Magnetic Properties of *Cancer magister* Hemocyanin†

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ABSTRACT: The magnetic susceptibilities of *Cancer magister* hemocyanin, oxyhemocyanin, and apohemocyanin were measured over the temperature range 1.5–77°K, and at 293°K, using a vibrating sample susceptometer and a modified Gouy balance. The samples were diamagnetic at all temperatures. The proton magnetic resonance spectra of oxygenated and deoxygenated hemocyanins were identical, within the limits of resolution of a 220-MHz spectrometer, when the samples were dissociated to fully active subunits of 80,000 molecular weight at 0.02 M sodium carbonate (pD 11.2). Thus, no difference in the magnetic state of oxygenated and deoxygenated hemocyanin was detectable under the conditions used.

Hemocyanin, a copper protein capable of reversible oxygenation, occurs widely among molluscs and arthropods. The *Cancer magister* protein has a molecular weight of 940,000–950,000 and contains 24–26 atoms of Cu (Thomson *et al.*, 1960; Ellerton *et al.*, 1970). The protein binds O_2 and CO in a ratio of one molecule per two copper atoms (Kubowitz, 1938; Vanneste and Mason, 1966; Redfield, 1934, 1950), and a unit containing two copper atoms appears to be the minimum active size (Pickett *et al.*, 1966; Schoot-Uiterkamp, 1972). For these reasons it is believed that the active site of hemocyanin may contain two adjacent copper atoms which participate together in O_2 binding. However, no ligand of either of the two copper atoms is known.

The oxygenated form of hemocyanin is reported to be no more paramagnetic than the deoxygenated form (Rawlinson, 1940; Bayer and Fiedler, 1962) but absolute paramagnetic susceptibilities are not known. Neither form gives an electron spin resonance (esr) signal (Nakamura and Mason, 1960). *Cancer magister* oxyhemocyanin has absorption bands at 575 nm, ϵ_{Cu} = 413 M^{-1} cm^{-1} (Thomson *et al.*, 1960; Simo, 1966), and at 340 nm, ϵ_{Cu} = 10,000 M^{-1} cm^{-1}. The spectrum can be resolved at −195° (Mason, 1964) or by circular dichroism at room temperature (Van Holde, 1967) into bands at 420, 566, and 625 nm. Various structures involving Cu(II) ions have been proposed for oxyhemocyanin (Orgel, 1958; Manwell, 1960; Morpurgo and Williams, 1968), based upon this spectroscopic, magnetic, and chemical evidence, but the issue remains unresolved. Since both susceptibility and proton magnetic resonance (pmr) line shifts and widths can be sensitive probes of the magnetic properties of coordinated metals, we have used these techniques in studies of *C. magister* hemocyanin in an effort to delineate the structure of the active site of this respiratory protein, and especially to verify whether or not the copper atoms and O_2 were sufficiently closely interacting to form a diamagnetic system.

Materials and Methods

Preparation of Hemocyanin. Hemocyanin was prepared from clotted hemolymph of the Pacific crab, *C. magister*, by the method of Thomson *et al.* (1960), and sterilized by passage through a Millipore filter. The centrifuged pellet was diluted in 0.10 M potassium phosphate buffer (pH 7.0) with or without 0.01 $MgCl_2$, as noted.

The hemocyanin purity, as judged by sodium dodecyl sulfate gel electrophoresis, Cu content, and O_2 binding (absorbance at 340 nm) was about 98% after centrifugation for 7 hr at 150,000*g*. $MgCl_2$ was required to give maximum O_2 binding at atmospheric O_2 pressure (pH 8.5). The following properties of the purified material were observed: A_{280nm} = 14.7 for a 1% solution of oxyhemocyanin at pH 7.0 to 8.5; A^{Cu}_{340nm} = 10,000 ± 1000 M^{-1} cm^{-1}, pH 8.5, 0.01 M $MgCl_2$. For routine assay, maximal O_2 binding capacity was represented by the ratio A_{340}/A_{280} = 0.20 at pH 8.5, 0.01 M $MgCl_2$.

