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Co(II)-substituted *Haemophilus influenzae* β -Carbonic Anhydrase: Spectral Evidence for Allosteric Regulation by pH and Bicarbonate Ion

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Abstract

Cobalt(II)-substituted *Haemophilus influenzae* β -carbonic anhydrase (HICA) has been produced by overexpression in minimal media supplemented with CoCl_2 , enabling kinetic, structural, and spectroscopic characterization. Co(II)-substituted HICA (Co-HICA) has comparable catalytic activity to that of wild-type enzyme with $k_{\text{cat}} = 82 \pm 19 \text{ ms}^{-1}$ (120% of wild-type). The X-ray crystal structure of Co-HICA was determined to 2.5 Å resolution, and is similar to the zinc enzyme. The absorption spectrum of Co-HICA is consistent with four-coordinate geometry. pH-dependent changes in the absorption spectrum of Co-HICA, including an increase in molar absorptivity and a red shift of a 580 nm peak with decreasing pH, correlate with the pH dependence of k_{cat}/K_m . The absence of isosbestic points in the pH-dependent absorption spectra suggest that more than two absorbing species are present. The addition of bicarbonate ion at pH 8.0 triggers spectral changes in the metal coordination sphere that mimic that of lowering pH, supporting its hypothesized role as an allosteric inhibitor of HICA. Homogeneously ($99 \pm 1\%$ Co) and heterogeneously ($52 \pm 5\%$ Co) substituted Co-HICA have distinctly different colors and absorption spectra, suggesting that the metal ions in the active sites in the allosteric dimer of Co-HICA engage in intersubunit communication.

Keywords

β -carbonic anhydrase; *Haemophilus influenzae*; bicarbonate; metalloenzyme; cobalt substitution; allostery

1. Introduction

Carbonic anhydrases (carbonate hydrolyase, EC 4.2.1.1) are metalloenzymes that catalyze the interconversion of CO_2 and bicarbonate:

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To date, there are five known convergently evolved forms of the enzyme: the α -form is found in animals and a few eubacteria [1]; the β -form is found in bacteria, yeast, and plant chloroplasts [2]; a γ -form is represented in an archaeobacterium [3]; the δ - [4] and ζ -forms [5] have been identified in a marine diatom. Most are active as zinc-metalloenzymes, although the γ - and ζ -forms can utilize Fe^{2+} [6] and Cd^{2+} [7], respectively. A variety of divalent metal ions (Co, Mn, Ni, Cu, Fe, Cd) may replace zinc *in vitro* in CA with variable activity [8, 9]. X-ray crystallography [7, 10-15] and/or EXAFS1 [16] reveals that most CA forms share a similar four-coordinate metal ion coordination sphere of the type $\text{His}_3(\text{H}_2\text{O})$ or $\text{Cys}_2\text{His}(\text{H}_2\text{O})$, where the ionizable water ligand serves an essential role in the catalytic mechanism. The latter metal coordination sphere is utilized by β -CAs, and exemplified by the X-ray crystal structures of *Pisum sativum* β -CA (PDB 1EKJ), and the D44N variant of *Haemophilus influenzae* β -CA (HICA, PDB 3E1V, [17]). The β -CAs can be further divided into two subclasses, “Type I” β -CAs [18] have a canonical $\text{Cys}_2\text{His}(\text{H}_2\text{O})$ zinc coordination site, with the fourth coordination site occupied by water, as in *P. sativum* [10, 11, 15]. “Type II” β -CAs [18], exemplified by HICA (PDB 2A8D), have a zinc coordination geometry where the catalytically essential water molecule has been replaced by an Asp residue, resulting in a “closed” $\text{Cys}_2\text{HisAsp}$ coordination sphere. The majority of the X-ray crystal structures of “Type II” β -CAs are crystallized in this form, which is hypothesized to represent an inactive, T-state [19] conformation of the enzyme [12-14, 20]. This suggests a basic model for structural inactivation whereby the enzyme achieves the T-state via the simple ligand exchange mechanism of Asp44 displacing the bound water molecule from the metal ligand sphere, an inactive state stabilized by the allosteric binding of HCO_3^- ion as an allosteric inhibitor (Figure 1).

Kinetics studies[13, 20-25] show that both “Type I” and “Type II” β -CAs are highly efficient catalysts for eq 1, with k_{cat} values on the order of 10^5 s^{-1} and k_{cat}/K_m values on the order of $10^8 \text{ M}^{-1} \text{ s}^{-1}$. Kinetics studies of HICA, however, confirm a cooperative inhibition by HCO_3^- ion [13], and exhibit a strong pH dependence consistent with the cooperative loss of multiple protons to achieve the active, basic form of the enzyme [13]. The pH-rate profile of k_{cat} is consistent with the sequential loss of two protons with $\text{p}K_a$ values of 8.3, and the pH-rate profile of k_{cat}/K_m are consistent with a model that requires the cooperative loss of four protons with a $\text{p}K_a$ of 7.7 in order to generate the active enzyme. The kinetics of HICA suggests that a dimer is the minimal allosteric unit [13].

