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SPECTROSCOPIC STUDIES OF LIGAND PERTURBATION EFFECTS ON THE HALF OXIDIZED ACTIVE SITE OF BUSYCON CANALICULATUM HEMOCYANIN

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SUMMARY: We report the preparation and characterization of an important new series of hemocyanin derivatives containing a variety of anions bound to the cupric ion in a singly oxidized active site. Spectroscopic studies on these half met-L derivatives, Cu(I)...Cu(II)-L, where L = CN^-, NO_2^-, $CH_3CO_2^-$, and N_3^- reveal that the unpaired electron is localized on one copper in an approximately tetragonal site with a $d_{x^2-y^2}$ ground state. Ligand variation is used as a perturbation on the spectral features to show that the small molecule (and by analogy, oxygen in oxyhemocyanin) is bound in the equatorial plane of the copper.

INTRODUCTION: The hemocyanins are respiratory proteins which bind oxygen in the ratio of one per two coppers.[1] Although it has been shown that the oxygen binds as peroxide,[2] the electronic structure of the binuclear copper unit is not well understood. The coppers are generally believed to be in the +2 oxidation state but exhibit no EPR signal.[1] This has been associated with antiferromagnetic coupling[3] between the coppers. Similarly, no EPR signals have been observed[4] for a number of different met (doubly oxidized) derivatives.[4,5] Treatment of the protein with nitric oxide produces a mixture of doubly and singly oxidized sites.[6] While this doubly oxidized derivative differs from the previous mets in that it shows an EPR signal, the dipolar nature of the signal does demonstrate that the coppers are in close

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proximity[7] (about 6 Å). The singly oxidized form is unique in that it eliminates the effects of the second copper(II) ion, thus providing a simplified system amenable to detailed studies.

MATERIALS AND METHODS: Hemocyanin was purified from the hemolymph of the marine snail, *Busycon canaliculatum*, by ultracentrifugation and the initial singly oxidized derivative was prepared as previously described.[6] Ligand substitution reactions were performed at pH = 6.3 using .1M added anion except for the cyanide reaction which was done at pH = 8.0. Double integration of the EPR spectra shows that in all cases approximately 75% of the sites are singly oxidized.

RESULTS AND DISCUSSION: The EPR signal obtained from *Busycon canaliculatum* hemocyanin treated with an excess of sodium nitrite is shown in Figure 1. We find this derivative undergoes ligand substitution reactions with a variety of anions producing the new series of hemocyanin derivatives: half met-L, where L = CN^-, NO_2^-, CH_3COO^- and N_3^-. In all cases, the ligand is tightly bound and can only be displaced by a large excess of a second anion and subsequent dialysis to remove the free ligand, CN^- being an exception in that it binds irreversibly. The original product of the nitrite preparation appears to contain coordinated nitrite as demonstrated through the recovery of the optical and EPR properties associated with the half met-NO_2^- form by addition of a small excess of $NaNO_2$ to a number of half met derivatives at both acidic and basic pH's. We have also determined that a second ligand binds in the presence of a hundred-fold excess of N_3^-, NO_2^-, and CN^-. Reduction of the half met derivatives with sodium dithionite yields regeneration of oxyhemocyanin as shown by an increase in absorption at 345 nm with the simultaneous loss of the EPR signal.[8]

A comparison of the EPR spectra shown in Figure 1 reveals that, while there are certainly significant changes with ligand, the variations can be viewed as perturbations on the active site.

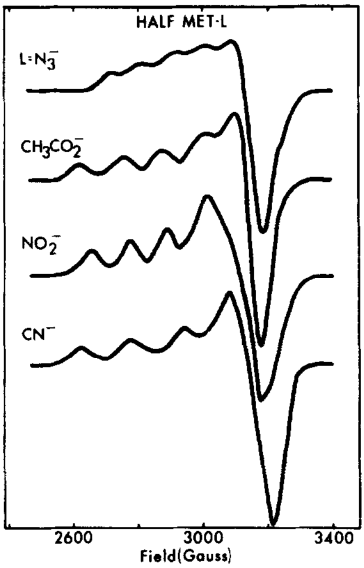

Figure 1. EPR spectra of half met-L derivatives recorded at -100°c using a Varian E-9 spectrometer operating at 9.10 GHz.

In all cases, $g_{\parallel} > g_{\perp} > 2.0023$, the spectra are approximately axial,[9] and $A_{\parallel}$ is in the range of 0.011 - 0.015cm^{-1}. These results and the optical spectra to be dicussed indicate that the copper(II) is in an approximately tetragonal site with a $^2B_1(d_{x^2-y^2})$ ground state.

The room and low temperature absorption and room temperature circular dichroism spectra of the series of half met-L derivatives in order of increasing ligand field strengh of L is shown in Figure 2. A general shift of absorption intensity to higher energy occurs going down the series. By comparison to extensive spectral data of simple copper complexes,[10] we assign the intense peak in the absorption spectrum as the $^2E \leftarrow {}^2B_1$ $(xz, yz \rightarrow x^2 - y^2)$ transition and the shoulder to lower energy as the $^2A_1 \leftarrow {}^2B_1$ $(z^2 \rightarrow x^2 - y^2)$ transition. The dominant positive peak in the circular dichroism spectra which shifts

