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CHEMICAL AND SPECTROSCOPIC CONFORMATION OF AN EXOGENOUS LIGAND BRIDGE IN HALF MET HEMOCYANIN

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SUMMARY: Half met-N_3^- hemocyanin is shown to undergo a unique change at the Cu(II)···Cu(I) active site with temperature, exhibiting class II mixed valent properties at low temperature (The appearance of an intense near IR intervalence-transfer transition and a delocalized EPR spectrum). This requires a Cu(II)-N=N=N-Cu(I) bridging geometry. The effects of CO coordination to half met-N_3^-, combined with the presence of a low energy $N_3^- \rightarrow$ Cu(II) charge transfer transition, demonstrate that azide is also bridging at room temperature. Finally, half met-N_3^- is found to be capable of coordination of a second N_3^- at the copper(II) site.

INTRODUCTION: In a previous communication (1) the preparation of a series of half met-L derivatives was reported. These contain a Cu(II)···Cu(I) active site to which a variety of ligands could be coordinated through rather complex ligand substitution chemistry. In particular, exogenous ligands were observed to bind extremely tightly to this active site as compared to the met apo derivative (2) from which the copper(I) was selectively removed yielding a Cu(II)···( ) active site. This was used to support ligand coordination to the copper(I) through a Cu(II)-L-Cu(I) bridge. In this communication we present definitive chemical and spectroscopic evidence which determines the geometry of N_3^- bridging to the binuclear copper site of half met-N_3^- and the ability of this site to coordinate additional ligands.

MATERIALS AND METHODS: Hemocyanin was purified from the hemolymph of the marine snail, Busycon canaliculatum, by ultracentrifugation (3). Half met-N_3^- was prepared by treatment of half met-NO_2^- (1, 4) with a 100 fold excess of NaN_3 for 24 hr followed by dialysis against pH = 6.3 .1M phosphate buffer for 24 hr. The carbon monoxide adduct of half met-N_3^- was prepared by exposure to 30 psi CO for 30 min at pH = 6.3 phosphate in a high pressure reaction vessel. Dialysis of the half met-N_3^- under CO pressure was accomplished by allowing one end of the dialysis tube to remain open to the CO atmosphere above the

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dialysate level. EPR spectra of frozen solutions were recorded on a Varian E-9 spectrometer at 9 GHz. Temperature dependent optical absorption spectra were obtained of buffered sucrose solutions using a Cary 17 spectrometer equipped with a Spectrim II cryocooler. Difference spectra of the charge transfer region (300 - 500 nm) of the half met-N_3^- forms were taken relative to half met-$CH_3CO_2^-$ which shows no absorption bands in this region (5).

RESULTS AND DISCUSSION: The half met-N_3^- form of mollusc hemocyanin is found to exhibit a unique temperature dependence in its EPR and optical spectra (Figure 1). Upon cooling, a discontinuous shift to higher energy by $\sim$ 1500 cm^{-1} is observed for the dd (800 → 710 nm) and charge transfer transitions (500 → 460 nm). This is associated with the appearance of a unique low temperature EPR spectrum and an intense transition in the near IR (λ = 1500 nm, $\varepsilon \sim 1000$ $M^{-1}cm^{-1}$). These spectral properties define the half met-N_3^- derivative to be a class II mixed valence system (6) at low temperature with the near IR absorption being an intervalence Cu(II)Cu(I) → Cu(I)Cu(II) transition. The high intensity of this transition requires a good pathway for inner sphere electron transfer between the two coppers and defines the bridging geometry as end to end for the azide. Intervalence-transfer transitions are also observed for half met-X derivatives where X = Cl^-, Br^- and I^-, the trend in intensity being consistent with the inner sphere electron transfer rates ($I^- > Br^- > Cl^-$) (7).

