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Building reactive copper center(s) in human carbonic anhydrase II

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Abstract

Re-engineering metalloproteins to generate new biologically relevant metal centers is an effective way to test our understanding of the structural and mechanistic features that steer chemical transformations in biological systems. Here we report thermodynamic data characterizing the formation of two type-2 (T2) copper sites in carbonic anhydrase and experimental evidence showing one of these new copper centers has characteristics similar to a variety of well-characterized copper centers in synthetic models and in enzymatic systems. Human CA II is known to bind two Cu²⁺ ions; herein, these binding events are explored using modern isothermal titration calorimetry (ITC) techniques that have become a proven method to accurately measure metal-binding thermodynamic parameters. The two Cu²⁺-binding events have different affinities ($K_a \sim 5 \times 10^{12}$ and 1×10^{10}) and both are enthalpically driven processes. Reconstituting these Cu²⁺ sites under a range of conditions has allowed us to assign the Cu²⁺-binding event to the three-histidine, native, metal binding site. Our initial efforts to characterize these Cu²⁺ sites have yielded data that show distinctive (and noncoupled) EPR signals associated with each copper-binding site, and that this reconstituted enzyme can activate hydrogen peroxide to catalyze the oxidation of 2-aminophenol.

Re-engineering metalloproteins to generate new biologically relevant metal centers is an effective means of exploring how the structure surrounding the metal center affects its reactivity.[1] Furthermore, these biological models offer the opportunity to develop new catalysts that maintain the extraordinary selectivity and fidelity often associated with enzymes. In some cases Nature provides us with these models, and we just have to be lucky enough to identify them.

Carbonic anhydrase (CA) is a robust, well-studied, zinc-dependent metalloenzyme, which has been suggested as a good macromolecular template to support a range of biophysical studies.[2] These properties also suggest CA would make an excellent template for the

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Electronic Supporting Materials: Experimental details as well as Figures A, B, and C can be found in the electronic supporting materials.

rational-design of new copper centers within a biological setting. Native CA utilizes a non-heme Zn^{2+} center, bound through three protein-derived histidine residues, which is used to catalyze the reversible hydrolysis of carbon dioxide to the bicarbonate ion. The metal-free form of CA (apoCA) is relatively stable and easy to generate using dipicolinic acid. CA has been reconstituted with a number of transition metals, including copper.[3, 4] Although there are several crystal structures of copper-substituted CA,[3, 5] the structure reported by Håkansson *et al.* shows two distinctive copper ions bound to CA (Figure 1),[3] which is consistent with the known stoichiometry of Cu^{2+} binding.[6] One of the copper binding sites appears to coordinate to His4 and His64, although the other structural elements, such as solvent or other protein-derived additional ligands, were not resolved in the crystal structure. We have termed this site CuA. The other Cu^{2+} metal ion binding site (CuB) is the traditional metal binding site of CA, where the Cu^{2+} is clearly coordinated with three protein-derived histidine residues (His94, His96, and His119). Interestingly in this structure, a water molecule and dioxygen were assigned to occupy the other coordination sites in the metal's trigonal bipyramidal geometry.[3] The copper centers in CA are approximately 13.6 Å apart, separated by a solvent accessible channel. The reconstituted CuB site in the dicopper carbonic anhydrase (Cu/Cu-CA) is structurally similar to the T2-active site in a variety of copper containing proteins and related model complexes.[7-9]