Magnetic Susceptometry Samples. Hemocyanin samples for the susceptibility measurements at temperatures below 77°K were centrifuged a second time at 150,000*g* for 7 hr. Pellets were dissolved in 0.05 M Tris–0.01 $MgCl_2$ buffer (pH 8) and dialyzed against 100 volumes of the following buffers for 24 hr each. For apohemocyanin: (1) 0.05 M Tris, 0.01 M $MgCl_2$, and 0.01 M KCN (pH 8); (2) number 1 repeated; (3) 0.05 M Tris, 0.01 M $MgCl_2$, and 0.001 M EDTA (pH 8.5); and (4) number 3 repeated. For hemocyanin itself, the same buffers were used, but without KCN. The final concentration of hemocyanin was approximately 140 mg/ml. Dialysis buffer 4 was used as the buffer control for magnetic susceptometry. Copper content was determined by atomic absorption spectroscopy, standard addition method (Willis, 1963). The exact properties of the samples studied are given in Table I.

Magnetic susceptibility measurements at temperatures below 77°K were made with a vibrating sample susceptometer (Redfield and Moleski, 1972) as described previously (Moss *et al.*, 1969), except that deoxygenation with glucose–glucose oxidase was omitted. The dissolved oxygen correction for the experiments was estimated by measuring the susceptibility of air-equilibrated buffer. Deoxygenated samples were transferred to susceptibility holders in an N_2-filled glove bag and frozen. After the measurements, samples were thawed and transferred to 1-mm path-length optical cells in the glove bag, the cells were sealed, and optical spectra were taken. Less than 3% conversion to oxyhemocyanin was detected. Optical spectra of oxygenated samples were also checked after the measurements. There was less than 5% loss of optical density at 575

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TABLE I: Properties of Hemocyanin (Hc) Preparations Used in Low-Temperature Magnetic Susceptibility Studies.

Sample	A_{340}/A_{280} after Oxygenation	[Cu][a] (mM)	g-atoms of Cu/mol of Protein[b]	[Cu(II)][c] (mM)
Oxygenated Hc	0.209	3.65	26.0	0.027
Deoxygenated Hc	0.209	3.91	26.0	
Oxygenated apoHc	0.011	0.075	0.52	<0.02
Deoxygenated apoHc		0.087	0.52	

[a] The total copper content was determined by atomic absorption spectroscopy. [b] Based on protein molecular weight of 940,000. [c] [Cu(II)] was estimated prior to the magnetic susceptibility measurement by comparison of the esr spectrum of the protein at −175° with that of Cu(II) EDTA standard, using the peak to trough amplitude as a measure of signal intensity.

nm. Double integration of derivative electron paramagnetic resonance (epr) spectra showed less than 1% conversion to epr detectable Cu for either oxy- or deoxyhemocyanin, after thawing and refreezing.

Susceptibilities were also checked at 293°K. Though the measurements are considerably more difficult at the higher temperature, the results are presented as a verification that the magnetic state of the protein does not change on freezing and also in order to put a lower limit on any exchange coupling in the oxyprotein which would lead to increasing paramagnetism with increasing temperature. The susceptometer balance used has been described in detail previously (Theorell and Ehrenberg, 1951; Ehrenberg *et al.*, 1962) and the procedure used was that of Ehrenberg *et al.* (1962) with the exception that the diamagnetism of the apohemocyanin was not determined.

Two separate air shipments of hemocyanin from Portland, Ore., to Stockholm, Sweden, were made. In both cases, the samples warmed to ambient temperature before arrival, probably accounting for the somewhat elevated (≃10% of total copper) level of epr detectable copper present in these compared to the samples used for the low temperature measurements. Magnetic measurements were made in terms of oxy minus deoxy values for each sample, however, so that the susceptibility of the epr detectable component should be constant and not interfere.

Upon arrival the samples were spun at 100K × *g* for 180 min; the pellets were resuspended in one-fifth volume of 0.15 M phosphate, Na^+ salt, pH 6.5, recentrifuged at 100K × *g* for 180 min and resuspended in phosphate buffer. The samples were then dialyzed against 0.15 M phosphate, Na^+ salt, pH 6.5, overnight. All above procedures were carried out at 4°.

Oxyhemocyanin was obtained by flowing 100% O_2 over a gently rocking sample of the hemocyanin immersed in an ice bath. The O_2 was bubbled through H_2O before passing over the sample of hemocyanin. Susceptibilities were determined after 30 and 60 min of equilibration with O_2; no differences were detected.

Deoxyhemocyanin was obtained by using N_2 instead of O_2 in the scheme described above. No OD at 600 mμ was observed with the deoxy sample.

