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A Designed, Redox Active Metalloprotein Which Models the Cu_{T2} site in Nitrite Reductase

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In this paper we describe the characterization of a redox active site in the *de novo* designed metallopeptide $Cu(TRIL23H)_3$ as a model of the Nitrite Reductase (NiR) enzyme, where both Cu(I) and Cu(II) are bound to the three histidines in the active site. Cu(I), bound to the $(TRIL23H)_3$ helix bundle with a 1:1 stoichiometry, is capable to bind CO with stretching frequencies remarkably close to those of the $Cu(I)(His)_3$ Type 2 sites of NiR and PHM enzymes. $Cu(II)(TRIL23H)_3^{2+}$ presents Vis absorption and EPR features which suggest a five coordinated $Cu(His)_3(H_2O)_2$ environment, and can be reduced to form $Cu(I)(TRIL23H)_3^{+}$ using ascorbate. The latter Cu(I) form can be then reoxidixed by addition of nitrite, and a Nitrite Reductase activity similar to the natural enzyme was demonstrated by recovery of the Cu(II) form and production of NO. Successive additions of ascorbate in presence of $Cu(II)(TRIL23H)_3^{2+}$ and an excess of nitrite allowed to cycle between the Cu(I) and Cu(II) form of the metallopeptide. The NiR activity showed a pH dependence similar to the enzyme, with maximum activity at pH 5.8 and negligible at pH 7.4. (181 words)

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Metalloproteins are involved in the most complex biomolecular processes in Nature. Copper metalloproteins are extraordinarily efficient molecular tools involved in many systems including biological denitrification pathways.[1] The catalytic conversion between nitrite (NO_2^-), nitrate (NO_3^-) and nitric oxide (NO) is often achieved using copper enzymes. Copper nitrite reductase (NiR) is a homotrimeric metalloenzyme which contains both Type 1 and Type 2 copper sites,

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carrying out dissimilatory reduction of nitrite to nitric oxide. The Type 2 copper is coordinated to three imidazoles and a water forming a distorted tetrahedral environment,[2-6] and it is the site where the catalytic conversion of nitrite into nitric oxide occurs ($NO_2^- + e^- + 2H^+ = NO + H_2O$). The electron necessary for the reduction of nitrogen from the (+3) to the (+2) oxidation state is provided by the reduced Type 1 copper, which in turn is reduced by a pseudoazurin.[7]

De novo designed metallopeptides provide a new approach for understanding the active site of metalloproteins, with the ultimate aim to construct new metalloproteins that reproduce the structure and function of native ones [8,9]. Two important examples of functional, de novo designed enzymes are the DueFerri (DF) and the zinc(II) TRI systems. The first utilizes Fe bound to a 4-helix bundle in a redox role to oxidize phenols,[10,11] while the second exploits Zn(II) to mimic very well the active site structure and hydrolytic chemistry of carbonic anhydrase, using a 3-stranded coiled coil.[12]

Among helix bundles containing copper binding sites, examples were reported of Cu(I)[13,14] and Cu(II)[15-22] peptides, but only a few examples of controlled binding of copper in $(His)_3$ binding sites are reported, [16,20,21] and only one refers to metal binding to a preassembled apo- three stranded coiled coil.[21] Surprisingly, none of these compounds were studied in terms of Cu(I)/(II) redox processes, which are the key reactions for the design of a functional copper redox protein.

In this paper we present the characterization of the *de novo* designed peptide Cu(**TRI**L23H)$_3^{+/2+}$ (**TRI**L23H=[Ac-G-(LKALEEK)$_3$(**H**KALEEK)-G-NH_2]). This peptide binds both Cu(I) and Cu(II) and, more importantly, forms a functional NiR Type 2 copper center. Thus, this metallopeptide represents the first example of a functional Cu(I)/(II) mimic of a catalytic Type 2 copper center embedded into a designed protein.

Results

The stability of (**TRI**L23H)$_3$ at pH 7.4 has been recently reported.[12] The three histidine residues are believed to bind to first row transition metals based upon the X-ray structure of the related system Hg(II)$_S$[Zn(II)$_N$(H_2O)](**CS**L9CL23H)$_3^+$. This parallel 3-stranded coiled coil contains a pseudo tetrahedral Zn(His)$_3$(H_2O) site (Figure 1). Since both Zn(II) and Cu(I) are d^{10} metals, we felt that the latter ion could be bound analogously as Zn(II) to the same $(His)_3$ site in (**TRI**L23H)$_3$. Therefore, we reacted the 3-stranded coiled coil with Cu(I) under inert atmosphere to avoid oxidation of the metal.

