Kinetics of Copper(II) Uptake by Apoazurin in Complexing Media*

Judith A. Blaszak, David R. McMillin, Andrew T. Thornton, and David L. Tennent

From the Department of Chemistry, Purdue University, West Lafayette, Indiana 47907

We have studied the kinetics of copper uptake by apoazurin in imidazole and 1-methylimidazole buffers in the pH range of 7–9 with $\mu = 0.5$ M and copper(II) in large excess. The reaction has been monitored by measuring the visible absorbance and circular dichroism as a function of time. The uptake occurs in a stepwise fashion, and at least two intermediates are implicated. Overall, the rate of uptake varies inversely with the concentration of the proton and the (complexing) buffer, but depends directly on the copper concentration. A model involving a weakly absorbing intermediate is proposed to rationalize the data taken at the lower end of the pH range. According to the model the intermediate forms with a second order rate constant of about $30 \text{ M}^{-1} \text{s}^{-1}$ and is probably described as a ternary complex of copper, buffer, and one or more of the histidine ligands of the binding site. This then decays by a pH-dependent process to give product. At higher pH values there is evidence that relaxation to product occurs via a second intermediate form in which the cysteine ligand is bound to copper. The relevance of these results to the question of how copper is selectively incorporated into the protein is considered. Finally, a milder, more reliable route to the preparation of apoazurin is described.

Azurin is bacterial protein which is believed to function as an electron transferase. The protein contains a single copper ion bound in a distorted tetrahedral site through an $\text{N}_2\text{SS}^\text{a}$ donor set derived from the side chains of cysteine, methionine, and histidine residues (1, 2). Although various metals can bind in the site (3, 4), with the possible exception of iron, only copper could be expected to exhibit a reduction potential in the physiologically useful range. The intracellular mechanism by which copper is selectively inserted into the protein is not known. In general the incorporation of metal ions into proteins seems to occur post-translationally, as is well established for catalase (5) and cytochrome c (6). In several other cases there is evidence that the apoprotein is synthesized in the absence of the metal ion (7–10). Although many factors may be involved including transport phenomena, relative ion abundances, redox considerations, and compartmentalization, the selective uptake of a particular metal ion must ultimately be explained in terms of kinetics and/or thermodynamics (11). The interrelationship between the two factors is apparent from Equation 1, the simplest model of metal uptake,

$$M + A \rightleftharpoons H$$

where $M$ denotes the metal ion, $A$ the apoprotein, and $H$ the holoprotein.

In this simple case the formation constant is directly proportional to the on-rate constant and inversely dependent upon the off-rate constant. However, "equilibrium" is not necessarily achieved in vivo.

We became interested in the kinetics of metal uptake by apoazurin when we noted that under comparable conditions Cu(II) was taken up much more rapidly than Co(II), Ni(II), or Mn(II) (3). Given that a pseudotetrahedral geometry is rare for Cu(II) and that thioether donors are poor ligands for Cu(II) (12, 13), it seemed plausible that the disparity among the on-rates might play a significant role in copper selection. Previous workers have studied the kinetics of the Cu(II) uptake by apoazurin (14, 15) and by a related protein tellacyanin (16) and have identified a first order step which involves a rearrangement of the protein moiety (14, 16). A limitation of those experiments is that the initial state of Cu(II) was not controlled. Here we report detailed studies in the physiological pH range where complexing buffers have been used to define the speciation. Depending on conditions, one or more intermediates appear during uptake, and no single step in the process is uniformly rate-limiting. Our results imply that any intramolecular rearrangements of the protein which may occur are unlikely to affect the rate of metal uptake under physiological conditions where, in fact, the rate of the release of copper from storage may be the limiting factor. Another significant result is that a new, more reliable method of preparing apoazurin has been developed.

**EXPERIMENTAL PROCEDURES**

**Materials**—The bacterial culture of *Pseudomonas aeruginosa* was obtained from the American Type Culture Collection (strain no. 10145). Highly purified imidazole, grade III (low fluorescence blank), was purchased from Sigma, as was cysteine and histidine. Thiourea (Aldrich) and ammonium thiocyanate (Matheson, Coleman, and Bell) were purified by recrystallization from ethanol or simply by passage through a Chelex 100 (Bio-Rad) column. The 1,10-phenanthroline, 2,2'-bipyridine, diethyldithiocarbamate, and 1-methylimidazole were all purchased from Aldrich and used without further purification. NaClO₃ was made from reagent grade NaClO₃ and concentrated HClO₃. All other buffers and solutions were prepared with reagent grade chemicals and generally passed through a Chelex 100 column before use.

