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REACTIONS AND INTERCONVERSION OF MET AND DIMER HEMOCYANIN

Richard S. Himmelwright, Nancy C. Eickman and Edward I. Solomon*

Department of Chemistry

Massachusetts Institute of Technology

Cambridge, Massachusetts 02139

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SUMMARY: The regenerable methemocyanin of Busycon canaliculatum is shown to be EPR-nondetectable. The small EPR signal present in met preparations is a nonregenerable binuclear cupric unit accounting for ∿ 6% of the active sites. A variety of anions are found to bind to met with the reactions being complicated by both reduction and competitive binding of buffer. The metastable dimer hemocyanin is shown to rearrange either directly to EPR-nondectable met or through an EPR-detectable regenerable binuclear cupric form obtained on reaction of dimer hemocyanin with azide. These results combined with previous results on half met derivatives are used to support the presence of an endogenous protein bridge between the two coppers in hemocyanin.

INTRODUCTION: The met and dimer derivatives of oxyhemocyanin are prepared by unrelated chemical reactions and exhibit very different ground state magnetic properties. While there is general agreement concerning the EPR-nondetectable nature of arthropod methemocyanin (1,2), there have been reports associating EPR signals with mollusc methemocyanin (3,4). Dimer hemocyanin, however, shows a broad intense EPR signal due to weak dipolar coupling between two cupric ions (5). This communication presents evidence showing that the regenerable methemocyanin of the mollusc Busycon canaliculatum is EPR-nondetectable, with the small EPR signals observed being due to only ∿ 6% of non-regenerable sites obtained in the met preparation. In addition, reactions of dimer hemocyanin and reactions leading to the interconversion of met and dimer are presented, along with a rationale for the different ground state magnetic properties exhibited by these binuclear cupric derivatives in terms of elimination of a protein ligand bridge in the dimer forms.

* To whom all correspondence should be sent.

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MATERIALS AND METHODS: Hemocyanin from the marine snail, Busycon canaliculatum, was obtained by ultracentrifugation (6). Met (7,8) and dimer (9) hemocyanin were prepared according to published procedures. EPR of frozen solutions were recorded at 77K using a Varian E-9 spectrometer operating at 9.1 GHz. Optical spectra were taken on a Cary 17 spectrophotometer.

RESULTS AND DISCUSSION: Generation of methemocyanin by treatment with H_2O_2 (7) or incubation with N_3^- (8) at 37°C (pH = 5.0 acetate buffer) results in the appearance of a weak EPR signal (Figure 1A). The intensity of this signal can only account for 6 ± 3% of total copper present, based on double integration of the signal and absorption at 280 nm. When the methemocyanin is then treated with a stoichiometric amount of H_2O_2, 90% of the oxyhemocyanin is regenerated when compared to the original oxy concentration. No loss in EPR intensity is observed upon treatment with peroxide at pH = 7.0 phosphate buffer and only slight reduction of the EPR intensity is observed when peroxide is added in pH = 5.0 acetate buffer. Therefore, the regenerable methemocyanin is EPR-nondetectable and the EPR signal observed for methemocyanin can be ascribed to a small percentage of sites obtained in the met preparation that are EPR-detectable and nonregenerable. These EPR-detectable nonregenerable sites are interesting, in that they are binuclear cupric sites and that the interaction between the copper ions is buffer, pH and anion dependent (10). This is demonstrated by addition of excess azide to the protein. While the intensity of the EPR signal does not increase, the resulting spectrum (Figure 1C) is that of weakly coupled cupric centers (g = 4 signal, broad g = 2 signal).

The effect of ligands such as N_3^- (cf. references 2, 4, 11), SCN^-, NO_2^-, and CN^- on the regenerable methemocyanin has been investigated. N_3^- and SCN^- are found to reversibly bind to the active site based on changes in the ligand field transitions [λ_{max} = 710 nm (N_3^-), 660 nm (SCN^-)] and the appearance of a charge transfer band associated with azide [λ_{max} = 360 nm (N_3^-)]. An optical titration of methemocyanin with N_3^- at pH = 5.0 acetate buffer is complicated as previously observed in the CD spectrum (4). This is due to competitive binding of acetate. However, at pH = 6.3 phosphate buffer one band is observed at $\sim$ 360 nm and the binding constant of N_3^- is calculated to be 1.5 mM^{-1}.