Cobalt(II) is a convenient probe for studying zinc(II) proteins: its ionic radius and polarizability are very similar to those of Zn(II). In contrast to Zn(II) (d^{10}), the d^7 electron configuration of Co(II) is accessible to electronic spectroscopic methods, [26] yielding information on the nature of the first coordination sphere of the metal. Co(II) has been shown to isomorphously replace Zn(II) in archeobacterial, bovine and human forms of CAs (α - and γ -CAs, [27-29]) and, as a result, spectroscopy of Co(II) substituted CA isozymes of the α - and γ -classes has been used to probe the environment of the metal ions in these active sites. [30, 31] For these CAs, preparation of cobalt-substituted enzyme is accomplished via a denaturant and/or metal chelator to form the apo-enzyme, followed by refolding and/or reconstitution with Co^{2+} . Previous attempts to metallosubstitute HICA or any β -CA in this manner have been unsuccessful due to ineffective metal removal using chelators such as 1-, 10-phenanthroline or 2,6-pyridinedicarboxylic acid.

¹CA, carbonic anhydrase; HICA, *Haemophilus influenzae* β -carbonic anhydrase; Co-HICA, Co(II)-substituted HICA; CoZn-HICA, heterogeneously substituted Co(II)-Zn(II) HICA; TB medium, Terrific Broth medium; IPTG, isopropyl- β -D-thiogalactoside; EDTA, *N,N,N',N'*-ethylenediaminetetraacetic acid; PEG-4000, polyethylene glycol 4000; HEPES, 4-(2-hydroxyethyl)piperazineethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-morpholinopropane-1-sulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; FPLC, fast protein liquid chromatography; ICP-OES, inductively coupled plasma-optical emission spectroscopy; LMCT, ligand to metal charge transfer.

Co-substituted α - and γ -CAs have been used to probe the coordination states of the metal ion in the active site [32, 33], the binding activity of inhibitors such as sulfonamides [28, 33], esterase activity [29], as well as the mechanism of action [32, 34, 35]. It would be useful to have a spectroscopic probe in β -CAs to validate the ligand sphere and geometry visualized in the crystal structures, and to actively monitor hypothesized changes in the ligand sphere induced by pH or allosteric inhibitor binding. In "Type II" (allosteric) β -CAs, it is especially significant to explore the proposed allosteric switching mechanism, since the immediate environment of the active site metal is significantly altered in the proposed R $\rightarrow$ T state transition, where Asp44 displaces the catalytic water. The ligand sphere change accompanying this allosteric transition should be spectroscopically observable, providing a method for probing the allosteric regulation in solution.

We report the overexpression and purification of Co(II)-substituted HICA (Co-HICA). The resulting enzyme has enzymatic activity comparable to wild-type HICA. The crystal structure of Co-HICA was determined to 2.5 Å, and is nearly identical to the previously solved HICA structure. Spectroscopic characterization of the Co-HICA is consistent with pseudotetrahedral coordination in a Cys₂HisX coordination sphere, where X is an oxygen ligand. Significantly, pH changes in the Co(II) spectrum correlate with changes in k_{cat} and k_{cat}/K_m in steady state kinetics, confirming the location of important species within the ligand binding sphere. Additionally, bicarbonate ion, hypothesized to be an allosteric inhibitor of HICA, can trigger the same spectral changes in the active site as lowering pH. Finally, spectral differences in homogeneously and heterogeneously substituted enzyme suggest that there is crosstalk between the metal ions in an allosteric dimer.

2. Experimental Methods

2.1 Overexpression and purification of HICA

Two methods were used to express Co-HICA. In the first method, HICA was overexpressed in TB medium supplemented with CoCl₂ at the time of induction. In the second method, enzyme was expressed in M9 minimal media containing M9 5 $\times$ salts, 10 μ M CoCl₂ and 0.25 mg/L thiamine in sterile, deionized water (17-18 M Ω) to minimize the presence of zinc in the growth medium. For both methods, cells were induced in exponential growth phase ($A_{600} = 0.6-1.0$) with 0.2 mM IPTG, and supplemented with 1 mM CoCl₂ post-induction. The first method resulted in heterogeneously Co(II)-substituted HICA; the second method resulted in homogeneously Co(II)-substituted enzyme. Purification, storage and quantification of Co-HICA was performed as described previously for the wild-type enzyme [13]. Briefly, crude homogenates of overexpressed protein were purified to homogeneity by ion exchange (Q-sepharose FF), hydrophobic interaction (butylsepharose FF), and gel exclusion chromatography (Superdex 200) using an AKTA FPLC (GE Healthcare) and quantified by inductively coupled plasma-optical emission spectroscopy (ICP-OES) (Perkin-Elmer Optima SC 3000) for cobalt at 228.616 nm and zinc at 213.856 nm. Concentrations of active protein were based on total Co + Zn concentration. Enzyme samples were extensively dialyzed against deionized water prior to analysis.

