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Observation of an Unprecedented Cu Bis-His Site: Crystal Structure of the H129V Mutant of Nitrite Reductase

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Copper nitrite reductases contain both an electron-transfer type 1 Cu site and a catalytic type 2 Cu site. We have mutated one of the type 2 copper ligating histidines to observe the effect on catalytic turnover. This mutation has created a unique site where Cu is ligated by 2 His Nϵ2 atoms alone.

Bis-His Cu compounds are known in two forms, where the preferred tetracoordination is maintained by coordination to either the amino or carboxylate groups in addition to the Nϵ2 atoms of the histidines.[1] A study by Hirasawa and coworkers[2] on a Cu(II) doped single crystal of L-histidine hydrochloride monohydrate incorporated the Cu ion but also included water, carboxylate, and chloride ions.[2] Cu(I) dimers have been reported where a Cu(I) dimer has been synthesized with both two- and four-coordinate sites;[3] again the Cu is ligated by the ring imidazole N atoms and a main chain N atom. We have observed in a high resolution (1.9 Å) protein crystal structure a novel bis-His Cu site where the T2Cu of native nitrite reductase has lost two ligands upon mutation forming a truly two-coordinate Cu site with ligation from only His Nϵ2 with no additional interactions.

Copper-containing dissimilatory nitrite reductases (CuNiRs) have been isolated and characterized from a number of bacterial sources.[4−7] CuNiRs contain both a type 1 copper site, presumed to mediate electron transfer, and a type 2 site ($Cu(His)_3 \cdot H_2O$) where catalytic turnover occurs.[8] CuNiRs are classified as either blue or green depending on their visible absorption spectra. The color arises primarily from a (Cys)S−Cu charge-transfer band characteristic of T1 Cu centers, with absorption bands at 600 and 450 nm. The ratio of the intensities of these bands and hence the color of the enzyme depend on the detailed geometry of the T1 Cu center. The two copper sites are physically connected in two ways. One is the His94 → Asp92 → His89 link which has been called the substrate-sensing loop while the Cys130-His129 bridge links the two metal centers in a "hard-wired" manner and has been implicated in electron transfer from the type 1 Cu site to the catalytic type 2 Cu site.[9,10]

To investigate electron transfer from the type 1 Cu to the type 2 Cu we mutated His129 to Val. The gene encoding the mutated protein was overexpressed, and the products were purified by methods described previously.[11] The mutant enzyme was unable to reduce nitrite using either Azurin I or the methyl viologen/dithionite mixed assay. ICP analysis showed that the enzyme as isolated contained 5.4 Cu atoms per trimer and this was confirmed by the X-ray fluorescence measurements which showed only the presence of Cu. These data are consistent with the full occupancy of the T1 and T2 sites with Cu. The mutant protein displays a light blue color and exhibits an absorbance peak at 597 nm (ϵ_{597} = 1.740 mM^{-1} cm^{-1}), which is significantly less intense than the native protein[4] where the extinction coefficients are ϵ_{470} = 1.600 mM^{-1} cm^{-1}, ϵ_{593} = 6.300 mM^{-1} cm^{-1}, ϵ_{770} = 3.00 mM^{-1} cm^{-1}. These changes reflect alterations in the geom-

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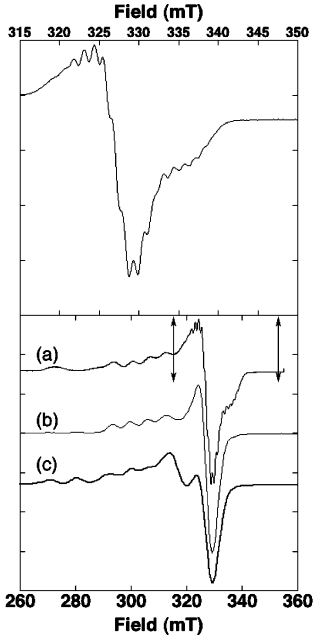