**Instrumentation**—Electronic absorption spectra were recorded on a Cary 17D or a McPherson EU-700D spectrophotometer. Most kinetic studies were run on the Cary 17D spectrometer equipped with a thermostatted cell compartment. Circular dichroic spectra and kinetics were recorded with the use of a Cary 61 CD spectrometer. A Hewlett-Packard 8450 A UV/VIS spectrophotometer was used to record time-resolved spectra. Stir-cool devices were purchased from Thermo-electrics, Unlimited, Inc., and ultratfiltration devices were obtained from Amicon. Fluorescence spectra and kinetics were recorded on a Perkin-Elmer MPF-44B fluorescence spectrophotometer.

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Temperature was controlled during the spectroscopic measurements using Lauda K-2/RD circulating constant temperature baths.

Methods—The bacteria were cultivated and native azurin was isolated as described previously (3). Apoazurin was prepared by a variety of methods, but generally using the following procedure. Native azurin was first reduced with ascorbate and then dialyzed against a solution of acetate or phosphate buffer (depending on the desired pH) containing 0.25 M NaCl and a 0.1 M concentration of a Cu(I) complexing agent for 12 h. A second dialysis of at least 12 h against 0.1 M phosphate buffer, pH 7, was required to remove the complexing agent and the copper complex. Protein purity was checked by UV spectral analysis. At this point the apoprotein was dialedyzed into the kinetics buffer. All dialyses were carried out in a fiber device under nitrogen, the temperature being controlled between 5 and 10 °C with a stir-cool unit.

Copper solutions for kinetic studies were made from a stock solution of CuSO₄, where the concentration was determined by the method of Felsenfeld (17), a NaClO₄ stock solution, and a weighed amount of buffer. A pKₐ value of 7.35 was assigned to the buffer in calculating ionic strengths. The ionic strength was controlled with NaClO₄, except in the case of 1-methylimidazole, where NaN₃ was used. The pH of each copper and each protein solution was adjusted using a microelectrode which was calibrated at the appropriate temperature by a strong acid/strong base titration at μ = 0.5 M (NaClO₄).

Kinetics were usually followed by measuring the change in absorbance at 626 nm after adding equal volumes of the buffered reagents to a small volume 1-cm cell. The solutions and cells were equilibrated to the appropriate temperature prior to mixing, and the solutions were mechanically stirred before starting data acquisition.

When the kinetics were simple first order, kobs, the experimental rate constant, was obtained from a plot of log[Ao - A(t)] versus t, the time. More complex kinetics, where an intermediate was involved, were modeled by numerically integrating the appropriate rate equations using an adaptation of the method of Gear (18).

RESULTS

Preparation of Apoazurin—During the initial stages of this work apoprotein was prepared by a variation of the procedure of Yamanaka et al. which, according to the authors, removes copper "by diaysis at 5 °C ... against 0.1 M phosphate buffer containing 0.5 M KCN, at pH 7..." (15). In order to contain HCN, we normally carried out the diaysis at room temperature in a fumehood. We assume that in fact Yamanaka et al. added KCN to a 0.1 M phosphate buffer, pH 7, yielding a final pH of approximately 11, since even a solution nominally containing 0.1 M H₃PO₃ and 0.5 M KCN exhibits a pH well above 7.

This procedure was abandoned when we found that the kinetics of copper(II) uptake varied depending on the number of times the protein was exposed to the cyanide solution. Moreover, as seen in Fig. 1B, the UV spectrum of the protein was affected by the procedure. After treatment, the protein absorbance was increased at 250 nm and the absorbance maximum which normally occurs around 280 nm was distinctly blue-shifted. Several other methods also proved unsatisfactory. Dialyzing oxidized azurin, Cu(I)Az, against histidine at pH 7 removed copper, but the method was slow and did not seem to produce apoprotein that could be readily reconstituted. Dialysis against diethyldithiocarbamate bleached the protein, but did not remove all traces of copper. Treatment of Cu(I)Az with 1,10-phenanthroline at pH 4 or 5 removed the copper, but the resulting apoprotein exhibited extraneous UV absorption, possibly due to retained 1,10-phenanthroline. A satisfactory method for copper removal was finally developed using thiourea at a pH around 4 or 5. The kinetics of Cu(II) uptake proved to be reproducible on recycling the procedure and the reconstituted protein exhibited a UV spectrum (Fig. 1A) which agreed with that of native protein.