2.2 Steady-State Kinetics Methods

Saturated solutions of CO₂ were prepared by bubbling CO₂ gas into water in a vessel maintained at 25.0 $\pm$ 0.1 °C, and dilutions prepared in the absence of air by coupling two gas-tight syringes as described by Khalifah [36]. CO₂ concentrations were calculated based on a 33.8 mM saturated solution at 25.0 °C [37].

All steady state kinetic measurements were made at 25.0 °C using a Hi-Tech SF-61DX2 stopped-flow spectrophotometer. Initial rates of CO₂ hydration were measured using the

changing pH-indicator method described previously [36-38]. All stopped-flow kinetic studies were carried out in the presence of 250 mM Na₂SO₄, which was required for maximum enzyme stability and activity in dilute solution [13, 39]. The pH-rate profiles of k_{cat} and k_{cat}/K_m were fit to equations 3 or 4 to be consistent with that of the wild type enzyme [13]. Equation 3 describes a pH dependence that requires the sequential loss of two protons to generate the active enzyme, where k_{obs} is the observed value of k_{cat} or k_{cat}/K_m , k_{max} is the maximal value of k_{cat} or k_{cat}/K_m , and K_{a1} and K_{a2} correspond to the dissociation constants for the stepwise ionization of the enzyme, $EH_2 \rightleftharpoons EH^- + H^+ \rightleftharpoons E^{2-} + 2 H^+$, where the doubly deprotonated form of the enzyme is the active species. For Co-HICA, pK_{a1} and pK_{a2} were highly co-dependent in the fitted data, so these were constrained to be equal to one another in order to obtain an average value and standard error.

$$k_{obs} = \frac{k_{max}}{\left(1 + \frac{[H^+]}{K_{a2}} + \frac{[H^+]^2}{K_{a1}K_{a2}}\right)} \quad (3)$$

Equation 4 describes a pH dependence that requires the apparent cooperative loss of four protons with identical pK_a values to generate the active enzyme species, *i.e.*,

$EH_4 \rightleftharpoons E^{4-} + 4 H^+$, where E^{4-} is the active species of enzyme, and k_{max} is the maximal value of k_{cat} or k_{cat}/K_m . Previous study of the wild-type enzyme suggests that this apparent 4-proton dependence is the result of two “crossed” 2-proton dependences [13].

$$k_{obs} = \frac{k_{max}}{\left(1 + \frac{[H^+]^4}{K_a^4}\right)} \quad (4)$$

Experimental data for k_{cat} and k_{cat}/K_m were fit to the logarithmic forms of equations 3-4 using non-linear least squares in Origin 7.0 (Microcal) and represent analyses of data from at least three separate reaction traces. Values of k_{cat} and k_{cat}/K_m are reported here on a per subunit basis.

2.3 Crystallographic Methods

Single, three-dimensional crystals of Co-HICA were grown over several days in 0.2 M sodium acetate, 0.1 M Tris-Cl, 0.1 M (NH₄)₂SO₄, and 27% PEG 4,000 at pH 8.5 and 10 mg/mL protein at 22°C using hanging drop vapor diffusion. Crystals were soaked in artificial mother liquor plus 30% glycerol for 30-60 seconds prior to flash cooling in liquid nitrogen. Data collection was carried out at beamline F1 of CHESS at a wavelength of 0.95 Å. Data was processed using MOSFLM [40, 41] and SCALA [42], and an initial molecular replacement solution was obtained by using a search model derived from a single dimer of wild-type HICA (PDB 2A8D) in Phaser [43]. The initial solution was subjected to multiple rounds of refinement using Refmac5 [44] and model-building in Coot [45, 46]. Final refinement incorporated TLS [47] with one TLS group per protein chain. Simulated annealing omit maps (2F_o-F_c and F_o-F_c difference Fourier maps) were used to verify the correctness of the model; data collection and refinement statistics are reported in Table 1.

2.4 Absorption spectroscopy of Co(II)-substituted HICA

UV-visible spectra of Co(II)-substituted HICA were measured at 25.0°C using an Ocean Optics USB-4000 spectrometer and a 1 cm path length, 40 µL microcell (Starna). The pH of the solution was controlled with 100mM concentrations of MES (pH 6.51), MOPS (pH 6.92)

– 7.53), HEPES (pH 7.75 – 8.00), Bicine (pH 8.25 – 9.00), or CHES (pH 9.25 – 10.50). Enzyme concentration was 143 μM .