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^{1}H NMR Spectroscopy. The addition of 1 eq. of [Cu(I)(CH_3CN)$_4$]BF_4 to an oxygen-free solution of (**TRI**L23H)$_3$ in D_2O at pH 7.4 caused the shift of ^{1}H NMR signals of the imidazole CH from 6.25-7.0 to 6.5-9.5 ppm (all amidic protons are not observed due to deuterium exchange). A similar

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behavior was observed at pH 6.0; however, a different set of slightly broader CH resonances compared to those at pH 7.4 appear (see Supporting Information). In the presence of Cu(I), no peaks corresponding to those in absence of metal at the same pH were observed.

IR Spectroscopy. A buffered Cu(I)(**TRI**L23H)$_3^+$ solution (pH 7.4) was fluxed with CO generating a spectrum with a peak at 2063 cm^{-1} (half-height width of 14 cm^{-1}) which disappears after sparging the solution with N_2 for 15 minutes (Supporting Information). This peak, in the region of the CO stretching frequency, is the only detectable absorption in the 2140-1700 cm^{-1}.

Visible and EPR spectroscopy. The addition of 1 eq. of copper(II) chloride to (**TRI**L23H)$_3$ at pH 7.4 (unbuffered) produced a blue solution with a single absorption band at 640 nm (ε = 132 M^{-1} cm^{-1}) associated with the copper(II) *d-d* transitions. Solid HEPES added to this solution to obtain a 200 mM HEPES buffer at pH 7.4 did not produce any significant change in the visible spectrum. An analogous behavior was observed in 200 mM MES buffer at pH 6.0, and the visible spectrum is not different from that at pH 7.4 (λ_{max} = 642 nm, 143 M^{-1} cm^{-1}, Figure 5). The X-band EPR spectrum at pH 7.4 presents typical Type 2 Cu(II) features ($g_{//}$ = 2.28 and $A_{//}$ = 166 G, Supporting Information), the $A_{//}$ value slightly larger than that observed for Type 2 centers in NiR enzymes (95-150 G).[4,23,24] The binding of Cu(II) to (**TRI**L23H)$_3$ was also studied using the metallochromic indicator pyrocatechol violet by visible competition titrations with the three stranded coiled coil yeilding a conditional log $K_{binding}$= 6.22(1) (K_d = 0.60(1) μM, Supporting Information) to the 3-stranded coiled coil..

Study of NiR reactivity. The reduced Cu(I)(**TRI**L23H)$_3^+$ form of the peptide could be obtained *in situ* by reduction of the Cu(II) peptide upon addition of (1 eq.) sodium ascorbate to a deoxygenated, MES buffered solution of Cu(II)(**TRI**L23H)$_3^{2+}$ at pH 5.8. The diappearance of the band at 640 nm occurred within the mixing time. No visible bands appeared during the next 24 hr after sample preparation with the sample stored in a rubber sealed quartz cuvette (Figure 2 left).

The Nitrite Reductase activity of the Cu(I)(**TRI**L23H)$_3^+$ was investigated by monitoring both the oxidation of copper(I) to copper(II), and by the production of NO through reduction of nitrite. The anaerobic oxidation of Cu(I)(**TRI**L23H)$_3^+$ to Cu(II)(**TRI**L23H)$_3^{2+}$ in the presence of an excess of nitrite (100 eq.) could be followed by addition of sodium ascorbate (1 eq.) which initiated the reaction by reducing the metallopeptide to Cu(I)(**TRI**L23H)$_3^+$ and to. Upon addition of ascorbate, the color and the absorption spectrum of the solution immediately disappeared, and progressive absorbance recovery was measured over time by collection of the visible spectrum every 3 min (Figure 2 center and right). The absorbance recovery at λ_{max} = 640 nm reached a plateau after ca. 50 min. After 70 min, 77 % of the initial absorbance was recovered. Three further additions of sodium

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ascorbate (1 eq. each) were made to the same sample. Absorbance recoveries of 74, 69 and 65 % of the initial sample were obtained at 640 nm 70 min after each addition (Figure 3).

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The NO produced by the reaction between Cu(I)(**TRI**L23H)$_3^+$ and nitrite (1:1) was stripped out of the reaction vessel by a nitrogen stream, and trapped into a quartz cuvette containing an [Fe(II)(EDTA)]$^{2-}$ solution. The NO produced could be quantitated by the intensity of the band at 432 nm of [Fe(NO)(EDTA)]$^{2-}$ (ε = 780 M^{-1} cm^{-1}).[25] The production of NO in the reaction of Cu(I)(**TRI**L23H)$_3^+$ with nitrite at pH 5.8 was demonstrated using both native Cu(I)(**TRI**L23H)$_3^+$ (obtained by addition of $[Cu(CH_3CN)_4]^+$ to (**TRI**L23H)$_3$) or by ascorbate reduced Cu(II)(**TRI**L23H)$_3^{2+}$. The recovered NO for native and reduced Cu(I) peptide respectively after 1 hr was 71 % and 47% of the control reaction of nitrite with $[Cu(CH_3CN)_4]^+$ (Figure 3).