Herein, we report a detailed thermodynamic description of the reconstitution of the Cu^{2+} sites in CA and report our preliminary characterization of this novel remetalated protein's electronic structure and reactivity. The thermodynamic parameters associated with Cu^{2+} reconstitution are measured by a series of isothermal titration calorimetry (ITC) experiments in which Cu^{2+} is added to apoCA *in vitro*. Isothermal titration calorimetry has become a widely used method to determine thermodynamic parameters (K_{ITC} and H_{ITC}), which can be used to calculate G_{ITC} of metal binding ($G_{\text{ITC}} = -RT \ln K_{\text{ITC}}$), and the entropy change for metal binding, S_{ITC} ($G_{\text{ITC}} = H_{\text{ITC}} - T S_{\text{ITC}}$). [10] A representative isotherm for the addition of Cu^{2+} to apoCA and the integrated change in enthalpy data are shown in Figure A in the electronic supporting materials. As expected, the ITC titration of Cu^{2+} into apoCA yields a complex set of equilibria that can be readily fit to a two-site binding model (Table 1). A high affinity Cu^{2+} -binding site coordinates a stoichiometric amount of Cu^{2+} with an equilibrium constant (K_{ITC}) of 2.3×10^8 . This value is nearly 500 times higher than the K low-affinity site ($\sim 4 \times 10^5$).

Unfortunately, the equilibrium constants and thermodynamic data extracted from the two-site fit of the ITC experiment does not allow us to assign these parameters to specific metal-binding events. Previous studies have reported an association constant for Cu^{2+} binding to apoCA of 5.9×10^{13} . This binding constant was attributed to Cu^{2+} binding to the traditional metal-binding site in CA.[11] Corresponding ITC experiments also ambitiously attributed a high-affinity binding event for Cu^{2+} binding to the three-histidine site, described here as CuB.[6] However this previous calorimetry study, neglected a number of important buffer effects that dramatically temper the ITC results.[12] To clarify this quandary, Cu^{2+} was titrated into Zn-CA to generate a Cu/Zn-CA form of the enzyme, where the traditional, metal-binding site is occupied by Zn^{2+} and effectively allows us to probe the thermodynamics of the CuA binding site. The shape of the isotherm (cf. Figure B in the

supporting materials) and the thermodynamic data (Table 1) are highly similar to the data collected for the high-affinity, Cu^{2+} binding site, which indicates the high-affinity binding site in apoCA is the CuA center. Curiously, this makes the native-metal binding site (CuB) the low-affinity site.

If we delve into the ITC data more carefully, it is clear the equilibria measured in the ITC have to be treated as a competition of equilibria between the buffers and apoCA for Cu^{2+} . The complex equilibria associated with Cu^{2+} with some common buffer have been documented, and can be used to help deconvolute the equilibria measured in the ITC. Specifically in this case, copper interacts with ACES buffer, (*N*-(2-acetamido)-2-aminoethanesulfonic acid, $\log K = 4.32$) and the reactions occurring in the ITC cell are well represented as equations 1 in Table 2.[13] The metal-buffer interactions occurring in our ITC experiments can be modeled using the thermodynamic cycles shown in Table 2. This allows us to calculate the intrinsic association constants (K_a) for Cu^{2+} binding to apoCA (Table 2, Equations 3). From the large favorable intrinsic free energy changes for the two Cu^{2+} binding events, it is clear that the copper ions bind strongly to apoCA *in vitro*, with K_a values of 4.9×10^{12} and 9.5×10^9 for the CuA and CuB sites, respectively. Interestingly native CA binds a stoichiometric quantity of Zn^{2+} with a K_a about 2×10^9 , [12] which suggests the three histidine, metal-binding site has a similar affinity to Cu^{2+} and Zn^{2+} .

