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Supporting Information

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A De Novo Designed Metalloenzyme for the Hydration of CO_2**

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Supporting Information

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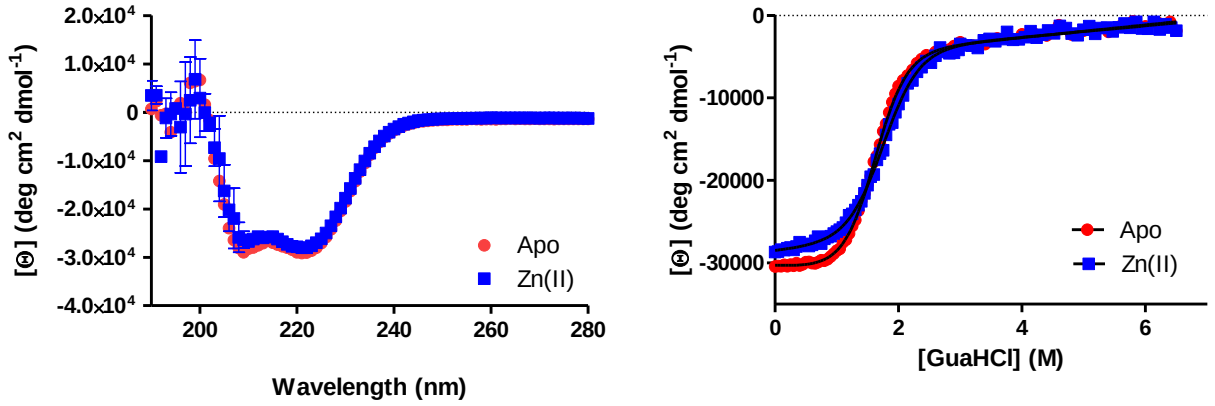

Figure S1. Circular dichroism spectroscopy for 5 μM α_3DH_3 (red) and 5 μM Zn(II)α_3DH_3 (blue) at pH 9.0. Left: Spectra showing α-helical nature of fully-folded peptides. Apo-α_3DH_3 and Zn(II)α_3DH_3 are initially 82% and 83% folded, respectively. Right: Guanidine hydrochloride denaturations. Fittings reveal ΔG_u = 3.4 kcal/mol for apo- α_3DH_3 and 3.5 kcal/mol for Zn(II)-α_3DH_3.

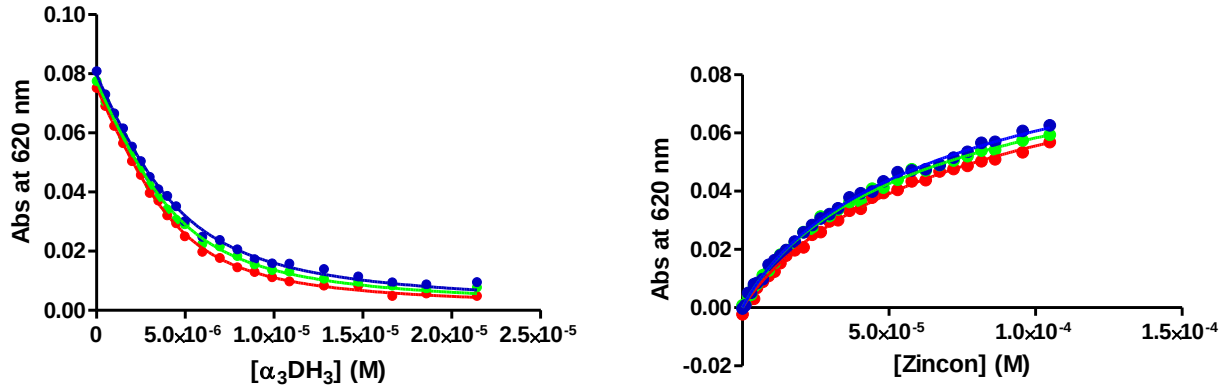

Figure S2. Competitive Zincon binding titrations at pH 7.5 with α_3DH_3. In triplicate, plots show the absorbance of Zn(II)-Zincon at 620 nm plotted against the titrant concentration. Left: Titration of α_3DH_3 into Zn(II)-Zincon (10 μM Zincon, 5 μM $ZnSO_4$, 50 mM HEPES). Right: Titration of Zincon into Zn(II)α_3DH_3 (10 μM α_3DH_3, 5 μM $ZnSO_4$, 50 mM HEPES).