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To determine the pH dependence of the NiR reaction, absorbance recovery experiments were carried out at pH 6.0, 6.5, 7.0 and 7.4 in 200 mM MES buffered solutions. The rate of reoxidation of copper(I) decreases as the pH increases, as shown in Figure 5. While at pH 6.0 the absorbance at 640 nm reaches a plateau in ca. 80 min and 68 % absorbance recovery, no plateau is reached at pH 6.5. For the latter sample, 38 % of initial absorbance is recovered in 80 min, while only 18 % recovery is observed at pH 7.0. At pH 7.4, no significant increase of absorbance starting from the spectrum immediately after addition of ascorbate is observed in 2 h. No activity was observed at pH 7.4 either using 200 mM HEPES or an unbuffered sample.

The pseudo-first order rate constants for the NiR reaction (k_i) were calculated from initial rates between pH 5.8 and 7.0 using the absorbance values at 640 nm (first 12-15 min of reaction). The k_i values resulted 3.073(6), 1.697(6), 0.4288(8) and 0.128(1) min^{-1} at pH 5.8, 6.0, 6.5 and 7.0, respectively. The pH dependence of the k_i values was analyzed as $\log_{10}(k_i) = a' - b\cdot$pH. The resultant a' and b parameters are 7.1(2) and 1.15(3), respectively.

Discussion

De novo metallopeptide design has as an objective the preparation of systems which exhibit structural, physical and functional similarities to known metalloenzymes. These synthetic constructs serve as a bridge between native systems which may be difficult to interogate because of their complexity and small molecule models whose utility may be diminished by the simplicity of the structure which may not allow for complete or accurate reproduction of the desired structure or chemical activity. For example, several copper complexes with nitrogen ligands as mimic for the Type 2 copper site of NiR were synthesized in recent years;[25-38]however, their use in the study of NiR activity has drawbacks such as poor solubility and poor control of the metal coordination environment. Copper nitrite reductase itself is complicated by having two distinct copper centers with different function and physical properties. *De novo* designed metallopeptides are soluble in

water, can enforce lower metal coordination spheres, inhibit unwanted dimerization while providing a hydrophobic environment that contains a single metal co-factor at which the desired chemistry may be performed. In a recent study, we have shown that a *de novo* design strategy is highly effective for providing mimicks of carbonic anhydrase using a mononuclear Zn(II) based hydrolytic system. In this report we explore a *de novo* designed redox based assembly in both the reduced and oxidized forms of a functional system exhibiting behavior similar to the Cu_{T2} site in Nitrite Reductase.

We felt that Cu(**TRI**L23H)$_3$ was an ideal system for these studies since the structure of Zn(II) bound to three histidines in the related designed peptide Hg_SZn_N(**CS** L9CL23H)$_3$ was known.[12] A comparison of the Zn(II) ion to that of Cu(I) in Cu_{T2}NIR is shown in Fig. 1. The overlayed metal sites show that the metal ion enzvironments are well matched and, in both structures, the metal ions are coordinated to the imidazole N_ε. A water molecule occupies the fourth coordination position, to give a tetrahedral environment in both cases which is slighlty distorted from that observed in NiR.

Copper site characterization. With this structural analogy as a starting point we believed that **TRI**L23H would be an excellent scaffold for building a Nitrite Reductase mimic. We first characterized the apo-(**TRI**L23H)$_3$ in D_2O using ^{1}H NMR in order to identify the non-exchangable C-H_δ and C-H_ε protons of the imidazole ring. The two major NMR singlets at pH 7.4 (Supporting Information) corresponding to the H_ε and H_δ protons of chemically equivalent imidazole rings. This spectrum is consistent with the presence of solely parallel three-stranded coiled coils in agreement with previous reports for TRI peptides at pH > 5.5.[39,40] Evaluation of the H_ε and H_δ protons as a function of pH supports the conversion of imidazolium to imidazole conversion over the pH range ??-??. The addition of 1 eq. of Cu(I) at pH 7.5 produces a change in the NMR spectrum with the appearance of multiple peaks in the range 6.6 - 8.7 ppm, which proves the coordination of Cu(I) at the imidazole site. The formation of Cu(I) 2-stranded coiled coils is ruled out by the absence of residual signals of the apo peptide, and an analogous behavior is observed at pH 6 (Supporting Information). At pH 6, both the spectra of the apo- and Cu(I)(**TRI**L23H)$_3^+$ present different set of NMR signals compared to pH 7.4, although the formation of 2-stranded coiled coils is again ruled out by the absence of residual signals of the apo peptide. The presence of multiple peaks in the spectra of Cu(I)(**TRI**L23H)$_3^+$, likely is the result of the presence of different similar energy conformations at the Cu(I)(His)$_3$ site, an observation consistent with the Cu(I) adduct of the 1-42 fragment of β-amyloid.[41]