The dialysis buffer was treated in the same manner as described above for each of the three hemocyanin samples. These buffers were then used in the determination of the volume magnetic susceptibilities of the proteins as described previously (Ehrenberg *et al.*, 1962).

Proton Magnetic Resonance Spectroscopy Samples. Pmr spectra of hemocyanin solutions were measured over the range 4–35° using a Varian HR220 pmr spectrometer. To obtain narrow line widths and high resolution, molecular weights below 20,000 are considered optimal for 220-MHz instruments. The smallest functional subunit molecular weight for *Cancer* hemocyanin is 80,000. We had hoped to use apohemocyanin as a copper-free control on native hemocyanin, but the dissociation of native hemocyanin to 80,000 molecular weight subunits required raising the pH above 10.0, and apohemocyanin (but not hemocyanin itself) began to denature and undergo irreversible aggregation at pH 8 in the absence of Mg^{2+}, and at pH 8.5 in the presence of Mg^{2+}. We thus had to be content with a pmr comparison of oxyhemocyanin and deoxyhemocyanin, without apohemocyanin. For proton magnetic resonance (pmr) studies, D_2O was used as a solvent. Subunit formation in D_2O was measured by Sephadex G-200 gel filtration. It was found that 90–95% dissociation of hemocyanin occurred in 0.02 M carbonate, pD 11.2, or in 0.05 M carbonate, pD 11.3. These results are consistent with previous observations on subunit formation (Ellerton *et al.*, 1970), since D_2O is a much weaker acid than H_2O, and p*K* values are generally 0.7 unit higher in D_2O (Robinson *et al.*, 1969). pD was measured with a glass electrode standardized in H_2O, using the correction factor, pD = pH + 0.4 (Covington *et al.*, 1968).

Hemocyanin, 20 mg/ml in 0.1 M phosphate buffer, pH 7.0, was centrifuged at 150,000 × *g* (Spinco 50 rotor) for 7 hr. Pellets were dissolved in D_2O and dialyzed against ten volumes of the following buffers in D_2O for 24 hr each: (1) 0.10 M Na_2CO_3–0.01 M EDTA (pD 10.5), (2) 0.02–0.05 M Na_2CO_3 (pD 10.8), (3) 0.02–0.05 M Na_2CO_3 (pD 11.2–11.3), and (4) repeat 3. Buffers were prepared by dissolving anhydrous Na_2CO_3 and Na_2EDTA in D_2O, and adjusting to the desired pD with 0.7 N DCl in D_2O. The final concentration of hemocyanin was about 90 mg/ml. The sample prepared at pD 11.2 retained 98% of its O_2 binding capacity measured at pH 8.5, while a sample prepared at pD 11.3 retained only 87% of its activity. O_2 binding at pD 11.2 was 90–95% of that measured at pH 8.5 by A_{340}. Deoxyhemocyanin was prepared by flushing with N_2.

Results

Magnetic Susceptibility. Figure 1 depicts low-temperature susceptibility results for oxyhemocyanin, oxygenated buffer, and calibrations. Figure 2 shows results for deoxyhemocyanin. Oxyhemocyanin susceptibility, taken as the slope of measured susceptibility with respect to $1/T$, was only 10% that expected from a spin $^1/_2$, $g = 2.0$, at the 3.65 mM concentration of Cu known to be in the sample (Figure 1). It was near values typically measured for O_2 dissolved in diamagnetic protein solutions, but slightly higher than the apohemocyanin or air-equilibrated dialysis buffer from these particular samples. The susceptibility of dissolved O_2 is difficult to control, probably due to complicated solubility properties near the freezing point of the solution and consequent aggregation effects dependent on other solutes, and freezing history. Deoxygenated

