPAH		Zn-cPAH		Co-cPAH	
T (K)	$10^{-3}k_u$ (s^{-1})	T (K)	$10^{-3}k_u$ (s^{-1})	T (K)	$10^{-3}k_u$ (s^{-1})	T (K)	$10^{-3}k_u$ (s^{-1})
319	3.60(5)	329	6.80(8)	317	1.00(1)	328	1.00(3)
321	6.10(1)	331	8.8(4)	331	5.80(6)	335	2.60(3)
323	6.60(1)	333	8.3(2)	333	7.40(8)	337	3.70(6)
325	7.10(3)	335	12.0(5)	335	11.0(1)	339	5.70(8)
327	7.9(3)	337	15.0(7)	337	12.0(2)	341	8.8(2)
335	20.0(2)	343	31.0(7)	339	17.0(5)	343	12.0(3)
339	27.0(3)	349	37(1)	349	58(2)		

	apo-cPAH	Fe-cPAH	Zn-cPAH	Co-cPAH
$\Delta H^{\ddagger}$ (kJ mol^{-1})	82 ± 8	84 ± 6	113 ± 3	157 ± 9
$\Delta S^{\ddagger}$ (J mol^{-1} K^{-1})	-34 ± 24	-32 ± 18	54 ± 9	175 ± 27
$\Delta G_{298}^{\ddagger}$ (kJ mol^{-1})	92 ± 8	94 ± 6	97 ± 3	105 ± 9

Table 2. Dissociation Constants and Thermodynamic Parameters for Binding of Metal Ions to Apo-cPAH at 37 °C

metal ion	K_d (nM)	ΔG_{ITC} (kJ mol^{-1})	ΔH_{ITC} (kJ mol^{-1})
Fe^{II}	860(170)	−32(6)	−20.0(0.4)
Zn^{II}	46(12)	−40(10)	−28.0(0.7)
Co^{II}	13(8)	−46(30)	−34(1)

values for all three titrations were positive and quite comparable. The cause of an increase in entropy is most likely liberation of water from the active site of the protein in order to accommodate metal coordination.

Fe^{II} titration experiments were carried out in the presence of 2.5 mM TCEP in order to keep the iron reduced. Performing Fe-cPAH titrations in the absence of TCEP did not yield any changes in binding enthalpies, most likely because of oxidation to Fe^{III} during the titration experiments (see the Supporting Information). The binding constant was acquired by one-site-model fitting of the difference data obtained by subtraction of a control ΔH_{ITC} value obtained without enzyme from the value of ΔH_{ITC} for Fe-cPAH. The X-ray structure of holo-cPAH shows one Fe per cPAH, which is inconsistent with our ITC result that yielded a stoichiometry of 3.0. We contend that the higher-than-expected N_{ITC} value is an artifact of subtracting two sets of data.

Discussion

More than 200 mutations that cause PKU have been discovered; the resulting phenotypes range from mild to severe PKU.[4] The mutations affect enzymatic activity, stability, and proper folding. Despite being only 35% homologous in sequence to human PAH, cPAH is nearly identical in structure to the catalytic domain of the human enzyme. cPAH can be superimposed with the corresponding residues in the catalytic domain of hPAH with an rms deviation of 1.2 Å.[2] Both enzymes adopt a mixed $\alpha\beta$ fold in a basket-like arrangement. Therefore, we have undertaken a study of cPAH unfolding kinetics and its dependence on the metal cofactor with the ultimate goal of understanding the role of the active-site metal in the molecular dynamics responsible for improper folding.

We have investigated the kinetics of thermal unfolding of apo- and holo-cPAH using circular dichroism over the temperature range 44−76 °C. The kinetic profiles for apo- and holo-cPAH showed a first-order dependence on protein concentration. The extrapolated unfolding rate constants (k_u^M) at ambient temperature followed the order apo > Fe > Zn ≫ Co. The transition-state parameters showed that the unfolding rates of Zn- and Co-cPAH were affected significantly by entropy while the unfolding rates of apo- and Fe-cPAH were dominated by enthalpy even at higher temperatures. The $\Delta S^{\ddagger}$ values for apo- and Fe-cPAH (the native form) were negative, close to zero, and similar within experimental uncertainty. Therefore, the enthalpic contribution to $\Delta G^{\ddagger}$ was dominant for apo- and Fe-cPAH. On the other hand, the $\Delta S^{\ddagger}$ values were large and positive for Zn- and Co-cPAH (54 and 175 J mol^{-1} K^{-1}, respectively) and contributed significantly to $\Delta G^{\ddagger}$, especially at higher temperatures. Even though the relative entropy of activation values suggest that the thermal unfolding of Co-cPAH (for which $\Delta S^{\ddagger}$ is more positive) is more favorable that that of apo- and Fe-cPAH, it is in fact $\Delta H^{\ddagger}$ that drives the reaction at physiological and ambient temperatures. The negative $\Delta S^{\ddagger}$ values for apo- and Fe-cPAH suggest a TS that is ordered compared with the native structure, akin to a molten globule.