The stretching frequency *v* of CO in the adduct with Cu(I)(**TRI**L23H)$_3^+$ is 2063 cm^{-1}, a value extremely close to those found for CO adducts with reduced PHM (2062-2075 cm^{-1}) and NiR (2050 cm^{-1}).[42] These values are indicative of a CO bound to an electron rich Cu(I)(His)$_3$ site,[43]

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matching these stretching frequencies better than any previously reported compound. These observations suggest that the imidazole groups embedded into Cu(I)(**TRI**L23H)$_3^+$ model very well the Cu(I)(His)$_3$ environment of the reduced form of NiR. .

We next characterized the oxidized form of the designed peptide. The visible spectrum of Cu(II)(**TRI**L23H)$_3^{2+}$ exhibits a single absorption maximum at 640 nm (ε = 132 M^{-1} cm^{-1}) which is assigned to the Cu(II) *d-d* transitions. The absorption maximum and extinction co-efficient are consistent with a "Cu(His)$_3$" site that could contain either one or two exogenous water ligands (predicted λ_{max} of 634 ± 11 nm [44]). The stability of the Cu(II)(His)$_3$ site as a function of the pH is proved by the visible spectrum which is almost identical at 7.4 in presence and absence of HEPES buffer, and at pH 6 in MES.

The EPR spectrum of Cu(II)(TRIL23H)$_3^{2+}$ has features typical of Type 2 copper centers. The observed g values ($g_{//}$ = 2.28) and hyperfine coupling constants ($A_{//}$ = 166 G) are somewhat larger than observed for NiR and PHM where a Cu(His)$_3$(OH$_2$) site is present. These parameters seem therefore more consistent with a 5 coordinate structure of the type Cu(His)$_3$(OH$_2$)$_2$, [28,45] and a distorted square pyramidal environment with three *quasi* in-plane imidazoled is also consistent with the observed *d-d* transition at 640 nm. Thus, we conclude that Cu(II) binds tightly to the three histidines, but that the coordination sphere most likely contains 5 rather than 4 ligands.

Before examining the reactivity of this system it was important to determine the affinity of the peptide for the Cu(II) ion and to estimate the redox potential of the system. The quantitative log $K_{binding}$= 6.22(1) (K_d = 0.60(1) μM indicates that > 97% of Cu(II) is bound to (**TRI**L23H)$_3$ for a 0.3 mM Cu(II)(**TRI**L23H)$_3^{2+}$ solution. Since we now have determined the binding constants for the Cu(I) and Cu(II) forms of the peptide we can estimate the reduction potential for this system at pH ??.

Having determined that Cu(I) and Cu(II) bind to the three histidines of (**TRI**L23H)$_3$ strongly, we next assessed the reactivity of these metal centers. As expected based on our predicted reduction potential, Cu(II)(**TRI**L23H)$_3^{2+}$ can be quickly reduced to Cu(I)(TRIL23H)$_3^+$ by addition of sodium ascorbate, as shown by the disappearance of the absorption *d-d* band of Cu(II)(**TRI**L23H)$_3^{2+}$ within the mixing time (Figure 2). We therefore decided to investigate nitrite reductase activity of Cu(I)(**TRI**L23H)$_3^+$, by assessing the production of NO and the possibility of cycling between the Cu(II) and Cu(I) forms of the metallopeptide in presence of the reductant (ascorbate) or oxidant (nitrite).

The kinetics of oxidation of Cu(I) was investigated by monitoring the appearance and increase over time of the *d-d* band of Cu(II) at 640 nm in samples containing ascorbate-reduced Cu(II)(**TRI**L23H)$_3^{2+}$ and nitrite in 100-fold excess (Fig. 2). The absorbance of the Cu(II)

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metallopeptide is only slightly higher in presence of an excess of nitrite, but at the same wavelength (640 nm, Figure 2). At pH 5.8, the recovery of the initial absorbance reached in 70 min and corresponding to a 77 % recovery can be accounted by the accumulation of NO in solution which promotes the stabilization of Cu(I) by virtue of the Cu(II) + NO = Cu(I) + NO_2^- + H^+ equilibria, as demonstrated for nitrite reductase, or by reacting with Cu(I). We, therefore, put forward the hypothesis that a stabilization of the Cu(I) form produces a ¾ total copper(I) reoxidation by nitrite. This seems confirmed by the three successive ascorbate additions to the same sample, where absorbance recovery diminishes to 65 % of the initial intensity at the fourth recovery cycle.