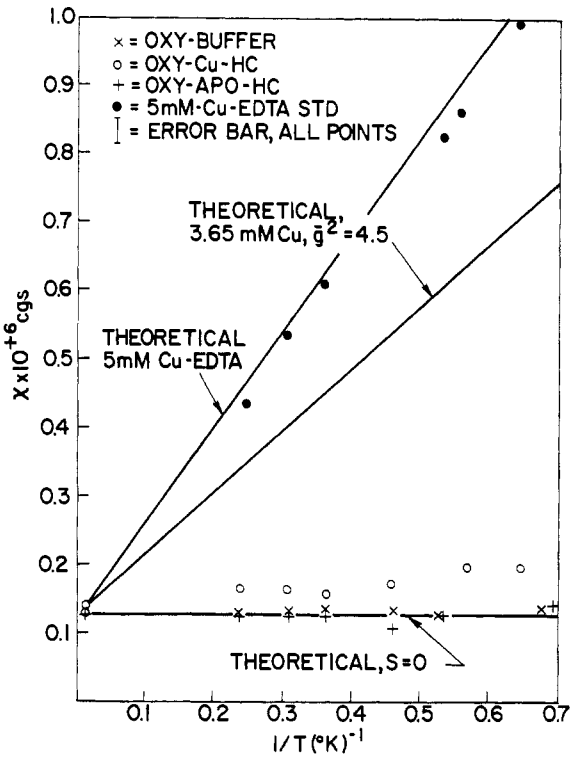

FIGURE 1: The temperature dependence of the susceptibility, χ, of oxygenated buffer, oxyhemocyanin (HC), oxygenated apohemocyanin, and a Cu(II) EDTA standard.

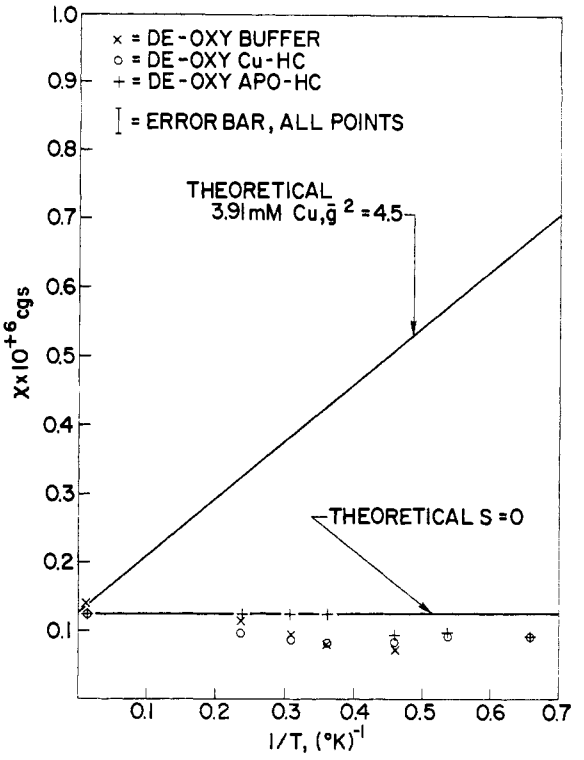

FIGURE 2: The temperature dependence of the susceptibility, χ, of deoxygenated buffer, deoxyhemocyanin, and deoxygenated apohemocyanin.

protein solutions did not show this small paramagnetism, so we do not attribute it to adventitiously bound metal impurities. Esr analysis (Table I) showed that the paramagnetic copper content was less than 1% and, therefore, too small to account for the magnetic susceptibility measured in the oxyhemocyanin samples. The small susceptibility increased with $1/T$ so it cannot represent a population of the magnetic excited state of an antiferromagnetically coupled pair. Measurements were repeated on three independently prepared samples with identical results, within experimental error. We conclude that the copper in oxyhemocyanin is diamagnetic. If the diamagnetism was due to an antiferromagnetic coupling of paramagnetic copper ions, our null result at low temperatures puts a definite lower limit of 5°K on the strength of the coupling.

Deoxygenated hemocyanin was also diamagnetic (Figure 2). There was no ambiguity due to dissolved oxygen. Buffer, apohemocyanin, and hemocyanin had identical susceptibilities, within experimental error. The small decrease in susceptibility at lowest temperatures was due to nuclear spins of the solid part of the sample holder, which displaces the sample during the reciprocating motion.

Oxy minus deoxy measurements were also performed at 293°K on three separate samples. The measurements averaged to very nearly zero susceptibility difference between the two hemocyanin forms, but because of the large experimental uncertainties in determining weak susceptibilities at high temperatures, we take the maximum error in the measurements, 55% of the susceptibility of free Cu^{2+} ions at the sample concentration, as the upper limit of the oxy minus deoxy value. If one supposed an exchange coupling between cupric ions in oxyhemocyanin, and diamagnetic cuprous ions in deoxyhemocyanin, this would raise our estimate of the lower limit on the exchange coupling (J) to 100–200°K.