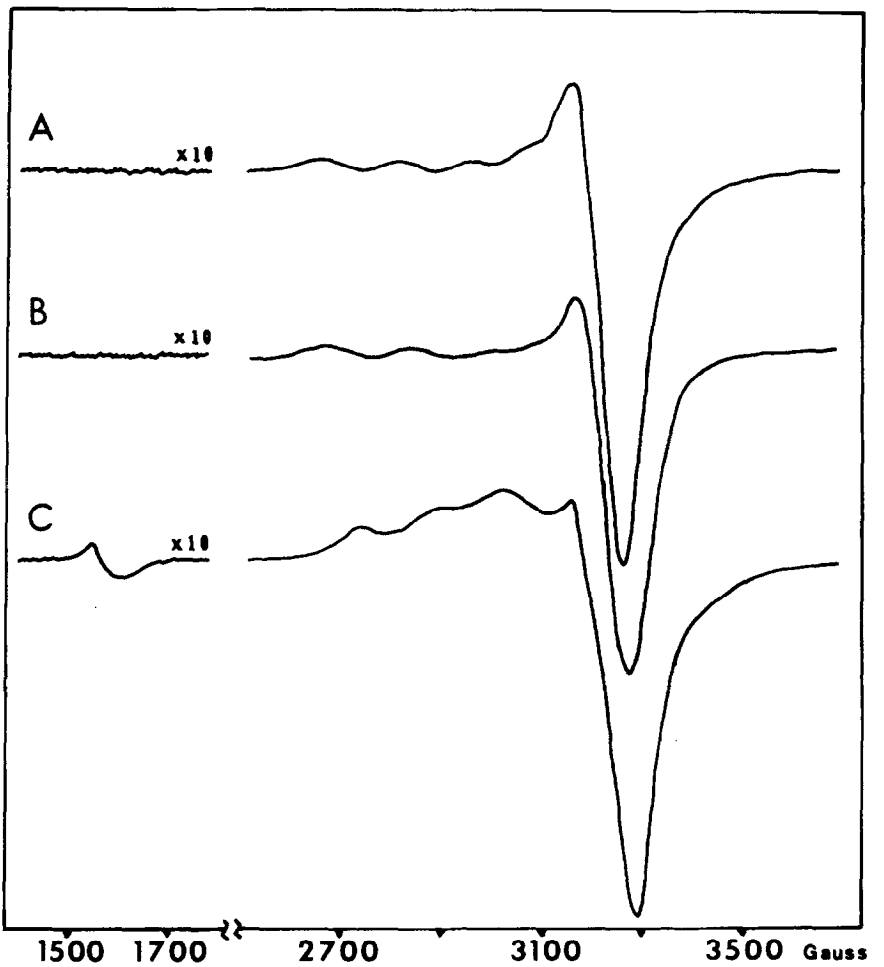


Figure 1. EPR spectra (pH = 5.0 .1M acetate buffer) of (A) Methemocyanin; (B) Methemocyanin after addition of stiochiometric amount of H_2O_2; (C) Methemocyanin after addition of 50 fold excess NaN_3. Relative receiver gains are indicated.

Reaction of NO_2^- and CN^- with met does not proceed by simple ligand substitution at the active site. At pH = 8.0, NO_2^- reversibly binds to methemocyanin as shown by small shifts in the d-d bands with no new EPR signal being obtained. At pH = 5.0 acetate buffer, however, addition of 100-fold excess $NaNO_2$ to met produces a clear solution of dimer hemocyanin (Figure 2B). Although nitrite chemistry under acid conditions is complex, one would expect primary generation of NO (12). The direct reaction of NO with met does, in