The development of NO by reaction of Cu(I)(**TRI**L23H)$_3^+$ with 1 eq. of nitrite at pH 5.8 was demonstrated by trapping the gas into a colorless $[Fe(EDTA)]^{2-}$ solution in citrate buffer to form $[Fe(NO)(EDTA)]^{2-}$.[25] The production of NO was observed at pH 5.8 starting both from native Cu(I)(**TRI**L23H)$_3^+$ (obtained by the Cu(I) precursor) and from Cu(II)(**TRI**L23H)$_3^{2+}$ that was reduced with ascorbate. After 1 h the amount of trapped NO is 71 and 48 %, respectively, of that from the control reaction using $[Cu(I)(CH_3CN)_4]^+$. Both the recovery of the Cu *d-d* absorption and the produced NO are not quantitative; however, these results demonstrate that both the oxidized metal site and NO are products of the reaction of Cu(I)(**TRI**L23H)$_3^+$ with nitrite. These data in concert demonstrate that we have made a functional mimic of the nitrite reductase reaction.

Finally, the NiR activity showed a pH dependence in the 5.8-7.0 pH range, the activity being higher at lower pH as found for the NiR enzyme.[46,47] This dependence of the pseudo-first order rate constant is linear with the pH, with a slope factor of 1.15(3) which suggests a first order dependence on $[H_3O^+]$.

Conclusions

The copper metallopeptide Cu(TRIL23H)$_3$ exist in two oxidation states with respect to the metal (+1 and +2). The possibility to cycle between these two oxidation states by addition of a reductant (ascorbate) or an oxidant (nitrite) provides a ground for these metallopeptides to be studied as models for Cu T2 in Nitrite Reductase. The reaction between the reduced Cu(I) form and nitrite leads to the evolution of nitric oxide, and the production of Cu(II)(**TRI**L23H)$_3^{2+}$. The nitrite reductase activity is strongly dependent on the acidity of the medium. While a significative activity was observed at pH 5.8, the metallopeptide does not exhibit a significant activity at pH 7.4. The initial rate of reaction is follows a first order dependence on the proton concentration and is consistent with the presence of a proton dissociation equilibria involving the free enzyme which affects the rate of reaction. The NMR and visible spectrophotometric characterization of the Cu(I)

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Comment [v23]: I think this section needs to be expanded. I would not say a dependence on H3O+ but on proton. We do not know whether it is water, a protonated substrate, a protonated protein ligand, a bound water, etc. We also at some point need to actually compare our peptides to NiR. Where have we succeeded, where have we failed to mimic the enzyme. This should also be discussed in the context of this pH dependence of activity. Is the change in activity due to change in our estimated potentials as a function of pH? I doubt it, but we need to address that this is really proton dependent for the reaction rather than simply changing the redox potential. Also, we need to point out that these reactions are slow (how much compared to the enzyme?) and that by lowering the redox potential we should be able to accelerate the rate. Can we say anyhting with respect to previous model studies? Are there any in water which we can compare? If so, what are their rates? Their pH dependencec? If there are no water soluble reactions with small molecules that would be fantastic. Then we would tout that and compare to reactions in non-acqueous solutions. Part of our justfication for doing this is that these should be better than scorpionates or other systems.

and Cu(II) forms of Cu(**TRI**L23H)$_3$ prove that in both forms the metal is coordinated to the imidazoles of the histidine residues. While the visible spectrum of the oxidized form suggests that Cu(II) is always (His)$_3$ coordinated in the range 6 – 7.4, differences in the proton NMR spectrum suggest that a change in the Cu(I) coordination of Cu(I) occurs in the same pH range. This behaviour has been interpreted in a possible change from di- to tricoordination of the copper(I) ion in the active site, to which corresond a different nitrite reductase activity.

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Experimental

General procedures

^{1}H NMR spectra were collected on a Varian MR400 spectrometer using gastight tubes where appropriate. The pH values were registered using Hamilton glass microelectrodes. The pH values in D_2O were corrected using the formula reported in the literature [48]. $[Cu(CH_3CN)_4]BF_4$ was synthesized as reported in the literature,REF. Oxygen-free aqueous solutions were prepared in the glove box using doubly distilled water sparged with an oxygen-free nitrogen stream for 6 hr. IR spectra were collected on a Perkin Elmer Spectrum BX FTIR spectrometer, using ZnSe cells. Visible spectra (400-900 nm) were collected on a Varian Cary 100 UV-Vis spectrophotometer provided with a thermostatting device, using matched quartz cells of 1 cm path length. EPR spectra were collected on a Bruker EMX X band EPR spectrometer provided with a cryostatting device.

