Kinetics of Copper Incorporation into an Engineered Purple Azurin

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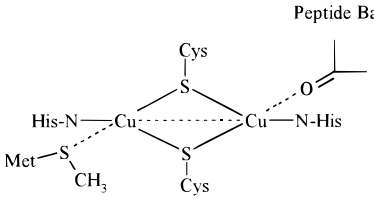
Purple Cu_A centers are mixed valence Cu_2S_2(Cys) units with each copper also coordinated to a histidine.[1] They mediate electron



transfer in both cytochrome *c* oxidase[2] and nitrous oxide reductase.[3] This new class of copper centers has been characterized by extensive spectroscopic[4−12] and X-ray crystallographic[13−15] work. Studies on water-soluble fragments containing Cu_A,[16−18] as well as synthetic[19−21] and protein analogues,[22−24] have shed light on the structure and function of purple copper centers. In our work, using loop-directed mutagenesis, we have engineered a purple copper center into a blue copper protein *Pseudomonas aeruginosa* azurin.[24] Comprehensive spectroscopic[7,25−27] and X-ray crystallographic[28] investigations of the engineered Cu_A center have demonstrated the striking similarity between purple azurin and native purple Cu_A centers. Studies of both blue and purple copper in the same azurin protein framework showed that Cu_A is the more efficient electron-transfer agent,[29] most likely because of lower nuclear reorganization in the delocalized binuclear structure.[7,10,30,31]

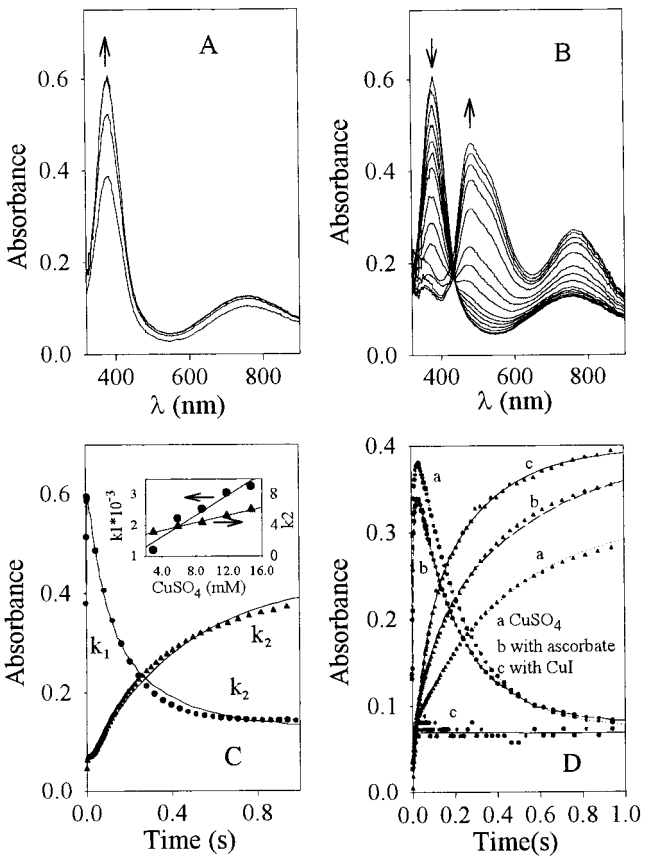


Figure 1. Kinetics of Cu^{2+} incorporation into the apo-form of engineered purple Cu_A protein before (A) and after (B) 10 ms, with absorbance change at (●) 386 nm and (▲) 485 nm shown in C. The final apo-protein concentration is 0.29 mM and $CuSO_4$ concentration is 2.9 mM. The insert in C shows the Cu^{2+} ion dependence of pseudo-first-order rate constants for intermediate (k_1) and product (k_2) formation. Panel D displays the effect of ascorbate and Cu^+ on Cu^{2+} addition, monitored at 386 nm (●) for intermediate formation and 485 nm (▲) for product formation: (a) addition of 2 equiv of $CuSO_4$ to apo-protein (The final apo-protein concentration is 0.18 mM); (b) addition of 1 equiv of ascorbate prior to the addition of Cu^{2+} in (a); and (c) addition of 1 equiv of CuI prior to the addition of Cu^{2+} in (a).

Even though much progress has been made in understanding the structure and function of Cu_A centers, the mechanism of copper ion incorporation is not known. In a previous paper,[24] we postulated that the free thiol groups of cysteines in some of the apo-proteins could serve as sacrificial reductants for the generation of Cu^+, which could then combine with Cu^{2+} to form the mixed valence center. Here we present the first kinetic study of copper incorporation into the apo-protein of the engineered purple Cu_A azurin using stopped-flow UV−vis spectroscopy. An interesting tetragonal Cu^{II}−S intermediate has been identified. A mechanism of copper ion incorporation is proposed, and its significance for the structure and function of Cu_A centers is discussed.