The complexity of this experiment and its interpretation increases if we consider that there may be protons released upon copper binding to apoCA. Due to the low solubility and lack of thermodynamic data for Cu^{2+} in common buffers such as phosphate, PIPES, HEPES, and MOPS, the number of protons released was not experimentally determined by performing similar experiments in other buffers having known ionization energies. However, the number of protons released can be estimated based on measured $\text{p}K_a$ values of metal-bound histidines. The $\text{p}K_a$ values associated with the copper-bound histidines, namely His64, His94, His96, and His119, have been determined to be less than 6 in apoCA, and the $\text{p}K_a$ of His4 is 7.06.[6, 14, 15] From these values we can estimate approximately 0.5 protons are released on average from the CuA site and 0 protons are released from the CuB site. If these estimates are correct, the pH- and buffer-independent ΔG and ΔH values for Cu^{2+} binding to the CuA site would be -17.4 and -14.5 kcal/mol, respectively. Since there is no proton dependent process associated with the CuB site, no correction is needed for the values listed in Tables 2, Equation 3 for the CuB site. Considering the changes in enthalpy associated with binding Cu^{2+} to the CuA and CuB sites, along with structure of the CuB site from 1RZC.pdb, it seems unlikely that the CuA site has only two protein-derived ligands. A possible explanation for this would be that there are more protein-based ligands bound to Cu^{2+} in the CuA site, but the additional ligands have not been resolved in the X-ray experiment. Additionally, the CuA site in 1RZC.pdb could be an artifact of the crystallization conditions, and the real binding site is somewhere else on CA. If either of these interpretations are indeed the case, the number of protons exchanged for Cu^{2+} binding to the CuA site may not be accurate. However, the ΔG term derived in Equation 3 for the CuA site should be reasonably close to the true value, which gives us an reasonable measure of how well copper ions interact with the CuA binding site in CA.

The two sites in Cu₂-CA have distinctive electronic structures. The EPR spectrum of 1.5 eq. Zn²⁺ and 0.75 eq of Cu²⁺ added to apoCA is shown in Figure 2A. Indicative of the formation of Cu/Zn-CA, the spectrum shows one copper species present in the CuA site with $g = (2.017, 2.067, 2.190)$ and hyperfine splitting of $A_z = 197$ G. The spin concentration of this species quantitatively agrees with the amount of added copper. The addition of 1.9 eq of Cu²⁺ to apoCA shows a spectrum with two species in equal amounts, whose total spin concentration agrees with the amount of Cu²⁺ added (Cu/Cu-CA, Figure 2B). Assuming that one of these species is the previously identified site in Cu/Zn-CA, we obtained the difference spectrum by subtracting the Cu/Zn-CA data from the Cu₂-CA spectrum (Figure 2C) to identify the EPR spectrum of the CuB site, which has $g = (2.029, 2.096, 2.326)$ and hyperfine splitting of $A_z = 127$ G. Similar g values have been reported for His-rich copper centers in biology.[16]

Combining the thermodynamic and electronic structural data provided above with the crystal structure of copper-substituted carbonic anhydrase, it is clearly demonstrated that a non-native (His)₃Cu²⁺ center is generated in carbonic anhydrase. Generally, the mechanisms of T2-mononuclear or noncoupled, dinuclear copper enzymes involve a reduced Cu(I) site that activates O₂ to generate reactive intermediates, such as cuperic-superoxides (Cu^{II}-O₂[•]) or hydroperoxides (Cu^{II}-OOH) species, which then attack an organic substrates.[7-9, 17] Since the Cu₂-CA is in a dicupric form, it does not activate O₂. However, if we use a two electron reduced form of O₂, namely hydrogen peroxide with Cu/Cu-CA (which is isoelectronic with a dicopper(I)-dioxygen complex) we can effectively oxidize 2-aminophenol.[18, 19] The 2-aminophenol oxidation assay is a commonly-used and well-studied assay to characterize the reactivity of a variety of copper-containing enzymes, including phenoxazinone synthase, peptidylglycine α -hydroxylating monooxygenase, and tyrosinase.[19] The reaction mechanism involves a catalytic step in which a 2-electron oxidation of 2-aminophenol to quinone imine takes place. The quinone imine then reacts with another 2-aminophenol to yield the yellow product 2-aminophenoxazinone ($\epsilon_{434} = 23\,200\text{ M}^{-1}\text{cm}^{-1}$).[18] These data clearly illustrate that H₂O₂ reactivity is dependent on a second Cu²⁺ bound to CA (cf. Figure C in the supporting materials) where the turn-over number was maximized at 0.09 s^{-1} . Although we hypothesize that the CuB site is the center of H₂O₂ activation, it is still unclear if CuB acts independently or if there is some supporting role for CuA in this chemistry. Similar shunt chemistry has been catalyzed by T2 centers in noncoupled dicopper proteins, where a side-on Cu²⁺-hydroperoxo intermediate has been reported as a discrete step in the catalytic mechanism.[9, 20] However, there are also several T2-mononuclear Cu²⁺ systems that can oxidize aromatic species using an end-on hydroperoxo moiety.[21] Clearly, further analysis of this Cu/Cu-CA system is required to understand the underlying mechanism of this H₂O₂-dependent chemistry. However the data reported here shows this remetallated protein is a compelling system that can be used as a starting point to probe biological reactions catalyzed by histidine-rich Cu²⁺ centers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Metal binding site of dicopper carbonic anhydrase. The native metal-binding site occupied with Cu^{2+} (highlighted in pink), whereas the novel, second, Cu^{2+} -binding site is associated with the N-terminus (highlighted in blue). Coordinates were used from the 1.62 Å structure, 1RZC.pdb.