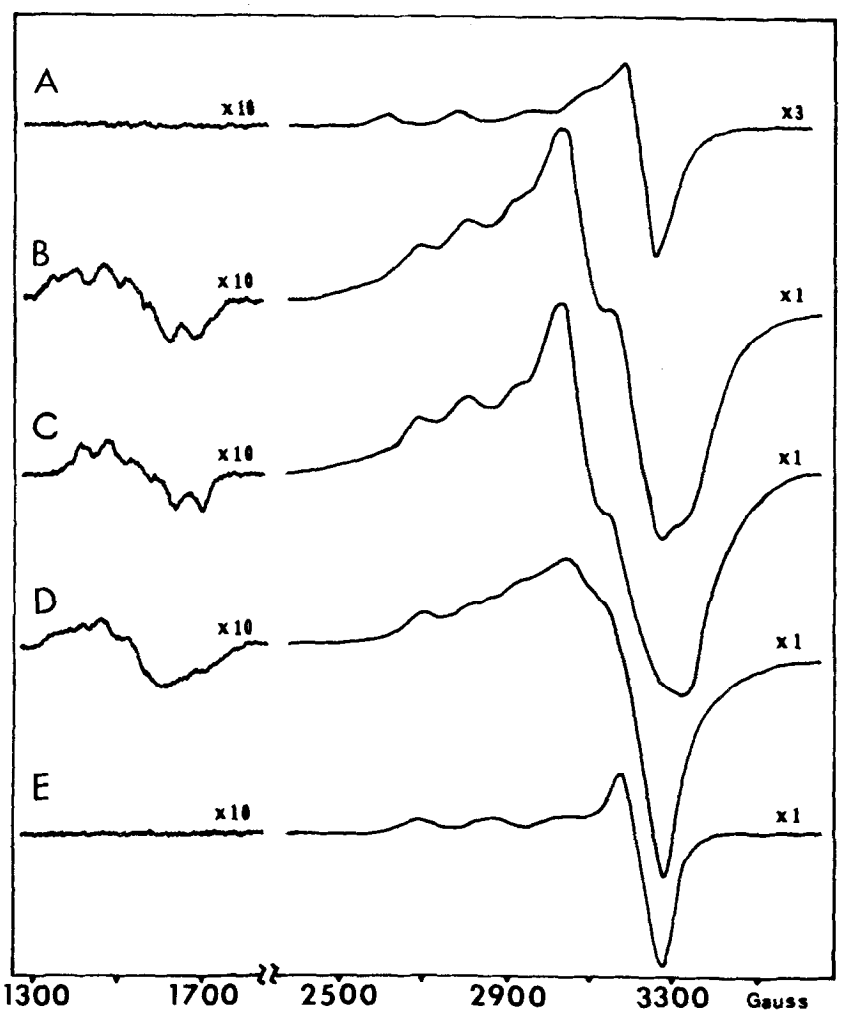
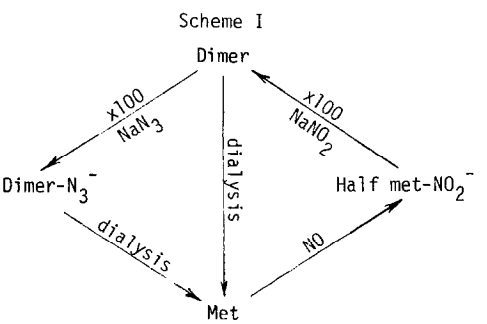


Figure 2. EPR spectra (pH = 5.7 .1M acetate buffer) of (A) Methemocyanin; (B) Methemocyanin after addition of 100 fold excess $NaNO_2$; (C) Dimer hemocyanin prepared by the action of NO on deoxy; (D) Dimer hemocyanin after addition of 100-fold excess NaN_3 (dimer-N_3^-); (E) Dimer-N_3^- after addition of 10 fold excess H_2O_2. Relative receiver gains are indicated.

fact, cause a one electron reduction, resulting in production of half met-NO_2^-. Finally, half met-NO_2^- in a 100 fold excess of $NaNO_2$ under anaerobic conditions produces dimer hemocyanin perhaps through an oxidation via nitrous acid. A one electron reduction to half met can also be accomplished by stoichiometric addition of NaCN (1:1 CN^-:Cu) to met at pH = 8.0 tris buffer yielding half met-CN^- (13).