Peptide Synthesis and Purification. **TRI**L23H (AcG-[LKALEEK]$_3$HKALEEKG-NH$_2$) was synthesized on an Applied Biosystems 433A peptide synthesizer using standard protocols [49] and purified and characterized as reported [50]. Solutions of the apopeptide were prepared by weight in doubly distilled water or in buffered solutions were appropriate.

NMR spectroscopy. Samples of (**TRI**L23H)$_3$ were prepared in 300 uL D_2O (*ca.* 9 mM). Sodium trimethylsilylpropanesulfonate (TSP) was added as the internal reference. The pH was corrected to 6 or 7.4 by addition of small aliquots of a NaOD solution in D_2O. Samples of Cu(I)(**TRI**L23H)$_3^+$ were prepared adding a proper amount of $[Cu(CH_3CN)_4]BF_4$ (stock solution) to a solution of (**TRI**L23H)$_3$ in D_2O (*ca.* 6.6 mM, 300 μL) at pH 6.0 or 7.4 (obtained adding NaOD solutions) previously sparged with a gentle flux of N_2 for 3 h.

IR Spectroscopy. IR samples were prepared in the glove box, using a deoxygenated 200 mM HEPES buffered D_2O solution (pH 7.4). A 5.9 mM solution of Cu(I)(**TRI**L23H)$_3$ (300 μL) was prepared in a rubber sealed glass vial by reacting **TRI**L23H (5.31 10^{-3} mmol) and $[Cu(CH_3CN)_4]BF_4$ (1.77 10^{-3} mmol from the stock solution. The final acetonitrile concentration in the samples was less than 4 %. The solution was sparged with 1 atm $CO_{(g)}$ for approx. 10 min. A 250 μL aliquot was injected through rubber septa into a previously CO-flushed solution IR cell with

ZnSe windows and the IR spectrum recorded. To check for reversibility of CO binding, the solution in the IR cell was re-transferred into the vial, the soluton sparged with N_2 for 15 min., then the IR spectrum collected. Spectra recorded were the average of 64 scans, 2 cm^{-1} resolution. The buffered D_2O solutions were used to collect background spectra.

Visible spectroscopy. Cu(II)(TRIL23H)$_3$ solutions (1.10 mM) at pH 6.0 or 7.4 (unbuffered) were prepared by mixing proper amounts of aqueous $CuCl_2 \cdot 2H_2O$ (*ca.* 0.03 M) and aqueous (TRIL23H)$_3$ solution (1.14 mM). The pH was corrected to 6.0 or 7.4 using small aliquots of conc. NaOH. Solid MES or HEPES were added to the solution to obtain a 200 mM solution, and the pH corrected to 6.0 or 7.4, respectively. The spectra were collected before and after buffer addition. The *in situ* reduction of Cu(II)(TRIL23H)$_3^{2+}$ into Cu(I)(TRIL23H)$_3^{+}$ was monitored by addition of sodium ascorbate (0.565 µmol, 1 eq.) to a 0.34 mM Cu(II)(TRIL23H)$_3^{2+}$ solution in deoxygenated 200 mM MES buffer (pH 6.0) obtained in 3 mL in a similar way as described above, in a rubber sealed quartz cuvette.

Binding constants determination: The binding constants of Cu(II) to (**TRI**L23H)$_3$ was determined at pH 7.4 by competition methods using pyrocatechol violet (PV) as the metallochromic indicator (50 mM MES). Cu(II)-indicator stability constants were determined by visible spectrophotometric titrations of PV with a Cu(II) solution at pH 7.4 (C_{PV} = 15.3 µM, Cu:PV = 0-3). Cu(II)- (TRIL23H)$_3$ binding constants were determined by back titration of Cu(II):PV = 1.78:1 solutions (C_{Cu} = 25.5 µM) with a 242 µM (TRIL23H)$_3$ solution up to Cu(II):(**TRI**L23H)$_3$ = 1. The visible spectra were treated with the Hyperquad 2006 program.

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EPR spectroscopy: The Cu(II)(**TRI**L23H)$_3$ solution (1.67 mM) was prepared by mixing proper amounts of aqueous $CuCl_2 \cdot 2H_2O$ (*ca.* 0.04 M) and a (**TRI**L23H)$_3$ solution (1.74 mM, 200 mM HEPES, pH 7.4). The spectra were registered at 77 K.