Pmr Studies. Pmr spectra of oxyhemocyanin and deoxyhemocyanin were obtained under conditions of dissociation of the proteins to subunits of 80,000 molecular weight. The spectra were largely structureless because of the relatively large subunit size and possible subunit associative equilibria. It was considered possible that even for the large subunit molecular weights involved, the presence of a Cu(II) center would be manifested by a broadening through dipolar relaxation of nearby protons. However, the envelopes of pmr absorption for the oxy and deoxy forms of hemocyanin were indistinguishable, indicating no differential broadening and, within the rather limited resolution achieved, no pmr detectable differences in the conformation or paramagnetism of the oxygenated and deoxygenated protein.

The appearance of contact shifted resonances was not considered a strong possibility because of the usually long electronic relaxation times (T_{1e}) exhibited by Cu(II). However, T_{1e} could be reduced substantially over normal values if the two Cu(II) ions were coupled by antiferromagnetic spin exchange. High- and low-field regions were searched for evidence of contact shifted resonances without success and no temperature-dependent resonances were observed. The failure to find evidence of even residual paramagnetism means that if Cu(II) centers are present (1) two Cu(II) are strongly antiferromagnetically coupled, (2) insufficient numbers of protons are broadened or contact shifted to yield a detectable influence on the absorption envelope, or (3) the Cu(II) centers have long electronic relaxation times.

Discussion

The low susceptibility of oxyhemocyanin, taken in conjunction with chemical (Kubowitz, 1938; Vanneste and Mason,

SCHEME I

$$-\text{Cu(I)}\quad \text{Cu(I)}- + O_2 \rightleftharpoons -\text{Cu(I)}\quad \begin{matrix} O \\ \| \\ O \end{matrix}\quad \text{Cu(I)}- \qquad (1)$$

$$-\text{Cu(I)}\quad \text{Cu(I)}- + O_2 \rightleftharpoons -\text{Cu}\uparrow\text{(II)}\quad \begin{matrix} O^- \\ | \\ O^- \end{matrix}\quad \text{Cu}\downarrow\text{(II)}- \qquad (2)$$

$$-\text{Cu(I)}\quad \text{Cu(I)}- + O_2 \rightleftharpoons -\text{Cu}\uparrow\text{(II)}\quad {}^{-}\text{O–O}^{-}\quad \text{Cu}\downarrow\text{(II)}- \qquad (3)$$

$$-\text{Cu(I)}\quad \text{Cu(I)}- + O_2 \rightleftharpoons -\text{Cu}\uparrow\text{(II)}\quad {}^{-}\text{O–O}\cdot\quad \downarrow\ \text{Cu(I)}- \qquad (4)$$

deoxyhemocyanin oxyhemocyanin

1966; Redfield, 1934, 1950; Pickett *et al.*, 1966; Schoot-Uiterkamp, 1972), spectroscopic (Ellerton *et al.*, 1970; Simo, 1966; Mason, 1964; Van Holde, 1967), and epr evidence (Schoot-Uiterkamp, 1972; Nakamura and Mason, 1960), indicates the probable presence of strong antiferromagnetic exchange coupling between a pair of closely linked copper atoms at its active site. The diamagnetism of deoxyhemocyanin indicates either exchange coupling in coupled Cu(II) ion pairs, or Cu(I) ions at the active site. The reasons for discarding alternate suggestions are discussed below.

Since isolated copper ions have either an odd number [$3d^9$, Cu(II)] or zero [$3d^{10}$, Cu(I)] unpaired spins, there is no possibility that the susceptibility measurements at low temperature arise from a nonmagnetic ground state of paramagnetic configuration, as is sometimes the case in even-numbered spin compounds, due to crystal field or spin-orbit coupling effects (Iizuka *et al.*, 1968). For both oxy and deoxyhemocyanin it remains, however, to consider the choice between (a) centers with exchange coupled pairs of paramagnetic cupric ions, or (b) with fully spin-paired (d^{10}) cuprous ions.