3. Results

3.1 Overexpression, purification and metal occupancy analysis of Co-HICA

Co-HICA was expressed in *E. coli* at a level of approximately 15% of total cellular protein estimated from scanning densitometry of crude lysates on SDS-PAGE gels. Purification and elution of the cobalt bound enzyme from a calibrated gel exclusion column (Superdex 200, GE healthcare) was identical to the native HICA, indicating a tetrameric holoenzyme in solution with a molecular weight of approximately 100 kDa. No decrease in protein concentration or coordinated metal population was observed after dialysis against deionized water.

Overexpression of HICA in TB medium supplemented with 1 mM CoCl_2 yielded a clear green enzyme solution; Overexpression M9 media supplemented with 1 mM CoCl_2 yielded a deep blue enzyme solution. This visible difference in enzyme species correlates with ICP-AES measurements of metal content: deep blue enzyme solution showed $99\% \pm 1\%$ cobalt incorporation (Co-HICA, no measurable zinc incorporation); the clear, green solutions of protein had $52 \pm 5\%$ incorporation of cobalt and $48 \pm 5\%$ zinc (CoZn-HICA). Since Zn(II) has no visible absorption, the absorbing species in the heterogeneously substituted solution must have cobalt in the smallest irreducible unit. In HICA, the smallest irreducible unit is the allosteric dimer [13]; if we take the dimer as the most relevant for statistical purposes, then statistical likelihood suggests the primary absorbing species for the heterogeneously substituted enzyme is Co-Zn dimers (69%), with Co-Co dimers making up the other 31% of absorbing species. In the homogeneously substituted enzyme the only significant absorbing species is Co-Co dimers; this preparation was used for further kinetic and structural studies.

3.2 Steady-State Kinetics

The pH -rate profiles of k_{cat} and k_{cat}/K_m for Co-HICA (figure 2) are similar to that of wild type HICA. [13] The pH -rate profile of k_{cat} for Co-HICA (figure 2A), as well as wild-type enzyme, can be fit by equation 3 which suggests that two H^+ ions must be lost to form the active form of the enzyme [13]. The pH -rate profile of k_{cat}/K_m of Co-HICA (figure 2B) is also quite similar to the pH dependence of wild-type HICA, including the rapid decrease of k_{cat}/K_m with decreasing pH , and all maximal activities are comparable to the wild-type enzyme. Maximal values of $k_{\text{cat}} = 82 \pm 19 \text{ ms}^{-1}$ and $k_{\text{cat}}/K_m = 10 \pm 1 \mu\text{M}^{-1}\text{s}^{-1}$ for Co-HICA are 120% and 230% of wild-type maximal values, respectively, and the data were modeled with the same curve fit parameters. The apparent pK_a values from Co-HICA k_{cat} measurements ($\text{pK}_{a1} = \text{pK}_{a2} = 7.73 \pm 0.09$) are indistinguishable from wild-type ($\text{pK}_{a1} = 8.3 \pm 0.6$ and $\text{pK}_{a2} = 8.4 \pm 0.6$) [13]; the apparent pK_a of the k_{cat}/K_m profile for Co-HICA (7.48 ± 0.03) is also indistinguishable from wild type enzyme ($\text{pK}_a = 7.74 \pm 0.4$) [13].

3.3 Structure of Co-HICA

The overall secondary and tertiary structure of Co-HICA differs from wild-type enzyme by 0.55 Å rmsd. The biological unit appears to be a dimer of dimers. A dimerization interface extends the β -sheet core across a pair of monomers that define a fundamental dimer, and is in close proximity to the active site cobalt ion (figure 3B). A tetramerization interface is orthogonal to the dimerization interface, and provides for the interaction and association of the fundamental dimers. This interface is characterized by a cluster of basic amino acid side chains: Arg160, Lys165, and Arg198 from one dimer, and Arg 124 from the neighboring dimer. Co-HICA crystallized in the same unit cell as wild-type HICA, with $1\frac{1}{2}$ tetrameric units in the asymmetric unit.

The cobalt ion is coordinated by residues Cys42, Cys101 and His98, with Asp44 replacing the catalytic water molecule (figure 3A). The pseudo-tetrahedral coordination is consistent with the wild-type enzyme zinc coordination sphere in the closed form, indicating that Co-HICA has crystallized in the inactive, T-state conformation observed for wild-type HICA (Figure 1). As with wild-type enzyme, the distance between cobalt ions in the allosteric dimer is approximately 23 Å, with active sites on the opposite sides of the shared β -sheet, separated by the dimerization interface. Co-HICA also shows four strong, approximately trigonal planar electron density regions near Asp74 on the tetramerization interface. These have been interpreted as acetic acid molecules, which are present in the crystallization solution. These molecules significantly improved phases when added to the model (Figure A.1).