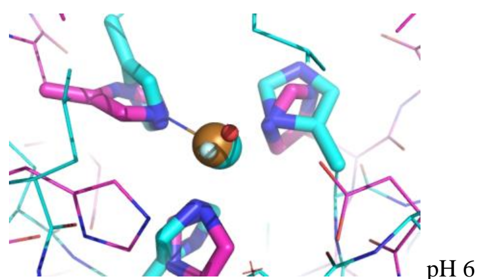
Nitrite reductase activity - NO production. Cu(I)(**TRI**L23H)$_3^{+}$ solutions (2.26 mM, 500 µL, 200 mM HEPES, pH 6.0) were prepared in rubber sealed vials by mixing 3.39 mmol of the solid peptide and 1.13 mmol of $[Cu(CH_3CN)_4]BF_4$ from the stock solution. A 0.01 M $[Fe(EDTA)]^{2-}$ solution was prepared from $FeSO_4 \cdot 7H_2O$ and H_2EDTA in deoxygenated 1 M citrate aqueous buffer at pH 5.0. The latter solution (3 mL) was put in a rubber sealed quartz cuvette, the spectrum registered, and then the cuvette connected to the peptide vial through a steel canula and kept in an ice bath at 0 °C throughout the entire experiment. The NiR reaction in the first solution was initiated by the addition of 1.13 10^{-3} mmol of $NaNO_2$ (from a 58.6 mM solution in 200 mM MES pH 6.0) to the copper/peptide solution, using a gas tight syringe. The produced NO was stripped out from the first vial through a gentle oxygen-free N_2 stream, and trapped in the cuvette as $[Fe(NO)(EDTA)]^{2-}$. The spectrum was collected after 1h. The NO produced was quantitated from the difference spectrum

($\varepsilon_{432\ nm}$ = 780 M^{-1} cm^{-1} for $[Fe(NO)(EDTA)]^{2-}$)[25]. The control reaction was performed using naked $[Cu(CH_3CN)_4]BF_4$, at the same conditions described above.

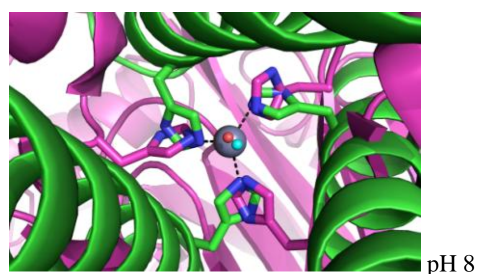
The production of NO starting from Cu(II)(**TRI**L23H)$_3^{2+}$ reduced *in situ* with ascorbate was performed using the same condition described above (200 mM MES pH 6.0). $CuCl_2 \cdot 2H_2O$ (1.13 mmol, *ca.* 0.07 M in water) was added to the peptide, followed by sodium ascorbate (0.565 mmol). All solutions were prepared in a glove box.

Nitrite reductase activity – Cu(I) oxidation. In the glove box, solutions of Cu(II)(**TRI**L23H)$_3^{2+}$ (0.33 mM, 3 mL, 200 mM MES at different pH) were prepared in a rubber sealed quartz cuvette from solid peptide, $CuCl_2 \cdot 2H_2O$ (*ca.* 0.07 M) and $NaNO_2$ (*ca.* 0.5349 M) solutions. Final Cu(II):(**TRI**L23H)$_3$:NO_2^- = 1:1:100. The reaction was initiated by addition of 1 eq. of sodium ascorbate. Aqueous MES buffer solutions (200 mM) at pH 6.0, 6.5, 7.0 and 7.4 were used, the pH corrected using deoxygenated aqueous NaOH (0.1 M) or $KHSO_4$ (0.5 M) solutions.

Calculations. Least square regression calculations were performed using SPSS 16.0.[51] The absorbance values of the *d-d* bands at 640 nm for the first 5 spectra collected for each nitrite reductase activity run were used in the calculations of the initial rates. The percentage of recovery of the initail sample absorbance at time t ($A^{640\ nm}_{t}$ / $A^{640\ nm}_{initial} \times 100$) was used for the calculations, where $A^{640\ nm}_{initial}$ is the absorbance at 640 nm of the sample prior the addition of ascorbate.



pH 6



pH 8

Figure 1. Overlayed representation of the X ray structures of the Zn(II) site in Hg_SZn_N(**CS L9CL23H**)$_3$ (green) and the Type 2 Cu(II) site in Nitrite Reductase (magenta, PDB 2dy2). Zn(II) is represented in gray and it is almost superimposed with Cu(II). The cyan and red spheres represents the coordinated water molecules to Zn and Cu, respectively.

Comment [v26]: We have a new nomenclature for this. I will ask Melissa to send you the final version of the paper so that you can modify this appropriately.

