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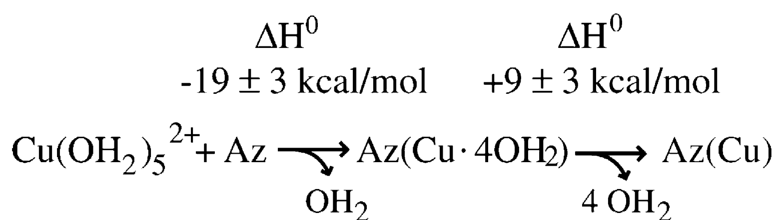


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# Kinetics and Thermodynamics of Copper(II) Binding to Apoazurin

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Metalloprotein function depends on the incorporation of an appropriate metal ion into a binding site within a protein.[1] Metal specificity can be dictated by either kinetic or thermodynamic factors. Type I copper proteins such as azurin function as electron carriers and must incorporate the redox-active copper metal ion for function. Azurin binds copper(II) quite tightly and exchanges metal ions very slowly so that detailed analysis of the thermodynamics of metal ion binding has been difficult. Isothermal titration calorimetry has the potential to reveal key thermodynamic parameters including both the standard free energy and enthalpy of metal binding.[2]

The binding of copper(II) to apoazurin was examined by this method.[3] Initial studies in HEPES and PIPES buffers produced very broad calorimetric traces indicative of slow metal binding; buffer effects on the kinetics of copper(II) binding to azurin have been observed previously.[4] In contrast, studies in cholamine[5] buffer, which has a very low affinity for metal ions, produced much sharper traces, suitable for detailed analysis (Figure 1).

The standard enthalpy for the overall reaction is $\Delta H° = -10.2 \pm 1.6$ kcal mol$^{-1}$. Closer examination of the calorimetric traces revealed two important features. First, despite the decrease in the maximum extent of the calorimetric traces as the titration progressed, binding is essentially stoichiometric as shown in Figure 1B; the decrease in peak extent is due to progress in the titration reflected in the peak shape. Second, the response after each injection of apoazurin shows an initial exothermic phase followed by an endothermic phase. The presence of these two phases reveals the occurrence of two kinetically separable steps in the binding reaction.

A two-step binding process, shown in Scheme 1, is suggested by these and previous observations.

In the first step, the apoprotein combines with copper(II) from solution, presumed to be[6] $Cu(OH_2)_5^{2+}$, in an exothermic reaction. The initial complex formed then rearranges to form the final product in an endothermic step.

To test this model, the calorimetric traces from each injection were isolated and integrated as shown in Figure 2A. These traces were then compared with the results from simulation of the data based in Scheme 1.[7] Four parameters were used in fitting these data: the two forward rate constants $k_1$ and $k_2$, and the standard enthalpies for the two steps, $\Delta H^0{}_1$ and $\Delta H^0{}_2$. Rate constants for the reverse reactions were not included since initial simulation revealed that both steps are essentially irreversible under the conditions used. Each of these variables was reasonably well defined by the data[7] with

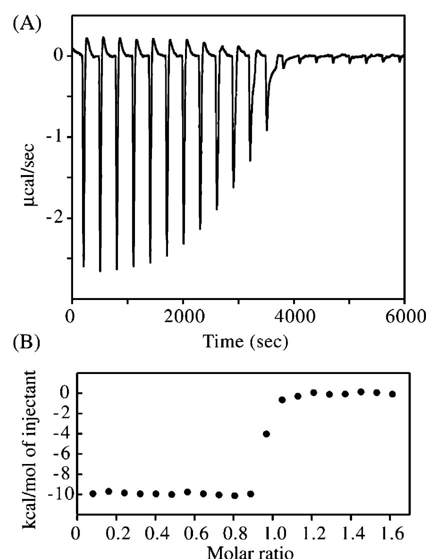


***Figure 1.*** (A) Calorimetric trace for titration of 50 nmols of apoazurin into Cu(II) in cholamine chloride buffer at pH 7.0. (B) Plot of net heat released as a function of the ratio of apoazurin to copper(II).