3.4. Spectroscopic characterization of Co-HICA and CoZn-HICA

The absorption spectrum (Figure 4A) of CoZn-HICA (principally Co-Zn dimers) at pH 8.0 has absorption bands at 410 nm ($\epsilon=1160 \text{ M}^{-1} \text{ cm}^{-1}$), 557 nm ($\epsilon=480 \text{ M}^{-1} \text{ cm}^{-1}$), 618 nm ($\epsilon=835 \text{ M}^{-1} \text{ cm}^{-1}$), and 703 nm ($\epsilon=350 \text{ M}^{-1} \text{ cm}^{-1}$). The longest wavelength absorption band is quite broad and may represent two overlapping *d-d* transitions. The absorbance maximum at 410 nm, combined with the minimum near 500 nm accounts for the green color of this protein. The absorption spectrum of Co-HICA (principally Co-Co dimers) at pH 8.0 is significantly different from CoZn-HICA. The absorption band at 410 nm present in CoZn-HICA is absent or shifted into the UV absorption tail, where there is a prominent shoulder at 340 nm with $\epsilon \approx 3500 \text{ M}^{-1} \text{ cm}^{-1}$. The visible absorption bands, 560 nm ($\epsilon=340 \text{ M}^{-1} \text{ cm}^{-1}$), 620 nm ($\epsilon=720 \text{ M}^{-1} \text{ cm}^{-1}$), and 703 nm ($\epsilon=290 \text{ M}^{-1} \text{ cm}^{-1}$), are slightly less intense. Again, the long wavelength absorption feature is quite broad and may represent two overlapping *d-d* transitions. The broad minimum in absorption between 400-500 nm (blue light) accounts for the blue color of this protein. When pH is lowered, the molar absorptivity of Co-HICA in the visible region markedly increases (figure 4B), and the absorption band at 560 nm undergoes a significant red shift from 560 nm to 580 nm (figure 4C). An absorption spectrum remarkably similar to the low pH spectrum of Co-HICA results from the addition of 25 mM sodium bicarbonate to the enzyme at pH 8.00 (figure 4A).

4. Discussion

4.1 Overexpression of Co(II)-substituted protein

Metallosubstitution of β -CA has proven to be elusive, because the typical approach, which involves chelation and removal of Zn(II) followed by reconstitution with Co(II), does not work. It seems likely that the presence of two thiolate ligands in the Cys₂HisAsp ligand sphere of β -CA, compared to the His₃H₂O ligand sphere of most other CAs results in much stronger chelation of the metal ion, inhibiting removal by external chelating agents.

Our method of producing Co-HICA, in which protein is overexpressed in *E. coli* in a zinc-depleted medium, has proven to be surprisingly efficient in both quantity of protein produced, and quality of Co(II)-substitution. A number of metallosubstituted enzyme have been produced this way [48-51], so it appears that this method may be generally suitable for the production of difficult-to-obtain metallosubstituted enzymes.

4.2 Kinetics of Co-HICA

The overall catalytic efficiency of Co-HICA is maximal at $pH > 8.0$ (Figure 2), suggesting that the metal-hydroxide form of the enzyme is the active species, as with wild type HICA. The substitution of cobalt for zinc has also not substantially changed the *pH*-rate cooperativity of the enzyme, consistent with the hypothesis that at low *pH*, the side chain of Asp44 can displace water to form the inactive, T-state of the enzyme. Specifically, the *pH*

dependence of k_{cat} (Figure 2A) can be accounted for by the sequential or simultaneous ionization of two groups to generate the active form of the enzyme, identical to HICA. In HICA, the pK_a values for these two groups are experimentally indistinguishable and have a value of approximately 8.3 [13]. Similarly, Co-HICA pH-rate profile data is fit with a sequential, two pK_a model as well, also having indistinguishable pK_a values of approximately 7.7. The pH profile of k_{cat}/K_m at steady state (Figure 2B) also closely resembles the wild type enzyme, modeled by equation 4, describing a cooperative 4-proton loss with a pK_a value of 7.48 ± 0.03 (compared to 7.7 ± 0.4 for the wild-type enzyme). The pK_a associated with the k_{cat}/K_m pH-rate profile should reflect the apparent pK_a of the metal-bound water molecule in the metal-hydroxide mechanism common to all carbonic anhydrases; the slightly more acidic pK_a value for Co-HICA is within one standard error of the wild-type enzyme, but may also reflect the increased acidity of Co(II)-bound water compared to Zn(II)-bound water [32, 52-54]. Cobalt derivatives of carbonic anhydrases in general approach the functional levels (50-200%) of zinc-bound enzymes [35, 55], our results (120% of wild-type) are consistent with other cobalt(II)-substituted zinc metalloenzymes.