Figure 1. Lower panel: the EPR spectra of (a) H129V (frequency 9.135 GHz, field width 295 ± 50 mT, microwave power 4 mW, temperature 120 K, protein concentration 50 mg/mL). The main contribution to the spectrum for H129V was simulated using typical type 1 Cu parameters, with $g_\perp = 2.052$, $g_\parallel = 2.221$, $A_\perp = 1.3$ mT, and $A_\parallel = 6.5$ mT. The super-hyperfine structure was accounted for by two equivalent nitrogen atoms from histidine ligands, with A_N(isotropic) = 1.0 mT. For comparison, the spectra are shown for the (b) type 2 Cu depleted native (T2D) and (c) fully Cu loaded native *Ax*NiR. The section (within arrows) for spectrum a is expanded in the upper panel to show the N-hyperfine splitting in the H129V spectrum clearly.

etry of the T1 Cu site in this mutant, indicating that the mutation of the type 2 Cu site bridging ligand, His 129, has had a significant effect on the geometry of the T1 center due to its direct linkage to Cys 130.

The EPR spectrum of the H129V enzyme (Figure 1a) differs markedly from that of native *Ax*NiR which has characteristic features of both T1 and T2 Cu centers (Figure 1c) and T2DNiR which has only T1 EPR features (Figure 1b). The EPR spectrum of H129V is dominated by the Cu hyperfine splitting of the T1Cu center and a strong N super-hyperfine structure. Underlying these, additional features are observed at around 270 and 290 mT, and a broad signal around 335 mT is present, which we presume arise from the type 2 Cu bis-His geometry. A detailed analysis of this enriched and complex EPR spectrum must await the isolation of T2D H129V mutant. The use of native T2D is precluded as the detailed geometry of the type 1 Cu site has changed significantly due to the mutation. The distinctive 11 line pattern of N super-hyperfine splitting arising from the novel Cu(bis-His) center revealed by the crystal structure is shown in the upper panel of Figure 1. Similar N super-hyperfine splitting was observed in the EPR spectrum of a single crystal of L-histidine hydrochloride monohydrate doped with Cu(II) by Hirasawa et al.[2]

Crystals of H129V were light blue in color, and the structure[13] revealed several small changes at the T1Cu site, compared to the native protein. These changes are minor, compared to the crystallographic ESU (estimated standard uncertainty), which is 0.152 Å based on *R*-factor and 0.144 Å based on the maximum likelihood method. The Cu−Cys130 Sγ distance has decreased by ∼0.1 Å compared to the native protein while the Cu−His89 Nδ1 distance has increased by ∼0.1 Å (Table 1). In the native protein, Met144 occupies a dual conformation with the Sδ atoms at distances of 2.45 and 4.26 Å from the Cu, while in H129V we see only one position for the Sδ, presumably because of limited resolution of the current structure.

Table 1. Copper−Ligand Distances in H129V and the Native 1.04 Å Resolution Structure of *Ax*NiR[12]

atom 1	atom 2	native 1.04 Å (Å)	H129V (Å)
Cu 501	His89 Nδ1	2.02	2.09
Cu 501	Cys130 Sγ	2.20	2.10
Cu 501	His139 Nδ1	2.03	2.05
Cu 501	Met144 Sδ	2.45/4.26	3.05
Cu 502	His94 Nϵ2	1.96	2.12
Cu 502	His129 Nϵ2 (Val Cγ1)	2.00	4.91
	(Val Cγ2)		4.11
Cu 502	His B249 Nϵ2	3.74	5.09
Cu 502	His B300 Nϵ2	2.00	2.39
Cu 502	Wat503	1.98	
Wat 503	Asp92 Oδ2	2.54	
Wat 600	Asp92Oδ1	2.88	

Several changes have occurred at the T2 Cu site (Figure 2), which has opened up with significant movements occurring in most of the ligand residues. The T2Cu-ligating water molecule plays an important role in maintaining the orientation of His249 in an unfavorable conformation in the wild-type protein. Loss of the water, among other factors, has caused a large increase (∼1.3 Å) in the distance between Cu and His249. The lack of the Cu-ligating water has allowed a reorientation of ∼40° in the His249 side chain. The Nϵ2 atom of His300 has moved by ∼0.4 Å away from Cu, and the imidazole ring of His94 has rotated by approximately 25°. The Cα and Cβ atoms of residue 129 have also moved considerably, by ∼0.3 Å, increasing the Cu−Cα and the Cu−Cβ distance by 0.2 Å. Asp92 has moved 0.33 Å with