Further transition-state analysis of the activation parameters in hand showed a correlation between $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for the different protein variants, suggesting (but not proving) that a single unfolding mechanism is shared by the apo and various holo forms of cPAH.[13] Figure 4 shows a plot of $\Delta H^{\ddagger}$ versus $\Delta S^{\ddagger}$. The linear correlation indicates an isokinetic relationship, and the slope affords an isokinetic temperature of 355 K, which corresponds to the temperature at which apo-cPAH and holo-cPAH with different metal cofactors (Fe, Zn, and Co) share a common unfolding rate constant. Isokinetic relationships have been observed for the unimolecular oxidoreduction reactions between mutant forms of cupriplastocyanin and zinc cytochrome c.[13b]

The isokinetic temperature (T_{iso}) can be observed directly from the Arrhenius plots (Figure 5) because its value is not very large (355 K, or 82 °C). It is worth noting that at temperatures higher than T_{iso}, the entropy of activation for Zn- and Co-cPAH becomes an important contributor to $\Delta G^{\ddagger}$, and their (predicted) rates of unfolding surpass those of apo- and Fe-cPAH (Figure 5). However, temperatures higher than 82 °C may not be physiologically relevant because the source

(13) (a) Espenson, J. H. *Chemical Kinetics and Reaction Mechanisms*, 2nd ed.; McGraw-Hill, Inc.: New York, 1995; p 164. (b) Ivkovic-Jensen, M. M.; Ullmann, G. M.; Young, S.; Hansson, O.; Crnogorac, M. M.; Ejdeback, M.; Kostic, N. M. *Biochemistry* **1998**, *37*, 9557–9569.

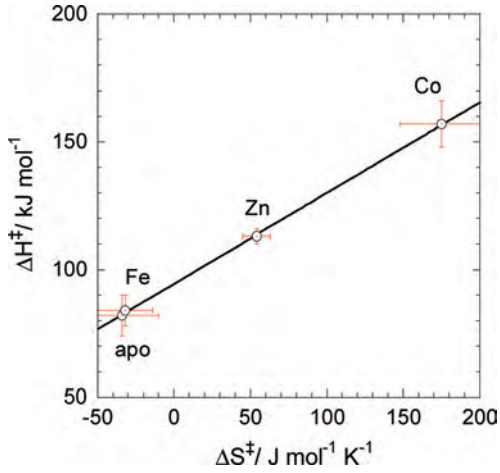


Figure 4. Plot of $\Delta H^{\ddagger}$ versus $\Delta S^{\ddagger}$ for thermal unfolding of apo- and holo-cPAH variants. The slope, which corresponds to the isokinetic temperature, is 355 ± 5 K.

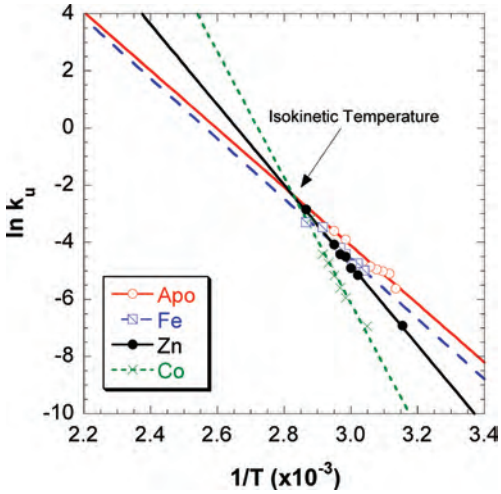
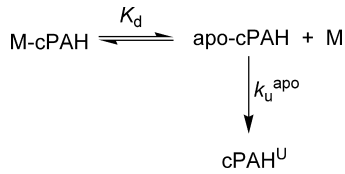
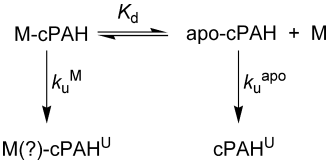


Figure 5. Arrhenius plots for thermal unfolding of apo-PAH and holo-cPAH with different metal cofactors (Fe, Zn, and Co).