Factors Affecting the Overall Kinetic Profile—Even qualitatively, the kinetics of Cu(II) uptake varied significantly with pH, temperature and, to a lesser extent, buffer concentration. At 20 °C or at lower pH there was a distinct lag phase in the kinetics (Fig. 2A). In contrast, at 37 °C or at a pH above about 8.5 the kinetics were generally biphasic (Fig. 2B). At 25 °C and at an intermediate pH the kinetics was simple and first order in the concentration of apoprotein (Cu(II) was in large excess). The pH at which first order kinetics was observed depended on the buffer system; with imidazole it occurred around pH 7 while in the case of 1-methylimidazole it occurred around pH 8.

For either buffer system the rate of Cu(II) uptake increased with increasing pH in the region of 7-9. While due to the complications described above the experimental rates cannot be rigorously described in terms of a single parameter, the log of the reciprocal half-life of reaction is found to depend inversely on log [H⁺], the slope being somewhat more positive than -1 (Fig. 3). The rate of uptake also increases monotonically as the temperature is increased from 20 to 37 °C, again with accompanying changes in the kinetic profile. In imidazole buffer with [H⁺] = 1.6 × 10⁻⁴ M and 1 mM total Cu(II), the pseudo-first order rate constant is 1.53 × 10⁻³ s⁻¹ at 25 °C and 9.6 × 10⁻³ s⁻¹ at 37 °C while the latter is calculated from the initial rate of the reaction. These results indicate the activation energy is approximately 120 kJ mol⁻¹.

Copper and Buffer Dependences—The copper dependence of the reaction has been studied in detail at 25 °C in μ = 0.5 M, 0.1 M imidazole buffer, pH 7. Based on the stability constants obtained from potentiometric data, we estimate that approximately 90% of the copper in these solutions exists as the tetrakis-imidazole complex, the only other species of significant concentration being the tris-imidazole complex. Under these conditions first order kinetics is obeyed, and the results are summarized in Table I. A plot of kobs, the observed rate constant, versus the concentration of Cu(II) is linear with a zero intercept (Fig. 4). The slope yields a second order rate constant of 0.97 M⁻¹ s⁻¹. We attempted to determine the copper dependence at higher temperatures as well but were unable to extract meaningful rate constants from the complex kinetic profiles.

The imidazole dependence of the reaction was examined under similar conditions except the Cu(II) concentration was maintained at 1 mM, the apoprotein concentration being held
Kinetics of Copper Uptake by Apozurin

Fig. 2. Absorbance (○) and log absorbance (△) data for the uptake of Cu(II) by apozurin. The total copper concentration was 1.0 mM and the protein concentration was around 20 μM in each case. The temperature was 25.0 °C and μ = 0.50 M. The lines are drawn to emphasize the deviations from first order behavior. A, The buffer was 0.10 M 1-methylimidazole, pH 7. B, The buffer was 0.10 M 1-methylimidazole, pH 9. The wavelength monitored was 626 nm.

Fig. 3 (left). A plot of the log of the reciprocal halflife (s⁻¹) of copper(II) uptake versus pH. The buffer is 0.10 M imidazole, copper concentration is 1.0 mM, μ = 0.50 M, and T = 25.0 °C.

Fig. 4 (center). A plot of kobs versus copper concentration. The data are taken in 0.10 M imidazole buffer, pH 7, μ = 0.50 M and T = 25.0 °C.

Fig. 5 (right). A plot of kobs versus reciprocal free imidazole concentration at pH 7 where the total copper concentration is 1.0 mM, μ = 0.50 M, and T = 25.0 °C.

around 2 × 10⁻⁵ M. The kinetics was simple pseudo-first order, except at 0.3 M (total) imidazole where the log plot was biphasic; the kobs values are presented in Table II. To analyze these results, we sought a relationship between kobs and [Im], the equilibrium concentration of free imidazole calculated from the pKₐ value after correcting for the amount of buffer which was complexed to copper. A double log plot of kobs versus [Im] had a slope of -1.25, suggesting an inverse dependence of an order greater than 1. As seen in Fig. 5, a plot of kobs versus [Im]⁻¹ could be fit to a curve of the form:

\[ k_{obs} = 1.07 \times 10^{-1}/[\text{Im}] + 1.78 \times 10^{-2}/[\text{Im}]^2 \]  

(2)
Further Spectral Studies—In an attempt to find evidence for possible reaction intermediates we measured time-resolved absorption spectra during copper uptake in 0.1 M 1-methylimidazole, pH 9 (Fig. 6). Under these conditions the absorbance change at 626 nm is biphasic. Note that the spectrum which is measured 0.2 min after mixing has an absorption maximum at approximately 600 nm and that the maximum shifts to longer wavelength as the reaction proceeds, finally ending at 626 nm, the wavelength maximum of native protein. These results indicate that an absorbing intermediate forms in route to the final product and that the spectrum of this species is slightly blue-shifted relative to native azurin.