Further confirmation of azide bridging in the half met-N_3^- form at room temperature comes from new chemical and spectroscopic results. Carbon monoxide is found to coordinate to half met-N_3^- under 30 psi of CO pressure. (Similar reactions have been observed for half met-Cl^- and half met-$CH_3CO_2^-$). The CO adduct is stable for approximately 2 hr after removal of CO pressure. This reaction produces the EPR spectral changes shown in Figure 2B. The temperature effect is no longer present and no mixed valence properties are observed for this form. Evacuation followed by flushing with nitrogen removes the CO and leads to the reappearance of the original low temperature half met-N_3^- EPR spectrum (Figure 2C). However, if the CO adduct of half met-N_3^- is dialyzed against pH = 6.3 phosphate buffer (.1M) under CO pressure the N_3^- is found to be completely removed from the active site after 28 hr resulting in

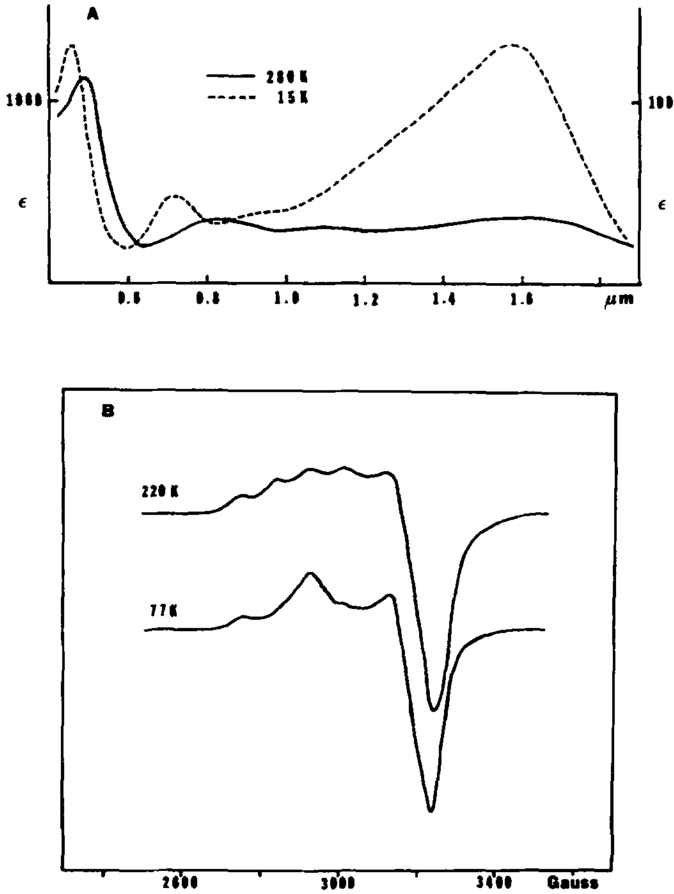


Figure 1. (A) Temperature dependence of the optical spectrum of half met-N_3^- recorded in buffered sucrose (pH = 6.3 .1M phosphate). (B) Temperature dependence of the EPR spectrum of half met-N_3^- recorded in pH = 6.3 phosphate buffer.

the half met-aquo EPR spectrum shown in Figure 2D. This is in strong contrast to the effects of dialyzing half met-N_3^- against phosphate buffer where no change is observed after > 100 hr of dialysis. This is, however, consistent with the dialysis times required to remove N_3^- from met apo-N_3^- ($\sim$ 24 hr). Thus, the N_3^- must be strongly coordinating to the copper(I). CO binding to the copper(I) then breaks or greatly weakens the N_3^--Cu(I) bond allowing the azide to be easily removed by dialysis. Coordination of the N_3^- to the copper(II) is demonstrated by the presence of a low energy ($\sim$ 500 nm) $N_3^- \rightarrow$