Scheme 2



Scheme 3



of cPAH, *C. violaceum*, is found in normal flora of water and soil in tropical to subtropical areas and has a growth range of 15−40 °C.[14]

In structural terms, a potential explanation for the existence of a common unfolding mechanism for apo- and holo-cPAH would be that in both cases, unfolding proceeds through a metal-free cPAH at the TS. The metalated protein is in dynamic equilibrium with the apo form, and the latter unfolds much more readily. Hence, the measured rate constant for holo-cPAH (k_u^M) would be a combination of the dissociation constant (K_d^M) and the unfolding rate constant for apo-cPAH (k_u^{apo}). This possibility is shown in Scheme 2, and its corresponding rate law is given in eq 4:

$$\frac{d[cPAH^U]/dt}{[PAH]_T} = k_{obs} = k_u^{apo}\frac{K_d}{K_d + [M]} \quad (4)$$

where $cPAH^U$ denotes unfolded (denatured) cPAH and $[PAH]_T$ represents the total PAH concentration. The presented hypothesis predicts that the rate of holo-cPAH unfolding would be dependent on the metal concentration in such a way that at metal concentrations well below K_d, we would observe kinetics comparable to that of apo-cPAH, and at metal concentrations well above K_d, we would see metal inhibition of the unfolding reaction. We have determined dissociation constants (K_d) for Fe-, Zn-, and Co-cPAH independently using isothermal titration calorimetry (Table 2). The K_d values for the three metals span 2 orders of magnitude (10 nM−1 μM). The value of K_d for Fe^{II} (native holo-cPAH) is consistent with K_d values reported for other three-substrate hydroxylases having similar non-heme active-site ligands, such as ACC oxidase.[15]

Simple calculations using the observed rate constants for unfolding (k_u, Table 1) give K_d values for the different metals that are markedly different (1−2 orders of magnitude) from those determined independently by ITC (Table 2). On this basis alone, Scheme 2 can be rejected. Furthermore, at metal concentrations ranging from 1 to 20 equiv (20 to 250 μM), which are much greater than the K_d values, the kinetics of unfolding showed no dependence on [M] (as shown in the Supporting Information). It is worth noting that using apo-cPAH and substoichiometric [M] afforded biphasic kinetics until the concentration of metal was equivalent to that of apo-cPAH, at which point a single-phase time profile was seen. This effect was most pronounced with cobalt because k_u^{Co} is an order of magnitude smaller than k_u^{apo}. For example, at a Co/cPAH ratio of 1:2, the biexponential time profile gave k_{fast} and k_{slow} values that corresponded reasonably well to k_u^{apo} and k_u^{Co}, respectively. These observations are consistent with a mechanism in which metalated (holo-) and apo-cPAH unfold with their own distinct rates, as shown in Scheme 3.

As depicted by the question mark in Scheme 3, the question of whether the metal remains bound to the denatured protein arises. Thermally unfolded cPAH aggregates with time and does not revert to the native (folded) state upon cooling. Since unfolding experiments on the holo enzyme conducted with excess metal gave single-phase time profiles, adventitious metal binding during the unfolding process is unlikely. Therefore, one may conclude that both apo- and holo-cPAH give rise to the same unfolded state ($cPAH^U$).

(14) Brazilian National Genome Project Consortium, *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 11660–11665.
(15) (a) Thrower, J. S.; Blalock, R., III; Klinman, J. P. *Biochemistry* **2001**, *40*, 9717–9724. (b) Brunhuber, N. M.; Mort, J. L.; Christoffersen, R. E.; Reich, N. O. *Biochemistry* **2000**, *39*, 10730–10738. (c) Zhang, Z.; Schofield, C. J.; Baldwin, J. E.; Thomas, P.; John, P. *Biochem. J.* **1995**, *307*, 77–85. (d) Pirrung, M. C.; Kaiser, L. M.; Chen, J. *Biochemistry* **1993**, *32*, 7445–7450.

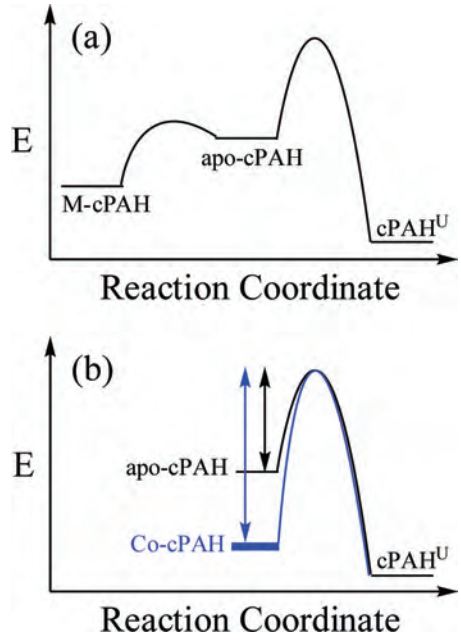


Figure 6. Reaction-coordinate representations of thermal unfolding of cPAH proceeding (a) via apo-cPAH exclusively and (b) from apo- and holo-cPAH independently through the same transition state.