4.3 Structure of Co-HICA

Co-HICA is isostructural with the wild-type enzyme (Figure 3B), with the classic pseudotetrahedral coordination of Type II β -CA and the closed Cys₂HisAsp metal coordination sphere characteristic of the inactive T-state (Figure 3A). The observed structural similarity, in conjunction with the kinetics measurements, strongly suggests that Co-HICA represents the wild-type enzyme well. Despite a crystallization pH value of 8.50, which is above the apparent pK_a of the metal bound water suggested by the pH-rate profile of k_{cat}/K_m , the enzyme has crystallized in the “closed” T-state conformation, rather than the “open” R-state conformation where a water or hydroxide ion replaces Asp44. We have previously observed this preference for crystal form in the wild-type enzyme and many variants crystallized at pH 7.50 [13, 17, 39], which is near the pH transition point for pH-rate kinetics. It seems likely that the T-state of the enzyme is less soluble than the R-state, and crystallizes preferentially. *E. coli* β -CA crystallizes in the “closed” T-state form even at pH 9.0 (J. D. Cronk, personal communication).

4.4 Spectroscopy of Co-HICA

Like other cobalt substituted enzymes with thiolate ligands [56, 57], Co-HICA shows a UV absorption with a prominent shoulder at 340 nm (not shown in figure 4) with a molar absorptivity, $\epsilon_{340} \approx 3,500 \text{ M}^{-1} \text{ cm}^{-1}$, which is probably a sulfur to cobalt LMCT band. The apparent molar absorptivity is slightly higher than the $1800\text{-}2600 \text{ M}^{-1} \text{ cm}^{-1}$ expected for a two-sulfur LMCT band [58], but the molar absorptivity is probably overestimated because of the overlapping UV tail. The absorbance peaks and molar absorptivities in the visible region (500-700 nm, all with $\epsilon > 200 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$) in Figure 4A are characteristic of the $d-d$ transitions for a four-coordinate Co(II) sphere [57, 59]. Higher coordination numbers for Co(II) would result in higher ligand-field stabilization, and be reflected in lower molar absorptivities ($<200 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ for pentacoordinate, a maximum of $30 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ for octahedral complexes) and shorter wavelengths of maximum absorption. A shift in the peaks themselves might reflect a bond angle or bond length change in the immediate pseudotetrahedral metal coordination sphere. Overall, the wavelengths of the absorbance maxima for Co-HICA are in agreement with the X-ray crystal structure and consistent with metal coordination in a pseudotetrahedral geometry comprising two sulfur ligands, one nitrogen ligand, and a fourth ligand position occupied with an oxygen atom in a molecule of water or aspartate, [56, 57]. Lowering the pH of Co-HICA enzyme results in an increase in the molar absorptivity of all $d-d$ absorption bands (Figure 4B), suggesting a significant change in the metal coordination sphere. This transition is too sharp to be accounted for by a

single proton dependence: it is modeled well by a highly cooperative, 4-proton-dependence transition as described by equation 5,

$$\varepsilon_{obs} = \varepsilon_{max} - \Delta\varepsilon / \left(1 + \left(\frac{[H^+]}{K_a} \right)^4 \right) \quad [5]$$

Where ε_{obs} is the observed molar absorptivity, ε_{max} is the maximal molar absorptivity at low pH, $\Delta\varepsilon$ is the maximal change (decrease) in molar absorptivity at high pH, and K_a is the apparent K_a of the transition. Figure 4C can be fit to a version of equation 5 where λ substitutes for ε . Alternative fits to 1- or 2-proton dependences are significantly poorer both qualitatively and quantitatively as measured by the residual error R^2 . A one-proton dependence fit is depicted in Figures 4B and 4C for reference. The cooperativity of this spectral transition mimics that of k_{cat}/K_m for HICA and Co-HICA (Figure 2B). The value of k_{cat}/K_m in the metal hydroxide mechanism of CA is a proxy for the relative fraction of enzyme in the metal-hydroxide form. The apparent pK_a value for spectral changes in Co-HICA (7.8-8.0) is slightly higher than the kinetically determined pK_a of 7.3, but it should be noted that the spectral data was collected at relatively low sulfate concentration (to avoid metal-anion complexation to the extent possible) whereas the kinetics data is collected at 250 mM sulfate as required for maximal activity [39]. Lowering the pH also results in an increase in, and a significant red-shift of the 560 to 580 nm peak, likewise suggesting a significant change in the metal coordination sphere.