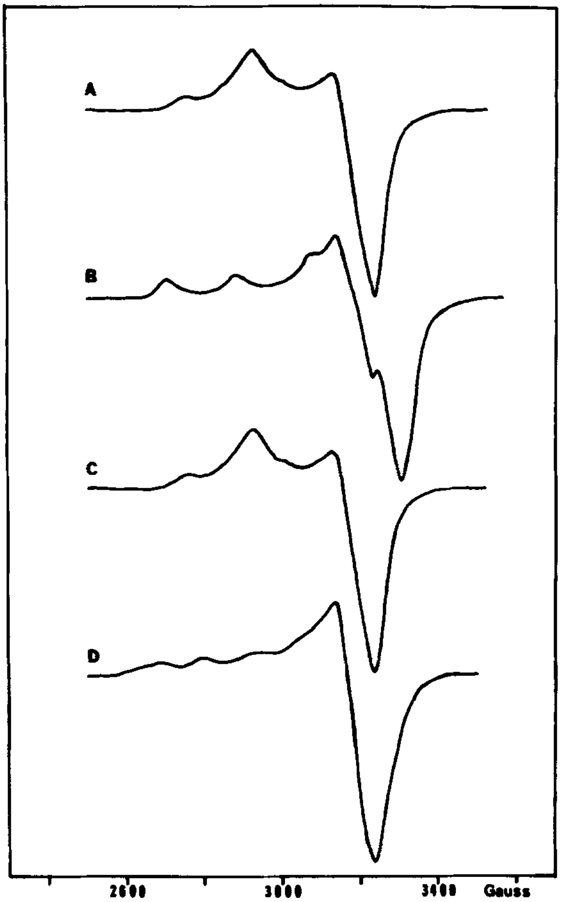


Figure 2. EPR spectra of half met-N_3^- recorded at 77K. (A) Half met-N_3^-, pH = 6.3 .1M phosphate buffer; (B) Half met-N_3^- + 30 psi CO; (C) Half met-N_3^- + CO after evacuation and flushing with nitrogen; (D) Half met-aquo obtained by dialyzing half met-N_3^- under CO pressure 28 hr.

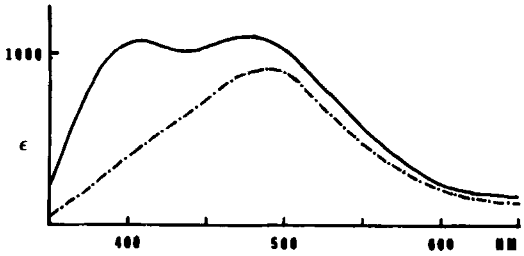


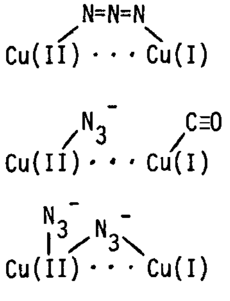
Figure 3. Difference spectra of the charge transfer region of half met-N_3^- recorded in pH = 6.3 .1M phosphate buffer at room temperature. (—·—·) Half met-N_3^-; (———) half met-N_3^- + 100X excess N_3^-. Spectra were taken versus half met-acetate, which has no acetate to copper charge transfer in this region.

Cu(II) charge transfer transition (Figure 3). Thus the N_3^- must also be bridging at room temperature. The temperature effect seems to be associated with a distortion at the copper(II) site (discontinuous shift in dd bands and charge transfer transitions) leading to a better pathway for electron delocalization between the coppers.

Finally, the half met-N_3^- is found to be capable of binding a second N_3^- to the copper(II) site. This is indicated by the appearance of a second $N_3^- \rightarrow$ Cu(II) charge transfer transition (Figure 3) upon addition of 100 fold excess azide. This charge transfer transition is at higher energy (λ = 410 nm, $\varepsilon \sim 1000\ M^{-1}cm^{-1}$) similar to that observed for met apo-N_3^- (λ = 420 nm, $\varepsilon \sim 2500\ M^{-1}cm^{-1}$) where the azide cannot be bridging. The half met-L forms where L = SCN^-, OCN^- and CN^- also coordinate a second exogenous ligand at the copper(II) site.

These experimentally determined modes of ligand binding are summarized in Scheme I.

SCHEME I



Half met-N_3^-

Half met-N_3^- + CO

Half met-N_3^- + 100X N_3^-

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