The lack of metal dependence of the unfolding kinetics and the K_d values for the different metals disprove the mechanism depicted in Scheme 2, and therefore, the reaction coordinate shown in Figure 6a can be rejected as a common underlying mechanism. However, the isokinetic relationship supports a common unfolding mechanism for apo- and holo-cPAH. Hence, the difference in the rate constants for apo- and holo-cPAH (k_u^{apo} and k_u^M, respectively) could be attributed to the greater stability of the latter, as depicted by the reaction coordinate in Figure 6b. Zn- and Co-cPAH in particular have higher activation energies than their apo and Fe analogs. It is interesting to note that the native state of the enzyme, Fe-cPAH, gave activation parameters comparable to those of the apo enzyme. Nature has selected a structural fold that is least disturbed by the native cofactor (Fe) compared with other biologically relevant metals (Zn and Co).

The reaction coordinate proposed in Figure 6b predicts a direct correlation between the activation energy for unfolding and the enthalpy of metal coordination to cPAH. The rate constants for unfolding of the holo-cPAH variants followed the same trend as the reaction enthalpies for metal coordination measured by ITC. The rate constants k_u decreased in the order Fe > Zn > Co, and ΔH_{ITC} for metal coordination was most favorable for cobalt(II) and least favorable for iron(II) in the series (Table 2). A plot of $\Delta H^{\ddagger}$ for thermal unfolding of holo-cPAH versus ΔH_{ITC} for metal coordination to cPAH afforded a linear correlation (Figure 7), supporting the notion that the cPAH variants share a common unfolding mechanism and variations in the enthalpies of metal coordination to cPAH account for the differences in their unfolding rate constants.

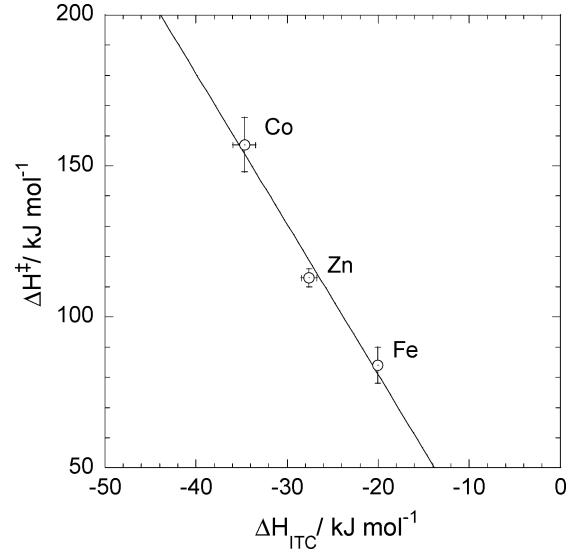


Figure 7. Correlation between $\Delta H^{\ddagger}$ for thermal unfolding of holo-cPAH and ΔH_{ITC} for metal coordination to cPAH ($R^2 = 0.98$). $\Delta H^{\ddagger}$ was measured by circular dichroism kinetics and ΔH_{ITC} by isothermal calorimetric titrations of the various metals with apo-cPAH.

Our studies have shown that cPAH is more stable in its metalated form at physiological temperatures. The trend in stability across the series of metals investigated follows the order Co > Zn > Fe at temperatures lower than T_{iso}. The fact that cPAH uses Fe^{2+} for catalysis but is stabilized to a greater extent by cobalt(II) and zinc(II) points to the existence of a metal-delivery system (chaperone) in *C. violaceum* that is able to discriminate among metals and bring the correct metal (Fe) to cPAH. Studies of transition-metal quotas of *E. coli* have shown that Fe and Zn are pooled in the cell to a concentration of 2×10^5 atoms per cell (0.1 mM) (Co is less abundant at 10 μM) before being distributed to their appropriate metalloproteins by metal-trafficking proteins.[16] In fact, a number of zinc-trafficking proteins, such as the Zn-sensing metalloregulatory proteins Zur and ZntR, have been identified in *E. coli*.[16] On the other hand, iron chaperones in bacteria have not yet been identified. Nevertheless, our findings in this work suggest that a discriminatory mechanism for delivery of iron to bacterial PAH is likely in order to avoid competition from Zn, which is present in the cytosol at concentrations comparable to that of iron.

Acknowledgment. The NSF is gratefully acknowledged for support of this work (CHE-0502391).

Supporting Information Available: Kinetic data for unfolding of apo- and holo-cPAH and its dependence on metal concentration, Arrhenius plots, and ITC data. This material is available free of charge via the Internet at http://pubs.acs.org.

IC800181Q

(16) (a) Outten, C. E.; O'Halloran, T. V. *Science* **2001**, *292*, 2488–2492. (b) Finney, L. A.; O'Halloran, T. V. *Science* **2003**, *300*, 931–936.