Kinetic studies of the wild-type enzyme have suggested that at high pH, the enzyme is characterized by the presence of metal-bound hydroxide ion, which is the active species (R-state). At low pH, the protonation of the metal-bound hydroxide ion to a metal-bound water facilitates its displacement by Asp44, transitioning to the inactive T state (Figure 5). The pH dependence of spectral changes in Co-HICA is consistent with the hypothesis of the hydroxide ion transitioning to the protonated water and further displacement by D44 at low pH. Further, the lack of clear isosbestic points observed in the pH dependence of Co-HICA spectra (and therefore the first coordination shell of the metal) may be explained by populations of all three species of Figure 5 being present at most pH values.

Figure 4A shows that 25 mM bicarbonate, the hypothesized allosteric inhibitor of HICA, at pH 8.0 can trigger a spectral change identical to that of lowering the pH of solution. This suggests that bicarbonate ion causes the same structural change as low pH, *i.e.* causing Asp44 to displace the metal-bound solvent molecule from the metal coordination sphere. It does not appear to be possible to trigger this spectral shift at pH values larger than 8.0, suggesting that only the accumulated H₂O-coordinated metal species at the active site allows displacement by Asp44, and that the hydroxide coordinated solvent form is not readily displaced by Asp44.

4.5 Spectroscopy of CoZn-HICA

The absorption spectrum of CoZn-HICA is similar to that of Co-HICA in the long wavelength (*d-d* transition) region. However, the sulfur to cobalt LMCT band has apparently shifted from 340 nm to approximately 400 nm (Figure 4A), and has a significantly smaller molar absorptivity. The LMCT shift is the primary factor for the readily discernable difference in color between Co-HICA (primarily Co-Co dimers) and CoZn-HICA (mostly heterogeneous Co-Zn dimers). Assuming there is a statistically random distribution of metal ion in the protein—and we cannot know otherwise—this result suggests the surprising conclusion that a Co(II) ion in one subunit of an allosteric dimer is sensitive to the nature of the metal ion, Co(II) or Zn(II), in the other subunit, some 23 Å distant. This result, although

surprising, is consistent with inter-subunit communication in the allosteric HICA dimer, and lends additional support for previously proposed model for the allosteric regulation within the functional dimer of regulated β -CAs. [13]

5. Conclusions

We have expressed a Co(II)-substituted β -carbonic anhydrase and confirmed the assignment of four-coordinate geometry around the Co(II) ion *in vitro* and in the crystal state, similar to the wild-type zinc-bound enzyme. Catalytic steady-state kinetics of Co-HICA are similar to HICA, suggesting that Co-HICA is good representative of the native enzyme. The metallosubstituted enzyme allows the detection of spectral changes in the coordination sphere for the first time, which correlate with a previously postulated allosteric regulation mechanism. Changes in the spectrum of Co-HICA can be induced by either pH or by the presence of allosteric bicarbonate ion; therefore, it should be possible to use Co-HICA to probe allosteric behavior of the enzyme in solution. Co(II) ions in the active site appear to be sensitive to the environment of identity of the neighboring active site in the allosteric dimer.

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Appendix

Figure A.1: $F_o - F_c$ omit map of bound acetic acid molecules deep in the tetramerization interface. The map is contoured at 4σ . The two acetic acid molecules and nearby amino acid residues are depicted as sticks. Each chain of the tetramer is shown with a different carbon color (red, yellow, orange, green); ligands are labeled where visible. Electron density around the acetic acid molecules and the bridging water (above the plane of the acetic acid molecules) is shown in grey mesh. Water molecules are shown as red spheres.

This unusual electron density, not seen in other HICA structures, has been interpreted as acetic acid, and not acetate, despite the solution pH of 8.5, because of electrostatic charge repulsion and lack of sufficient hydrogen bonding partners for acetate ions compared to acetic acid. Acetate is present in the crystallization medium. The well-described symmetric molecules coordinate one of the acetic acid oxygens, presumably the carbonyl oxygen, in a position to accept hydrogen bonds (at 2.69Å each) from two aspartate carboxylic acid groups. The other carboxylic acid OH of the acetic acid is donates a hydrogen bond to the backbone carbonyl of Phe75. It is possible that the acetic acid methyl and carbonyl oxygen atoms could be reversed, but the proposed arrangement maximizes hydrogen bonding interactions with Asp75, Phe75 carbonyl, and a the bridging water molecule.

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Figure 1.

Structural schematic of key active site and noncatalytic bicarbonate binding site interactions in the hypothesized active (R-state) and inactive (T-state) conformations of HICA [13].