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(13) A high multiplicity (3.5) data set with 99.7% completeness with high quality (R_{merge} = 4.7%) was used for finding a molecular replacement solution using MOLREP,[14−16] with the 2.1 Å[9] native enzyme structure as the starting model, yielding an *R*-factor of 34.0% and a correlation coefficient of 72.1%. The H129V model was subject to a cycle of positional and individual *B*-factor refinement in REFMAC5[14,17] using data from 20.0 to 2.50 Å. This yielded an *R*-factor of 24.6% (*R*-free 26.1%). Several cycles of rebuilding in "O" and inclusion of 174 waters reduced the *R*-factor to 21.1% (*R*-free 23.2% for all data from 50 to 1.95 Å). The final model consisted of 2571 protein atoms, 174 waters, and two Cu atoms. The Ramachandran plot[18] shows 89.0% of residues to be in the core regions, with 10% in the additionally allowed regions and the remaining 0.4% in the generously allowed regions. The X-ray crystallographic data are deposited in the protein data bank (1wae and 1waesf).

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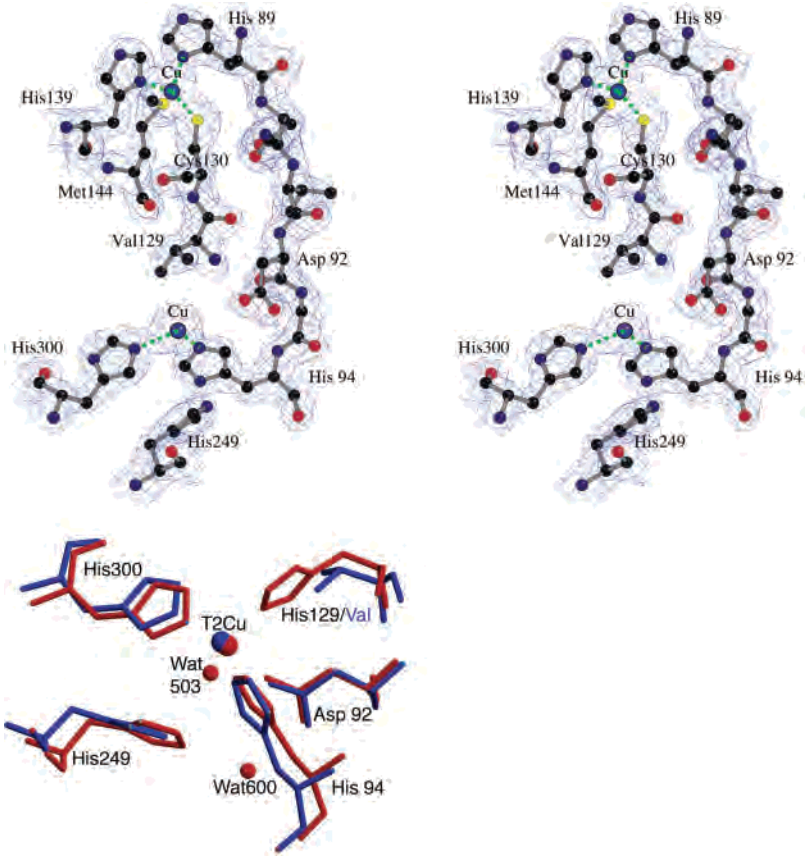

Figure 2. Stereo image (top) of the type 1 and type 2 Cu sites of H129V. The 2Fo-Fc electron density map clearly shows the absence of a Cu-ligating water at the T2Cu site. The superposition (above) of the native protein (red) with H129V (blue) clearly shows the changes that have occurred at the T2Cu site with the mutation of His129. Also, the superposition shows where the waters near the T2Cu in the native protein are and that they are absent in H129V.

respect to its position in the native structure, and both the Cu-ligating water and the water leading into the proton channel have been lost (Figure 2). The T2 Cu is also significantly more disordered than the T1 Cu by about 30% as judged by *B*-values. This compares to the native protein where the T1 Cu is typically 5−10% more disordered than the T2 Cu. The increase in disorder is probably due to the loss of two ligands resulting in an increased lability of the T2 Cu ion. This is seen in the map where the 2Fo-Fc electron density is elongated in the direction of the cavity (Figure 2). The consequence of the loss of His129 and water from the Cu ion is to generate a novel Cu bisHis coordination of Cu(II). This, to our knowledge, is the first 'truly' bisHis Cu(II) site observed in a biological system.

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