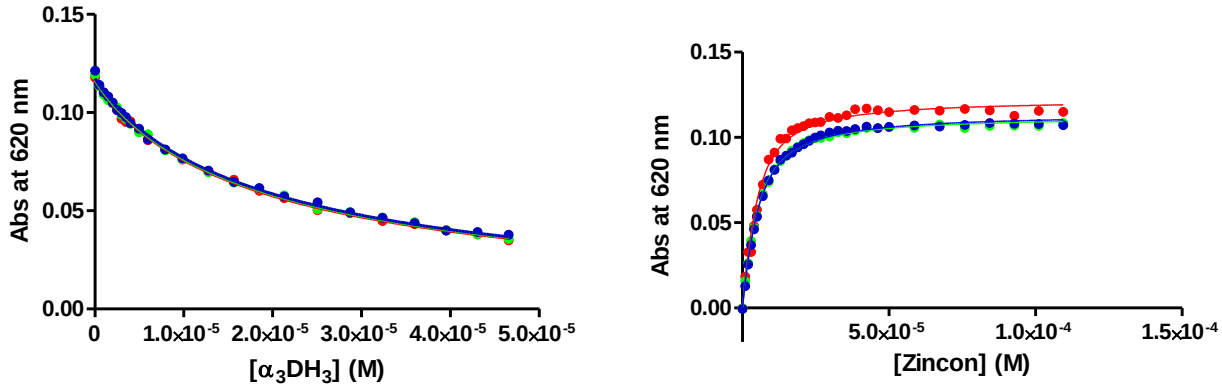

Figure S3. Competitive Zincon binding titrations at pH 9.0 with α_3DH_3. In triplicate, plots show the absorbance of Zn(II)-Zincon at 620 nm plotted against the titrant concentration. Left: Titration of α_3DH_3 into Zn(II)-Zincon (10 μM Zincon, 5 μM $ZnSO_4$, 50 mM CHES). Right: Titration of Zincon into Zn(II)α_3DH_3 (10 μM α_3DH_3, 5 μM $ZnSO_4$, 50 mM CHES).

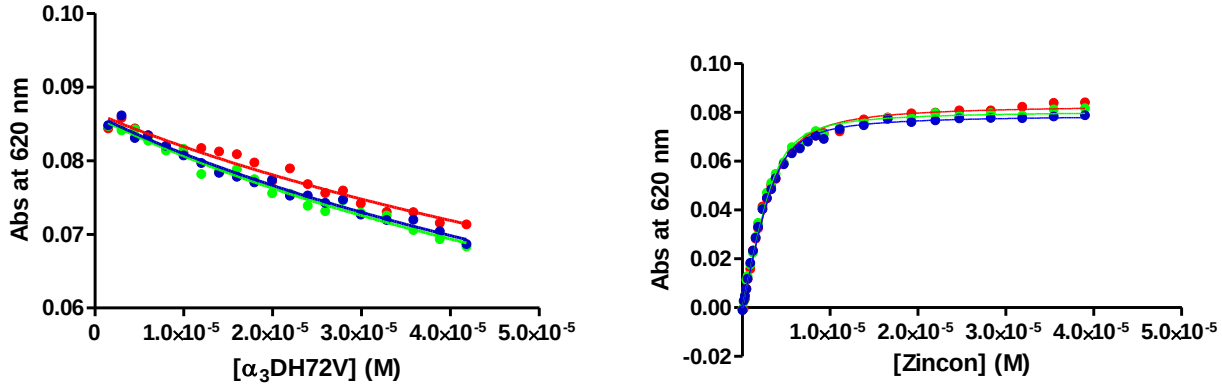

Figure S4. Competitive Zincon binding titrations at pH 9.0 with α_3DH72V. In triplicate, plots show the absorbance of Zn(II)-Zincon at 620 nm plotted against the titrant concentration. Left: Titration of α_3DH72V into Zn(II)-Zincon (8 μM Zincon, 4 μM $ZnSO_4$, 50 mM CHES). Right: Titration of Zincon into Zn(II)α_3DH72V (40 μM α_3DH72V, 4 μM $ZnSO_4$, 50 mM CHES).