We also followed the kinetics of Cu(II) uptake using circular dichroic spectroscopy at three different wavelengths: 467, 523, and 626 nm. First we followed uptake in 0.1 M 1-methylimidazole, pH 7, at \( \mu = 0.5 \) M with a copper concentration of 2 mM. Lag phase kinetics was observed at all three wavelengths, consistent with the absorption studies described previously.

In a similar buffer at pH 9 a biphasic response was observed at 467 and 626 nm, again consistent with the absorption studies; however, simple first order kinetics was observed at 523 nm (Fig. 7). A \( k_{obs} \) value of \( 2.6 \times 10^{-3} \) s\(^{-1}\) was calculated from the first order plot of the 523 nm data which agreed with the initial rates observed at 467 and 626 nm.

**TABLE I**

<table>
<thead>
<tr>
<th>[Cu] (mM)</th>
<th>( 10^3 k_{obs} ) (s(^{-1}))</th>
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<td>1.98</td>
</tr>
<tr>
<td>1.50</td>
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<tr>
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<td>1.02</td>
</tr>
<tr>
<td>0.50</td>
<td>0.94</td>
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**TABLE II**

<table>
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<th>[Im] (M)</th>
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<td>1.18</td>
</tr>
<tr>
<td>0.20</td>
<td>0.71</td>
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</table>

*Total imidazole concentration.

**FIG. 6.** Time-resolved spectra taken during copper(II) uptake in 0.1 M 1-methylimidazole buffer, pH 9, where the total copper concentration is 2.0 mM, \( \mu = 0.50 \) M, and \( T = 25.0 \) °C. The relative maximum around 460 nm reflects in part baseline curvature.

**FIG. 7.** Plots of the CD signal (●) and log CD signal (△) versus time for copper(II) uptake in 0.10 M 1-methylimidazole, pH 9. The total copper concentration is 2.0 mM, \( \mu = 0.50 \) M, and \( T = 25.0 \) °C. A, signal was measured at 523 nm. B, signal was measured at 626 nm.

**DISCUSSION**

While the use of complexing buffers has led to complicated kinetics, the conditions are of interest since proteins do not encounter a pool of "free" copper ions in the physiological milieu. From a more practical point of view these media allow us to avoid the formation of insoluble hydroxides and to monitor the kinetics under pseudo-first order conditions in copper. With copper in excess there is the complication that it may bind to multiple sites of the protein, but this problem should be minimized by the fact that the buffer competes for the metal ion. One of the most obvious effects of the buffer ligands is to slow copper uptake. This is not surprising since the requirement of dissociating the buffer would be expected to increase the overall activation energy. In accord with this reasoning we find that the uptake of copper(II) by apoazurin in 0.1 M imidazole buffer has an activation energy of \( \sim 120 \) kJ mol\(^{-1}\) or about 50% greater than that reported by Marks and Miller (pH?) who worked in weakly complexing media (14). The complications in our kinetics results can be attributed to reaction intermediates.

**Evidence for Intermediates**—Actually, since apoazurin is essentially a complicated multidentate ligand and since, as a rule, the latter attach to metals in a stepwise fashion (19, 20), the appearance of intermediate forms during the uptake process might be anticipated. In point of fact the lag phase, which
is observed in the absorbance change at low pH values, is most readily explained in terms of an induction period which is required for the build-up of a weakly absorbing intermediate complex of apoazurin and copper(II). An alternative explanation arises if the uptake is autocatalytic, but the introduction of remetalated azurin had no effect on the kinetics. Since the proposed intermediate exhibits no appreciable absorbance, it is unlikely that either the methionine or the cysteine sulfur is bound as intense charge transfer transitions would be associated with these chromophores (21, 22).