$$k_1 = 2.2 \pm 1.0 \times 10^3\ \mathrm{M^{-1}\,s^{-1}}$$

$$k_2 = 0.024 \pm 0.010\ \mathrm{s^{-1}}$$

$$\Delta H_1° = -19 \pm 3\ \mathrm{kcal\ mol^{-1}}$$

$$\Delta H_2° = +9 \pm 3\ \mathrm{kcal\ mol^{-1}}$$

These parameters can be interpreted on the basis of other characteristics of the azurin system. Most importantly, apoazurin adopts a well-defined structure very similar to that for Cu(II)−azurin; the only significant difference involves the position of one of the metal-coordinating His residues.[8] Thus, little change in protein conformation is associated with copper(II) binding. Because of this, the overall entropy change for metal binding can be estimated on the basis of two terms, developed for analysis of Zn(II) binding to a zinc finger peptide[9] and applied[9] to results for Zn(II) binding to carbonic anhydrase.[10] First, the Cu(II) is completely dehydrated when it binds to apoazurin, leading to the release of five water molecules into solution. The entropy of water release from metal ions has been estimated to be[11] 9.5 cal mol$^{-1}$ K$^{-1}$ (per water molecule). Second, the number of free particles (not including water) is reduced from two to one, leading to an entropy decrease


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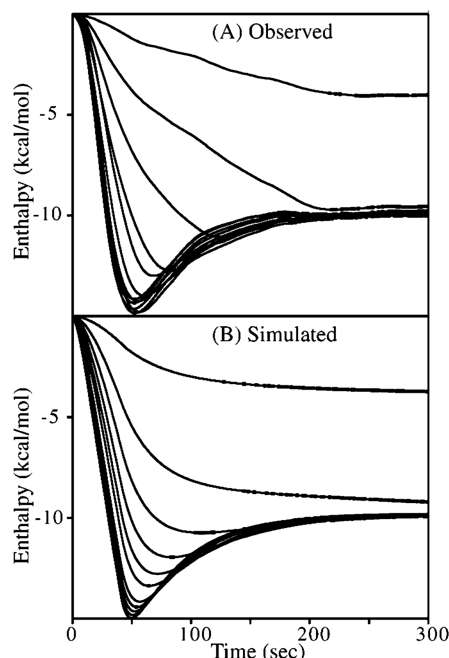



***Figure 2.*** (A) Integrated calorimetric traces from Figure 1, translated to begin at time equals zero. (B) Simulation of the results from (A) with the use of Scheme 1 with the parameters shown below.

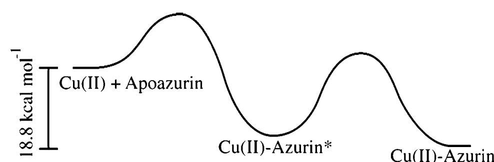
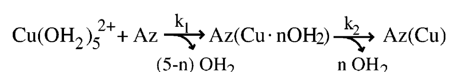



***Figure 3.*** A free-energy profile for Cu(II) binding to apoazurin.

***Scheme 1***



$$Cu(OH_2)_5^{2+} + Az \xrightarrow[(5-n)\,OH_2]{k_1} Az(Cu \cdot nOH_2) \xrightarrow[n\,OH_2]{k_2} Az(Cu)$$

estimated to be[9,12] $-19$ cal mol$^{-1}$ K$^{-1}$. Thus, the overall entropy change is $5 \times 9.5 - 19 = +29$ cal mol$^{-1}$ K$^{-1}$. On the basis of this value, the standard free energy change for Cu(II) binding is determined to be $-10.2$ kcal mol$^{-1}$ $-$ (298 K)(29 cal mol$^{-1}$ K$^{-1}$) $= -18.8$ kcal mol$^{-1}$, corresponding to a dissociation constant of 25 fM.