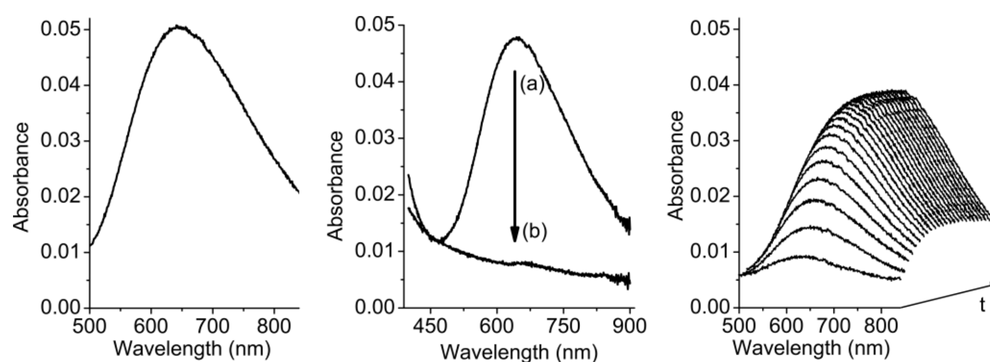


Figure 2. Left: Cu(II)(**TRI**L23H)$_3^{2+}$ (0.34 mM) before (a) and after (b) the addition of 1 eq. of sodium ascorbate (200 mM MES, pH 5.8). Center: Cu(II)(**TRI**L23H)$_3^{2+}$ (0.33 mM) in presence of 100 eq. of sodium nitrite (200 mM MES, pH 5.8). Right: Absorbance recovery after addition of 1 eq. of sodium ascorbate to the previous solution (3 min between each scan).

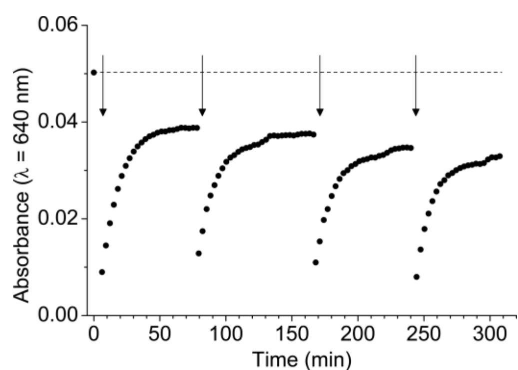


Figure 3. Absorbance recovery at 640 nm of Cu(II)(**TRI**L23H)$_3^{2+}$ (0.33 mM) in presence of 100 eq., reduced with 1 eq. of sodium ascorbate in correspondence of the arrows (200 mM MES, pH 5.8). The straight line indicates the absorbance of the initial sample.

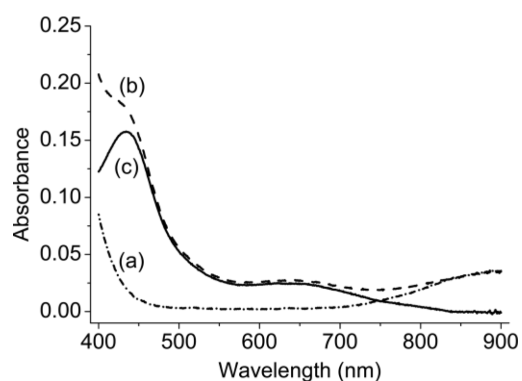


Figure 4. Spectra of $[Fe(EDTA)]^{2-}$ (0.01 M in buffer citrate 1 M, pH 5) before (a) and after (b) the reaction with NO evolved by the reaction of Cu(I)(**TRI**L23H)$_3^{+}$ with 1 eq. of sodium nitrite at pH 6.0. Spectrum (c) is the difference spectrum of the two previous spectra.

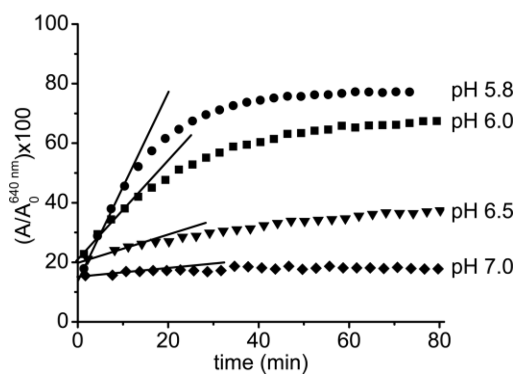


Figure 5. Relative recovered absorbance over time at 640 nm of the solution of Cu(II)(**TRI**L23H)$_3^{2+}$ (0.33 mM) in presence of 100 eq. of sodium nitrite after addition of 1 eq. of ascorbate (200 mM MES). Slope of the straight lines is the initial rate (v_i).

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