Addition of 10 equiv of $CuSO_4$ to 0.29 mM apo-Cu_A azurin resulted in a spectrum consisting of absorption around 386 and 765 nm, which developed within 10 ms with $k_{1obs} = 1.2 \times 10^3$ s^{-1} (Figure 1A).[32] Those absorption bands then decreased with concomitant increase of the final characteristic purple Cu_A

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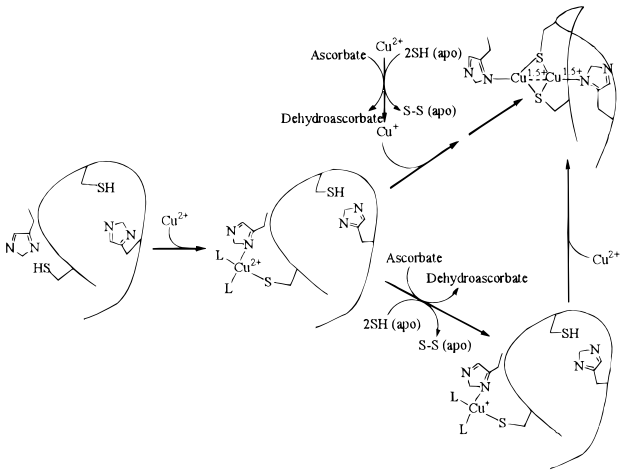
spectrum with absorption bands around 485, 530, and 770 nm (Figure 1B). The rate of absorption decrease at 386 nm is approximately the same as the rate of absorption increase at 485 nm ($k_{2obs} = 3.1\ s^{-1}$, Figure 1C), with an isosbestic point at 441 nm. These results indicate that an intermediate with absorption bands around 386 and 765 nm was formed first, which then converted to the final purple Cu_A center. Since the rate of intermediate decay and the rate of product formation were approximately the same, we used a kinetic model of A → B → C to fit our data. Figure 1C shows that the rates of both intermediate (k_1) and product (k_2) formation are linearly proportional to the concentration of added Cu^{2+} ions.

The absorptions at 386 nm ($\epsilon \sim 4200\ M^{-1}\ cm^{-1}$)[33] and at 765 nm ($\epsilon \sim 930\ M^{-1}\ cm^{-1}$)[33] for the intermediate are indicative of S-to-Cu^{II} charge transfer and d−d transition band, respectively, for a Cu^{II}−thiolate center in a tetragonal geometry.[34−37] The same intermediate and final product formation was observed when $CuSO_4$ was added to apo-protein in the presence of an external reductant such as ascorbate (data not shown). Interestingly, 1 equiv of ascorbate causes 11% less intermediate accumulation and 26% more product formation than the same reaction without any external reductants (compare Figure 1Da and 1Db).

On the basis of the above observation, we propose the following mechanism for the Cu(II) ion incorporation into apo-Cu_A azurin:



In this mechanism, Cu^{2+} is incorporated into the apo-protein to form an intermediate containing a tetragonal Cu^{II}−S(Cys) center and therefore displaying a strong S-to-Cu^{II} CT band at 386 nm. This intermediate is then converted to the final Cu_A center after Cu^+ is generated from reduction of Cu^{2+} either by free cysteines from nearby apo-proteins (which act as sacrificial reductants) or by external reductants such as ascorbate. Reduction of Cu^{2+} could occur either on the free Cu^{2+} or on Cu^{2+} in the intermediate. Since copper ions are involved in both intermediate and product formation, their rates (k_1 and k_2) should increase with Cu^{2+} concentration as observed. Since Cu^+ incorporation is a key step in intermediate conversion to product, the presence of external reductants such as ascorbate could accelerate this step, therefore reducing the intermediate accumulation. Consistent with this mechanism, the rate of product formation was found to be dependent on ascorbate concentration (see Supporting Information). More importantly, ascorbate allows it less likely to sacrifice apo-protein for Cu^{2+} reduction, making more apo-protein available for final product formation.

Going through an intermediate containing a tetragonal Cu^{II}−S(Cys) is not surprising because the tetragonal site is the preferred geometry for Cu(II) ions,[38] and this preferred geometry could be changed by the protein matrix to lower the reorganization energy during electron transfer.[7,10,30,31] Biphasic kinetics of Cu^{2+} incorporation into native blue copper azurin involving an intermediate that is very similar to the final blue copper center was also observed.[39] Furthermore, evidence for reduction of Cu^{2+} by the thiol group of Cys112 has been found during the study of unfolding of native azurin.[40,41] Consistent with the above mechanism, no intermediate accumulation and a 38% increase in final product formation was observed when 1 equiv of Cu^+ is added to apo-protein prior to Cu^{2+} addition (Figure 1Dc).

In summary, we have identified an intermediate containing a tetragonal Cu^{II}−S(Cys) center during the Cu^{2+} ion incorporation into apo-Cu_A azurin. Our study indicates that Cu^+, available directly or through reduction of Cu^{2+} by exogenous reductants, is important in copper ion incorporation into the Cu_A centers of cytochrome *c* oxidase and nitrous oxide reductase.

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Supporting Information Available: Figure S1 showing the relationship between the observed rate constants for the formation of the final product as a function of ascorbate concentration (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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