The initial bimolecular reaction is strongly exothermic with $\Delta H_1° = -19 \pm 3$ kcal mol$^{-1}$. In contrast, the second isomerization step is endothermic with $\Delta H_2° = +9 \pm 3$ kcal mol$^{-1}$. Since the second step proceeds to completion, this step must be entropically favorable. Assuming that $\Delta G_2° \leq -2$ kcal mol$^{-1}$ (corresponding to the reaction proceeding to greater than 96% completion), then $-T\Delta S_2° \leq -11 \pm 3$ kcal mol$^{-1}$ and so $\Delta S_2° \geq +37 \pm 10$ cal mol$^{-1}$ K$^{-1}$. The most likely source of this favorable entropy change is the release of water molecules from the Cu(II) ion. On the basis of 9.5 cal mol$^{-1}$ K$^{-1}$ per water molecule, this suggests that $4 \pm 1$ waters are released in this step ($n = 4$ in Scheme 1). On the basis of these estimates, the standard entropy change for the initial bimolecular step is $\Delta S_1° = -8 \pm 10$ cal mol$^{-1}$ K$^{-1}$. These data can be combined with the rate constants[13] determined to generate a free-energy profile for the Cu(II)-binding reaction (Figure 3).

These parameters provide some details for the reactions in Scheme 1. $Cu(II)(OH_2)_5^{2+}$ binds to apoazurin with the release of one to two water molecules. These Cu(II)-$OH_2$ bonds are replaced by stronger or additional bonds to the protein, leading to the large, negative enthalpy for this step. The rate constant for this process is relatively low but is comparable to second-order rate constants for other proteins that have highly structured metal-free forms such as the Zn(II) enzymes carbonic anhydrase and alcohol dehydrogenase.[14] The intermediate formed then spontaneously rearranges to generate the final Cu(II)−azurin product, driven by the release of the remaining waters from the Cu(II) ion. This rearrangement is quite slow and is likely to involve changes in protein conformation that allow the Cu(II) to fully engage the azurin site. This mechanism is similar to those proposed earlier for Cu(II) binding to apoazurin under different buffer conditions, but differs in that the first step is not reversible.[4] A fortunate combination of standard enthalpy changes and rate constants has allowed the mechanism of this protein metalation reaction to be elucidated in considerable detail.

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(3) Azurin from *Pseudomonas aeruginosa* was expressed in *Escherichia coli*. The plasmid encoding azurin construct (pET9a/azurin) was transformed into a BL21/DE3 cells by electroporation. Cu(II)−azurin was purified to homogeneity; the final sample had ratio of absorbances at 626 and 280 nm was greater than 0.50 Azurin was quantitated by its $\epsilon_{280} = 9800$ M$^{-1}$ cm$^{-1}$ absorbance. The azurin was demetalated by the method of Blaszak et al.[4] All apoazurin manipulations were performed in an anaerobic atmosphere to minimize cysteine and methionine oxidation. Buffers were all degassed extensively with helium prior to use. The titration experiments were performed on an Omega Titration Calorimeter (MicroCal, Inc., Northampton, MA) with a Keithly preamplifier. All titration buffers were prepared at 200 mM, pH 7.0, 50 mM NaCl. Copper(II) chloride (Aldrich) stock solutions were prepared in buffer and then standardized by EDTA titrations. The concentration of apoazurin (in the syringe) ranged from 1.0 to 2.2 mM, while the concentrations of Cu(II) solution (in the calorimeter cell) ranged from 28 to 55 $\mu$M. Each titration began with a peptide titration into buffer for the purposes of determining dilution effects not related to the metal binding. Each titration used 4−6 nmol of apoazurin per titration point. The results given are the average from three titrations.

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(7) The kinetics derived were simulated using Berkeley Madonna (www.berkeleymadonna.com). Differential equations derived from Scheme 1 were integrated to yield concentrations for apoazurin, the intermediate, and Cu(II)−azurin as a function of time. These concentrations were used to calculate the expected heat released as a function of time by multiplication by the standard enthalpy changes for the two steps. These data were corrected for the time response of the calorimeter by smoothing the results with a 35 s sliding window. This time constant was determined by examination of buffer dilution titrations. The relatively slow time response limits the applicability of this approach to systems with relaxation times in the range of 10 s. The simulated results were compared with the observed data. The sum of the squares of the differences between the simulated results and the observed data was calculated using each of the 12 curves shown in Figure 2. This sum was minimized through variation of the four parameters. The standard deviations in these parameters were estimated by determining the change in each parameter necessary to double this sum.

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(13) The free energies of activation ($\Delta G^{\ddagger}$) were determined through the use of the transition-state theory equation $k = (k_BT/h)\exp(-\Delta G^{\ddagger}/RT)$. The standard state was assumed to be 1 M for the Cu(II) solution.

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