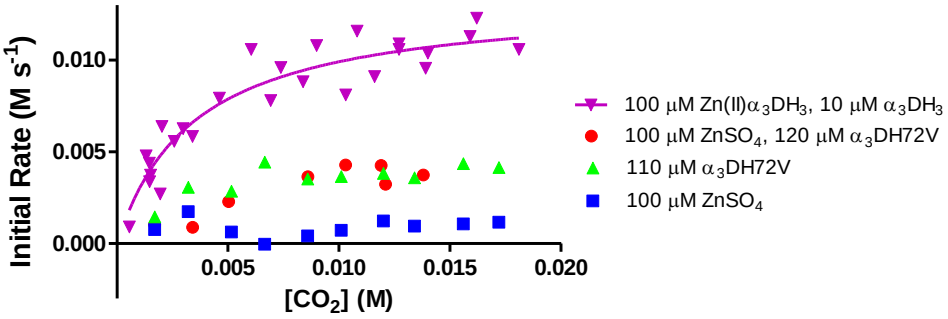

Figure S5. CO_2 hydration rates plotted versus substrate concentration at pH 9.5. The activity of Zn(II)α_3DH$_3$ (purple triangles) is significantly higher than any of the controls, showing that the activity results from the Zn(II)N_3O site. Apo-α_3DH72V shows some activity (green triangles), but it is not enhanced by the addition of $ZnSO_4$ (red circles), showing that adventitiously-bound Zn(II) does not contribute to the activity. Free Zn(II) shows minimal activity (blue squares).

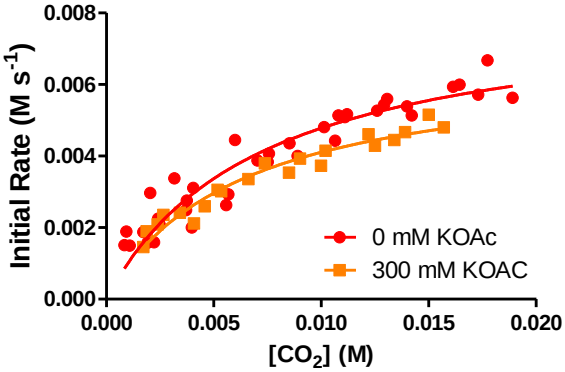

Figure S6. Michaelis-Menten plot for the hydration catalysis of CO_2 hydration by 100 μM Zn(II)α_3DH$_3$ at pH 8.5 in the absence and presence of potassium acetate inhibitor.

Table S1. Kinetic parameters for the catalysis of CO_2 hydration by Zn(II)α_3DH$_3$ at pH 8.5 in the absence and presence of potassium acetate inhibitor.

KOAC (mM)	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($M^{-1}s^{-1}$)
0	82 ± 6	7.2 ± 1.1	11,400 ± 1,100
300	67 ± 4	6.2 ± 0.8	10,700 ± 800

Experimental Section

Expression and Purification of α_3DH_3

The synthetic DNA for α_3DH_3 was cloned into a pET-15b vector (Celtek Genes) and expressed in *E. coli* BL21(DE3) competent cells (Invitrogen) in self-inducing media. The cells were lysed by sonication and the soluble protein was isolated after heat denaturation (55 °C) and acidification to pH 1.9. The lyophilized powder was redissolved and purified using reverse-phase HPLC with a linear gradient of H_2O to 9:1 CH_3CN/H_2O over 50 minutes. All HPLC solvents contained 0.1% trifluoroacetic acid. The purified protein had a yield of 230 mg/L and a molecular weight of 8283.5 Da by ESI-MS (collected on a Micromass LCT Time-of-Flight Mass Spectrometer). This compares well to the calculated mass of 8283.1 Da for α_3DH_3 lacking formyl-methionine. The concentration of the protein was determined by measuring the absorbance at 280 nm using the measured molar extinction coefficient of 7849 $mol^{-1}cm^{-1}$.

Expression and Purification of α_3DH72V

Using the GeneArt® Site-Directed Mutagenesis System (Invitrogen), the codon for His72 was mutated to that for Val within the α_3D gene (pET-15a vector). The resulting plasmid was expressed and purified as described above with a yield of 100 mg/L. The molecular weight of the pure protein was determined to be 7938.4 Da by ESI-MS, which compares well to the calculated mass of 7938.9 Da for α_3DH72V lacking formyl-methionine.

Circular Dichroism (CD) Spectroscopy

CD spectra were collected on an Aviv 202 CD Spectrometer at 25 °C using 1 cm path length open top quartz cuvettes. Samples were prepared at pH 9.0 in 10 mM potassium phosphate (ion source) and contained either 5 μM peptide or 5 μM peptide and 15 μM $ZnSO_4$. The percent folding was based on the presence of 59 helical residues and was determined using previously reported procedures.[1,2] Guanidine hydrochloride (GuaHCl) titrations were carried out using a Microlab 500 series syringe-pump automatic titrator controlled by Aviv software. Into a solution of 5 μM α_3DH_3 or 5 μM Zn(II)-α_3DH_3 in 10 mM KPhos at pH 9.0, GuaHCl was titrated to a concentration of 6.5 M, while keeping the protein concentration constant. The change in molar ellipticity upon titration was fit to a two-state unfolding model.[3]