Figure 2.

pH-rate profiles of (A) k_{cat} and (B) k_{cat}/K_m for Co-HICA (■) and HICA (□) [13]. A was fit to equation 3 with $k_{max} = 82 \pm 19 \text{ ms}^{-1}$, $pK_{a1} = pK_{a2} = 7.73 \pm 0.09$ (Co-HICA) and $k_{max} = 69 \pm 29 \text{ ms}^{-1}$, $pK_{a1} = 8.3 \pm 0.6$ and $pK_{a2} = 8.4 \pm 0.6$ (HICA). B was fit to equation 4, with $k_{max} = 10 \pm 1 \text{ } \mu\text{M}^{-1}\text{s}^{-1}$ and $pK_a = 7.48 \pm 0.03$ (Co-HICA) and $k_{max} = 4.3 \pm 0.8 \text{ } \mu\text{M}^{-1}\text{s}^{-1}$ and $pK_a = 7.74 \pm 0.04$ (HICA). Reactions were carried out at 25°C in 40 mM buffer (1-methylimidazole, pH 7.0-8.25; 1,2-dimethylimidazole, pH 8.25-9.25), 30-200 μM indicator

dye (*p*-nitrophenol with 1-methylimidazole; *m*-cresol purple with 1,2-dimethylimidazole), 10 μ M EDTA, 250 mM Na₂SO₄ and 0.4- 15 μ M enzyme.

Figure 3. Structure of Co-HICA. (A) 2Fo-Fc omit map of cobalt ion binding site in chain F. contoured at 4σ. Cobalt ion is colored blue, protein residues used to construct omit map are depicted as sticks. (B) The allosteric dimer, colored grey (chain D) and green (chain F). Cobalt ions are colored blue; active site metal ligands, and a sulfate ion on the dimerization interface, are depicted as sticks. Water molecules are red spheres. Figure was constructed using Pymol [61].

Figure 4.

UV-visible spectral features of Co-HICA and CoZn-HICA. (A) Absorption spectra: CoZn-HICA, pH 8.00 (green); Co-HICA, pH 8.00 (blue); Co-HICA, pH 7.53 (red); Co-HICA, pH 8.00 + 25 mM HCO_3^- (black). pH was controlled using 100mM buffer as described in Experimental Procedures. (B) pH dependence of molar absorptivity for Co-HICA: ϵ_{580} ($\square$);

ϵ_{700} (■); data (solid lines) was fit to equation 5 (see Discussion), with $\epsilon_{\max} = 513 \pm 8 \text{ L mol}^{-1} \text{ cm}^{-1}$, $\Delta\epsilon = 260 \pm 10 \text{ L mol}^{-1} \text{ cm}^{-1}$, $pK_a = 7.82 \pm 0.03$ (580 nm) and $\epsilon_{\max} = 585 \pm 29 \text{ L mol}^{-1} \text{ cm}^{-1}$, $\Delta\epsilon = 275 \pm 36 \text{ L mol}^{-1} \text{ cm}^{-1}$, $pK_a = 7.80 \pm 0.09$ (700 nm); dotted lines indicate best fit one-proton pH dependence titration curves; (C) pH dependence of $\lambda_{\max}$ for 580 nm peak of Co-HICA (▲); data was fit to a version of equation 5 (see Discussion) with $\lambda_{\max} = 577 \pm 2 \text{ nm}$, $\Delta\epsilon = 29 \pm 2 \text{ nm}$, $pK_a = 7.99 \pm 0.05$; dotted lines indicate best fit for one-proton pH dependence titration curves. Enzyme concentration used for all data was 143 μM , temperature was 25°C

Figure 5. Structural schematic of key active site distinctions in the hypothesized populations of metal-ligand species of HICA [13].

Figure A.1.

Table 1

Data Collection and Refinement Statistics Cobalt substituted HICA (PDB 3MF3)

Data Collection Statistics	
source	CHESS beamline F1
wavelength (Å)	0.918
space group	C121
cell parameters (Å)	229.492, 144.929, 52.215
(deg)	90, 93.77, 90
resolution (Å)	29.87–2.50 (2.64–2.50)
unique reflections	58139
redundancy	4.1 (4.1)
completeness	99.0 (98.7)
R_{sym} (%)	0.058 (0.236)
$\langle I \rangle / \langle \sigma I \rangle^b$	10.7 (2.5)
Refinement Statistics	
reflections in test set	2941
R_{work} (%)	0.178
R_{free} (%)	0.231
number of atoms	
protein	10483
ligand	6
ion	15
solvent	202
rmsd from ideal ^c	
bond distance (Å)	0.018
bond angle (deg)	1.674
Ramachandran plot outliers (%) ^d	0.2%

^aValues in parentheses represent data for the highest resolution shell.^bReported as $\langle \langle I \rangle / \sigma \langle I \rangle \rangle$ in SCALA.^cIdeal values from Engh and Huber [60].^dCalculated using a strict boundary Ramachandran plot [46].