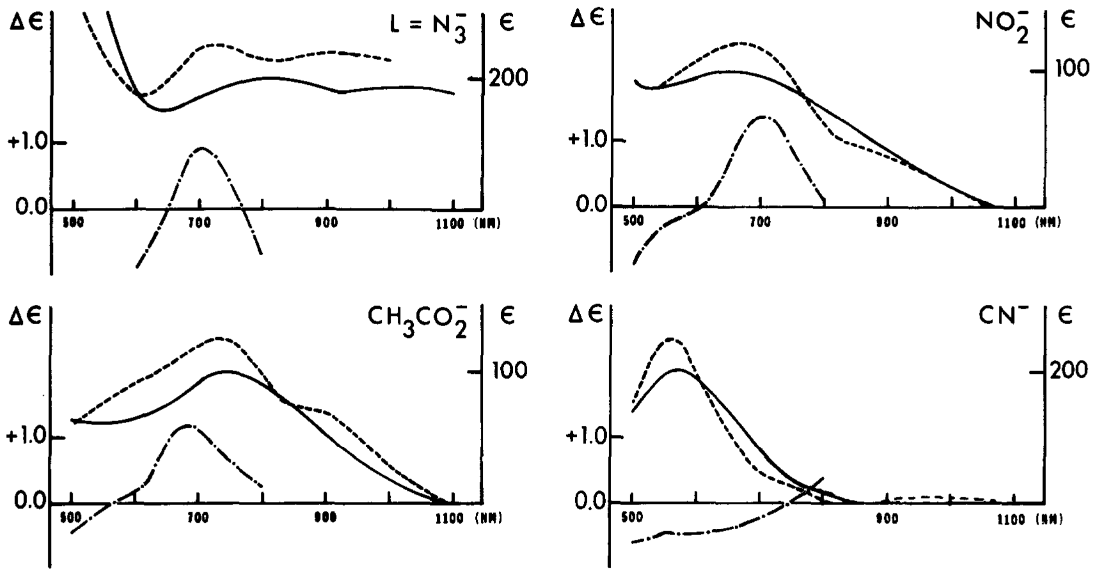

Figure 2. Optical spectra of half met-L derivatives. 280°K (solid line) and 15°K (broken line) absorption spectra were taken of proteins in buffered sucrose solutions on a Cary 17 using a Spectrum II cryocooler. Solution circular dichroism spectra (dot-dash line) were taken on an Instruments, S.A. dichrograph.

with change in ligand independently of the absorption maximum is most reasonably assigned as the ${}^2B_2 \leftarrow {}^2B_1$ ($xy \rightarrow x^2 - y^2$) transition, based on its large anisotropy factor.[11]

Thus, we see that as the ligand field strength of the varied anion increases, the bands in absorption shifts toward higher energy[12] and the energy of the ${}^2A_1 \leftarrow {}^2B_1$ transition approaches that of the ${}^2E \leftarrow {}^2B_1$. The band associated with the ${}^2B_2 \leftarrow {}^2B_1$ transition appears to move to lower energy relative to the intense absorption maximum, finally being resolved as a weak low energy band in the half met-cyanide derivative at low temperatures.

All of these observations can readily be interpreted based on a simple ligand field model with an increasing tetragonal

TABLE I

EPR Parameters and Electronic Energy Levels
of Half Met-L Derivatives

HALF MET-L L =	$g_{/\!/}$	$\sim g_{\perp}$	$A_{/\!/}$ $10^{-4}cm^{-1}$	E_{B_2} cm^{-1}	E_E cm^{-1}	E_{A_1} cm^{-1}	λ/λ_0
N_3^-	2.277	2.082	108	14300	12500	9700	.599
$CH_3CO_2^-$	2.318	2.080	141	14700	13700	11100	.707
NO_2^-	2.302	2.096	125	14100	14700	11600	.644
CN^-	2.291	2.069	150	10500	18700	16400	.462

distortion of the copper site with increasing ligand field strength. This requires that the varied ligand coordinate in the equatorial plane of the tetragonal copper site. The qualitative trends in covalency as given by the $g_{/\!/}$ orbital reduction factor (λ/λ_0) in Table 1 further support the specific band assignments and our overall interpretation. Extension of these results to oxyhemocyanin strongly suggests that the oxygen binds (as peroxide) through inplane coordination to at least one of the coppers.

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REFERENCES:

1. Lontie, R. and Vanguickenborne, L. (1974) Metal Ions in Biological Systems, Vol.3, H. Sigel, pp 183-200, Marcel Dekker, Inc. New York.
2. Loehr, J.S., Freedman, T.B., and Loehr, T.M. (1974) Biochem. Biophys. Res. Comm. 56, 510-515.
3. Solomon, E.I., Dooley, D.M., Wang, R.H., Gray, H.B., Cerdonio, M., Mogno, F., and Romani, G.L. (1976) J. Amer. Chem. Soc. 98, 1029-31.
4. Eickman, N., Himmelwright R.S., and Solomon, E.I., to be published.
5. Witters, R. and Lontie, R. (1975) FEBS Letters 60, 400-403.
6. Schoot Uiterkamp, A.J.M. (1972) FEBS Letter 20, 93-96.
7. Schoot Uiterkamp, A.J.M.,van der Deen, H., Berendsen, J., and Boas, J.F. (1974) Biochem. Biophys. Res. Comm. 372, 407-425.
8. Regeneration has also been accomplished with hydroxylamine by van der Deen, H. and Hoving, H. (1977) Biochemistry 16, 3519-3525.
9. While some rhombic distortion is present, at least in the half met-NO_2^- form, it is not a significant feature in either the optical or EPR spectra.
10. Hathaway, B.J. and Billing, D.E. (1970) Coord. Chem. Rev. 5, 143-207.
11. Yeh, C-Y. and Richardson, F.S. (1976) Inorg. Chem. 15, 682-690.
12. Due to unique effects observed upon lowering the temperature, only the room temperature EPR and optical spectra could be used for comparison with the data from other forms.