Figure 2.

EPR spectra of 0.85 mM apoCa, 25 mM ACES, pH 7, with (A) 1.5 eq. Zn^{2+} , 0.75 eq of Cu^{2+} ; (B) 1.9 eq of Cu^{2+} . (C) Difference spectrum B-A. Microwaves: 20 μW at 9.64 GHz. Temperature: 21 K. The simulations (black lines) are least-squares fits for $S=1/2$, $I=3/2$, with g and A_z and concentrations given in text.

Table 1
ITC Best Fit Values for Cu^{2+} Binding to apoCA and Zn-CA in 20 mM ACES, pH=7.0

	n_{ITC}	K_{ITC}	H_{ITC} (kcal/mol)	G_{ITC} (kcal/mol)	$-T S_{\text{ITC}}$ (kcal/mol)
Cu^{2+} into apoCA	1.0	$2.3 (\pm 0.3) \times 10^8$	-4.1 ± 0.0	-11.4 ± 0.1	-7.3 ± 0.1
	1.0	$4.2 (\pm 0.3) \times 10^5$	-6.0 ± 0.1	-7.7 ± 0.0	-1.7 ± 0.1
Cu^{2+} into ZnCA	1.0	$1.3 (\pm 0.3) \times 10^8$	-3.3 ± 0.0	-11.1 ± 0.1	-7.8 ± 0.1

Table 2Thermodynamic Cycle for Cu²⁺ Binding to apoCA in 20 mM ACES, pH = 7.0.

	Equation.	G° (kcal/mol)	H° (kcal/mol)	-T S° (kcal/mol)
High-affinity binding event (CuA)	1 Cu(ACES) ²⁺ + H ⁺ _x (CA) ↔ Cu(CA) ²⁺ + x H ⁺ (ACES) + (1-x) ACES ^a	-11.4 ^b	-4.1 ^b	-7.3
	2 Cu(ACES) ²⁺ ↔ Cu ²⁺ + ACES	5.9 ^d	11.5 ^c	-5.6
	3 Cu ²⁺ + H ⁺ _x (CA) + ACES ↔ Cu(CA) ²⁺ + x H ⁺ (ACES) + (1-x) ACES	-17.3	-15.6	-1.7
Low-affinity binding event (CuB)	1 Cu(ACES) ²⁺ + H ⁺ _y (CuCA) ↔ Cu ₂ (CA) ²⁺ + y H ⁺ (ACES) + (1-y) ACES ^a	-7.7 ^b	-6.0 ^b	-1.7
	2 Cu(ACES) ²⁺ ↔ Cu ²⁺ + ACES	5.9 ^d	11.5 ^c	-5.6
	3 Cu ²⁺ + H ⁺ _y (CuCA) ↔ Cu ₂ (CA) ²⁺ + y H ⁺ (ACES) + (1-y) ACES	-13.6	-17.5	3.9

^a x and y represent the number of protons released upon Cu²⁺ binding.^b from Table 1.^c Determined as part of this study via displacement experiments with EDTA.^d from ref 12