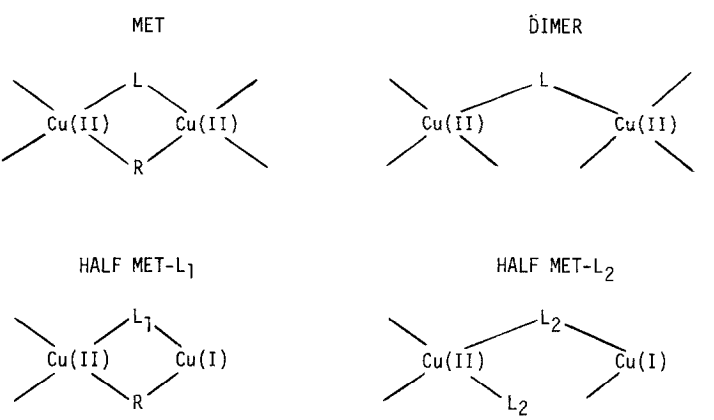
Figure 2C presents the EPR spectrum of dimer hemocyanin obtained by the action of NO on deoxy in the presence of trace amounts of O_2. Addition of a 100 fold excess of NaN_3 to the dimer changes the EPR spectrum to that shown in Figure 2D (dimer-N_3^-). The large g = 4 signal and broad g = 2 region indicate that this is also a binuclear cupric derivative. Dialysis of this dimer-N_3^- or allowing the preparation to sit for 72 hr results in complete loss of the g = 4 signal and the broad spectral features at g $\sim$ 2 (Figure 2E). (The residual g = 2 signal has $\sim$ 20% of the original intensity and is associated with unconverted half met obtained in the original dimer preparation). In contrast to the dimer and the nonregenerable EPR-detectable form produced in the met preparation, addition of 10 fold excess H_2O_2 to the dimer-N_3^- eliminates the dimer-N_3^- EPR spectrum and results in regeneration of oxyhemocyanin. Further, the EPR-nondetectable form obtained on dialyzing dimer-N_3^- to pH = 5.7 acetate buffer for 24 hr shows ligand field transitions characteristic of methemocyanin. This, coupled with the regeneration by H_2O_2, indicates that dimer-N_3^- has been converted to met. In addition, dimer hemocyanin is found to undergo reactions with other anions such as cyanide and thiocyanate. Finally, if the original dimer is dialyzed immediately after preparation, it also converts to methemocyanin. After dialysis, no dimer EPR spectral features are observed but the methemocyanin ligand field transitions are present and oxyhemocyanin is regenerated by treatment with H_2O_2. Therefore, both the original dimer and dimer-N_3^- forms of Busycon canaliculatum are metastable and convert to EPR-nondetectable met. The chemistry of met and dimer hemocyanin is summarized in Scheme I.

Scheme I



The met and dimer derivative both contain binuclear cupric active sites but exhibit very different ground state magnetic properties. The EPR spectrum, coupled with the position of the d-d bands (λ_{max} = 710 nm) observed for dimer, indicate that the active site contains two tetragonal copper(II)'s. A comparison of the d-d spectral region of met (λ_{max} = 680 nm) to that of dimer shows only small changes indicating that met also contains two tetragonal copper(II)'s with relatively weak interactions between the coppers as their molecular orbital transition energies are similar. The lack of an EPR signal in met hemocyanin must then be ascribed to antiferromagnetic coupling between the two coppers. The difference between met and dimer can now be related to the differences observed between Group 1 and Group 2 half met [Cu(II)···Cu(I)] derivatives (13,14). Group 1 derivatives (half met-L_1 where L_1 = NO_2^-, $CH_3CO_2^-$, Cl^-, Br^- and I^-) coordinate only one exogenous ligand in bridging modes which require the Cu(II)-Cu(I) separation to be between 2.5 - 4 Å depending on ligand. Group 2 derivatives (half met-L_2 where L_2 = CN^-, N_3^-, SCN^-) have the ability to bind a second ligand at the Cu(II). The bridging modes of L_2 ligands place the Cu(II)-Cu(I) separation at > 5 Å. Thus, L_2 ligands force the coppers apart, rupturing an endogenous bridge which leaves open an additional coordination position on the copper(II).(15)(See Scheme II). For dimer hemocy-

Scheme II



R=Endogenous protein ligand

anin, the 6 Å Cu-Cu separation obtained from computer simulation of the dipolar coupled EPR spectrum (5) requires that the endogenous bridge be broken in analogy to Group 2 half met forms. Protein residues containing phenolate or carboxylate would provide an effective superexchange pathway for antiferromagnetism with a Cu-Cu separation of < 4 Å. Thus if the Cu-Cu distance in the met derivative is < 4 Å, in analogy to the half met Group 1 series, the endogenous bridge can reform providing the pathway for superexchange coupling between the two copper(II)'s and eliminating the EPR signal.

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15. For the met apo form where only one copper is present at the active site the endogenous ligand remains coordinated and again only one exhangeable position is present on the Cu(II).