Zn(II) Binding Constant Determination

The dissociation constants for Zn(II) to α_3DH_3 and α_3DH72V were determined at pH 7.5 and 9.0 at 25 °C following our previously reported procedures.[4]

CO_2 Hydration Kinetics

The initial rates for CO_2 hydration catalyzed by Zn(II)-α_3DH_3 were measured at 25.0 °C on an OLIS RSM Stopped Flow Spectrophotometer using Khalifah's changing pH-indicator method.[5] The substrate stock solution was prepared by bubbling CO_2 (Matheson Tri-Gas, Inc., Research Grade) through deionized water for at least 20 minutes and then diluted with deionized water in a gas-tight syringe. This substrate solution was mixed by the stopped-flow with either a control solution (containing only buffer, indicator, and apo-α_3DH_3) or a catalyst solution (containing buffer, indicator, Zn(II)-α_3DH_3, and apo-α_3DH_3). After 1:1 mixing with the substrate solution, each shot contained 50 mM buffer and 25 μM indicator and were maintained at 0.1 M ionic strength with Na_2SO_4. Typically, the control shots contained 10 μM apo-α_3DH_3, while the catalyst shots contained 110 μM α_3DH_3 and 100 μM $ZnSO_4$, ensuring a final Zn(II)α_3DH_3 concentration of 100 μM. The buffer indicator pairs used were TAPS/*m*-cresol purple (pH 8-8.75, λ = 578 nm, $\Delta\epsilon$ = 35,000 $M^{-1}cm^{-1}$), AMPSO/thymol blue (pH 8.75-9.25, λ = 590 nm, $\Delta\epsilon$ = 40,500 $M^{-1}cm^{-1}$), and CHES/thymol blue (pH 9.25-9.5, λ = 590 nm, $\Delta\epsilon$ = 40,500 $M^{-1}cm^{-1}$). The experimental buffer factors

were close to those which were calculated theoretically, allowing for the CO_2 concentrations to be checked throughout the experiment.[5] Upon rapid mixing of the substrate solution with the buffer/indicator solution by the stopped-flow spectrophotometer, several seconds of absorbance data was collected. Initial rates were determined from the initial linear portion of the reaction (usually less than the first 10%). The rates from 6-11 replicates were averaged and, at each substrate concentration, the rate for the control solution was subtracted from that of the catalyst. The difference, reflecting the initial rate of the catalyst, was plotted as a function of [CO_2] and fitted using the Michaelis-Menten equation (in GraphPad Prism 5). Inhibition experiments were carried out as described above, with the addition of 300 mM KOAc to the buffer/indicator solution at pH 8.5.

EXAFS

Samples for EXAFS were prepared with final concentrations of 1 mM $ZnSO_4$, 1.5 mM α_3DH_3, 50 mM CHES buffer, and 4.1 M glycerol at pH 9.0. At these concentrations, >99.9% of the Zn(II) is bound to α_3DH_3. The samples were rapidly frozen in liquid N_2 and maintained at ~10K throughout the measurements. EXAFS data were measured at SSRL Beamline 7-3 using a Si(220) double crystal monochromator and a Rh-coated vertically collimating harmonic rejection monochromator. Data were measured as fluorescence excitation spectra using a 30-element energy-resolving Ge detector. Data were collected using 10 eV steps in the pre-edge region (9350 - 9640 eV), 0.25 eV for the edge region (9640 - 9690 eV), and 0.05 $Å^{-1}$ increments for the extended x-ray absorption fine structure (EXAFS) region to k=13 $Å^{-1}$, with integration times of 1 s in the pre-edge and edge regions and 1-20 s (k^3 weighted) in the EXAFS region for a total scan time of ~40 min.

Raw data were converted to EXAFS and fit using the EXAFSPAK[6] suite of programs, with theoretical amplitude and phase parameters calculated using FEFF 9[7] using two metal-ligand distances, the Debye-Waller factors for the Zn-O and Zn-histidine shells (1.6 x 10^{-3} $Å^2$ and 5.6 x 10^{-3} $Å^2$, respectively) and $\Delta E0$ (-8.3 eV) as the only adjustable parameters. The remaining histidine distances assuming a rigid imidazole ring with distances and angles equal to the average of Zn-histidine models found in the Cambridge Crystallographic database and the imidazole Debye-Waller factors were defined by assuming that the σ^2 value for each scattering path increased proportionally from those calculated by Bunker and Dimakis for an ordered imidazole.[8]

References

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