As has already been pointed out, there is direct evidence for another intermediate, one which absorbs significantly at 626 nm, in Fig. 6. The existence of this intermediate can explain the biphasic kinetics which are observed at higher pH. The first “phase” in the absorbance change is attributed to the formation of the absorbing intermediate, the second with its conversion to fully remetalated protein. From the fact that the absorption spectra of the intermediate and native azurin are very similar, we infer that the two copper environments are closely related and that, in particular, both involve the ligation of cysteine sulfur. Further evidence for this intermediate is found in the CD results since it is difficult to rationalize the first order kinetic plot at pH 9 except in terms of a sequential mechanism involving two species which have an isosbestic point at 523 nm. The agreement between the calculated first order rate constant and the initial rates which were obtained for the CD signals at 467 and 626 nm lends additional support to this interpretation. Note that even though the transition centered around 523 nm in the CD spectrum of azurin has been assigned to methionine sulfur (21), it does not follow that the methionine ligand is present because the cysteine bands are blue-shifted in the spectrum of the intermediate (Fig. 6). Since (at least) two intermediates are involved, there are too many parameters to attempt a complete mechanistic analysis, but we do gain valuable insight by attempting to model some of the kinetic data.

A Partial Kinetic Model—In the absence of a molar absorptivity for the absorbing intermediate we will ignore the high pH data and will only attempt to rationalize the transition from lag phase to first order behavior. In Scheme 1 we present the minimal reaction scheme which is consistent with the observed dependences on buffer, pH, and copper ion and which allows for the growth and decay of a weakly absorbing intermediate denoted as IH⁺. AH₂⁺ denotes apoazurin with two ligands protonated. The copper dependence shows up in Equation 5, and since Cu¹⁺L₄ is assumed to be the reactive form, the kinetics will reflect an inverse dependence on the buffer concentration via Equation 3. (Presumably a solvent molecule substitutes for the displaced buffer in Equation 3, but this will not affect the kinetic arguments and is ignored for notational convenience.)

Equations 4 and 6 of the scheme introduce a proton dependence. In principle the increase in the experimental rate of reaction with pH could reflect the formation of a reactive hydroxide complex of copper, but it is more reasonable to attribute it to the deprotonation of protein groups which subsequently bind to copper, e.g., as in Equation 4. Since there is no evidence to the contrary, we assume that the initial attack (Equation 5) involves a ligand of the copper binding site and leads to IH⁺. Since the sulfur ligands are excluded, the attachment presumably begins at one (or both) of the histidine ligands, His-46 or His-117. Note that the rate acceleration expected at higher pH values due to Equation 4 is offset to some extent by the fact that there are fewer protons available to compete for the ligand L. This means that less of the reactive copper species is available via Equation 3. For this reason and in order to account for the regular increase in rate as the pH increases in the range of 7–9 (which includes pH values well above the pK of a typical histidine) we also invoke the deprotonation of Cys-112 (Equation 6). Following this is the last step in the scheme, the formation of fully reconstituted azurin along with the release of any buffer which remains coordinated to copper (Equation 7).

If the rates involved in Equations 3, 4, and 6 are assumed to be rapid and these steps are treated as equilibria, there are five parameters to be defined. An estimate of K₁ is available from our own studies in imidazole buffer at μ = 0.5 where K₁ = 3.8 × 10⁻² M⁻¹. The other equilibrium constants have not been measured, but reasonable estimates suffice for our purposes. We let K₄ = 1.6 × 10⁻⁶ M, a value measured for the acid dissociation constant of one of the histidines of apoazurin (23), and K₅ = 4 × 10⁻¹⁰ M, a typical value for the deprotonation of a cysteine side chain (24). The two rate constants k₃ and k₄ have been varied systematically in order to fit the data presented in Fig. 8. Fair agreement with experiment is obtained using k₃ = 30 M⁻¹ s⁻¹ and k₄ = 0.3 s⁻¹. At pH 7 neither the k₅ step nor the k₆ step is rate-limiting, and as a result a slow build-up in the concentration of IH⁺ occurs (Fig. 8A). The concentration of IH⁺ is predicted to maximize about 20 min after mixing and there is a definite lag in the development of product. On the other hand, at pH 8 the rate of decay of IH⁺ is significantly greater, and the k₆ step becomes rate-limiting. Hence there is no appreciable build-up of IH⁺ and essentially first order kinetics are observed. Thus, the model nicely accounts for the shift from lag phase to first order kinetics with changing pH. It should be noted that our estimates of k₅ and k₆ are biased by a number of assumptions. For example, using a smaller estimate for K₅ would lead to a larger estimate for k₆. For illustrative purposes a representation of the basic reaction sequence, including the second intermediate, is given in Scheme 2 where, for the sake of simplicity, only four short segments of the peptide are depicted and protons are ignored. The absorbing intermediate is labeled as I2. An unidentified ligand X is included in the structure of IH⁺ to allow for the possibility of chelation to copper, vide infra.

Supporting Evidence for the Proposed Scheme—First we note that in broad outline the mechanism proposed above is consistent with observations regarding several related systems. Thus, if a weakly complexing buffer were used, this mechanism would, in the limit where the k₅ step is rate-determining yield a simple rate law consistent with the results of previous investigators (14, 16). Moreover, in our own studies of copper(II) uptake by apoaazurin we have observed lag phase kinetics when the copper(II) concentration exceeds about 0.5 mM in a 0.1 M imidazole buffer, pH 7. On the other hand, if the copper(II) concentration is held at 0.5 mM, lag phase kinetics occur when the imidazole concentration falls below about 0.02 M. In other words we see a deviation from pseudo-first order kinetics at high copper levels or low imida-
zole levels. These results are readily explained in terms of Scheme 1: pseudo-first order kinetics is observed as long as the $k_0$ step is rate-limiting, but at high copper(II) or low imidazole levels the kinetics reflects both the $k_0$ and the $k_5$ steps. The idea that an intermediate can build-up during uptake also finds support from studies of heme iron incorporation into cytochrome $c$ (25). Likewise, in studies of metal extraction from cobalt-substituted carbonic anhydrase, a ternary intermediate involving cobalt(II), protein, and a chelating ligand has been observed (26). While no intermediates have been detected during the uptake of zinc(II) by apocarbonic anhydrase, it has been suggested that the increase in the rate of zinc uptake with pH can be linked with the deprotonation of two sites of the protein (27), i.e. steps analogous to Equations 4 and 6 above. Marks and Miller (14) have independently proposed that the deprotonation of a histidine residue increases the rate of copper uptake by apozurin.

Another interesting aspect of the carbonic anhydrase study was that the observed second order rate constant was significantly smaller than those typical of the reaction of zinc(II) with small molecules (27). The same is true of our estimated $k_0$ value. As copper is found several angstroms beneath the surface of azurin (2), this may in part reflect the “buried” nature of the ligating groups. At the same time, the $k_0$ value is probably better viewed as an effective rate constant which reflects a more complex process than simple ligand replacement at copper. In particular, we suspect that the protein is chelated in some way to copper in IH'; otherwise, the formation of the intermediate could hardly be so favorable. This follows since the enthalpy associated with the replacement of a buffer molecule by an imidazole group in the binding pocket would, of itself, be small.

*Future Considerations*—A more detailed treatment of the kinetics of copper uptake in these media would have to consider: 1) the term in the rate law reflecting an inverse second order dependence on buffer concentration, 2) the inevitability of a $k_2$ step, 3) the possibility that the proton transfer steps cannot be treated as equilibria, 4) the fact that an absorbing intermediate is formed, and 5) the possibility that other intermediate forms and/or parallel paths are present.

One boundary condition is that, whatever magnitude $k_3$ may have, the $k_3/k_5$ ratio must be large in order to account for the level of IH' which forms. Additional insight which would facilitate analysis will probably come once the three-dimensional structure of the apoprotein is known. Supporting studies using kinetically inert metal complexes such as Pt(en) ($\text{OH}_2$)$_2^{2+}$ to label the point of initial attack might also prove helpful. A caveat for workers in this field is, as demonstrated by our results, that it is necessary to define carefully the state of the apoprotein. In particular one should check that consistent kinetics are observed after cycling through the reconstituted form.

We close with some remarks about copper selectivity. Our work reveals how strongly the kinetics of insertion—and by inference the effective binding constant of copper—depend on the initial state of the metal, and this conclusion is valid whether copper is taken up in its divalent or univalent form (28). In view of the building evidence that intracellular copper stores are tightly bound (29, 30), there may have to be a step...
in the chain of synthesis which is dedicated to the release of the copper stores, e.g. proteolysis of the storage complex. This point not withstanding, it would also seem to be required that the binding site of apoazurin be kinetically selective for copper since metal substitution reactions of the protein appear to be quite slow (3). It is widely held that the blue copper binding site is ideally constructed for its electron transfer function (1); the molecular engineering is the more remarkable if the same structure promotes the selective uptake of copper.

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