The arguments supporting a cupric configuration as at least part of a "resonance" structure for oxyhemocyanin have been based on the similarity of the visible optical spectrum to those of known cupric proteins (Morpurgo and Williams, 1968; Van Holde, 1967). Many have bands at about 600 nm, with weaker companion transitions near 450 and 750 nm. These can be accounted for by d-d transitions of Cu(II) ions in distorted tetragonal ligand fields (Brill and Bryce, 1968; Blumberg, 1966). An alternative explanation, that the bands in hemocyanin are due to simple Cu(I) ions involved in ligand charge transfer transitions (Orgel, 1958), leaves unexplained the spectral analogy with the other Cu(II) proteins, even though charge transfer complexes are known to be acutely sensitive to the properties and bonding of the ligand involved. For the colorless deoxyhemocyanin, an active site containing Cu(I) ions is likely, again on the basis of spectral analogy, in this case with the large class of colorless Cu(I) complexes. In addition, apohemocyanin can be reconstituted only with cuprous copper (Kubowitz, 1938).

With the most probable states given by the spectroscopic arguments above, proposed structures for deoxyhemocyanin and oxyhemocyanin (omitting possible water molecules) typically include those of Scheme I (arrows denote spin direction).

With or without the magnetic data, as long as not even one protein ligand nor even the symmetry of the site is known, there is little basis other than the spectroscopic evidence on which to choose from these models. One very general principle relating to the magnetic properties can be stated, however: the degeneracy of the Π_{Z^*} and Π_{X^*} antibonding orbitals of

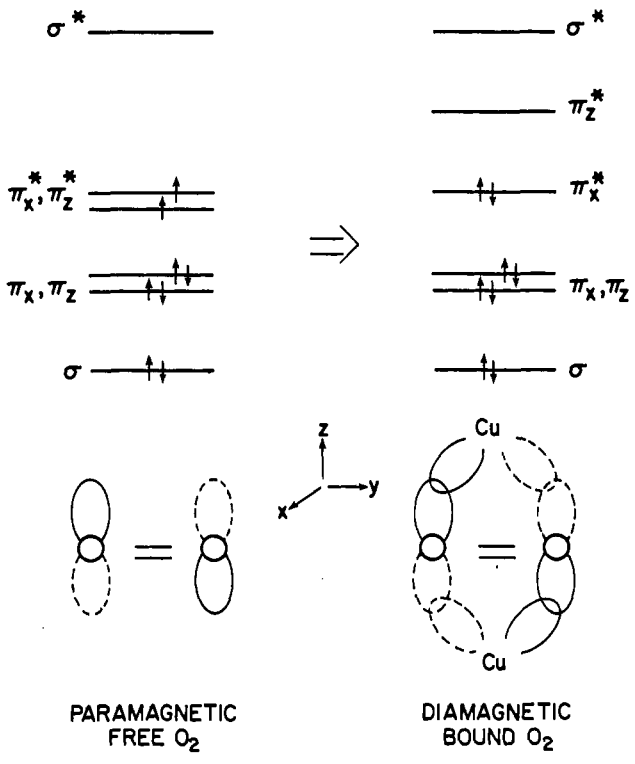

FIGURE 3: Comparison of molecular orbital schemes for paramagnetic free oxygen and diamagnetic bound oxygen complexes.

molecular O_2 is likely to be removed when it is associated with hemocyanin (Griffith, 1956) (Figure 3), simply because the cylindrical symmetry about the oxygen–oxygen axis will be removed. It is this degeneracy which accounts for the paramagnetism of molecular O_2. In a model with bridging O_2 and the axis as shown in Figure 3, the Π_{Z^*} orbital will be destabilized relative to Π_{X^*}. (The same basic argument also holds for the geometry of structures in eq 3 and 4 of Scheme I. Both Π^* electrons of O_2 will tend to pair in Π_{X^*} and there can then be an empty Π_{Z^*} orbital available to accept electrons from the copper atoms. For this kind of symmetrical complex any degree of Cu–O_2 covalency through the Π_{Z^*} orbital (Cu oxidation) will stabilize unpaired copper spins in the antiferromagnetic arrangement as well as leaving the oxygen spins paired (Figure 4). This statement rests only on the observation that if one copper atom donates a fraction of a particular spin to the oxygen Π_{Z^*} orbital, the exclusion principle dictates that it is energetically favorable for the opposite copper atom to donate the opposite spin. The residual spins on the copper atoms will then be antiparallel. The strength of the coupling of the residual spins depends on the amount of

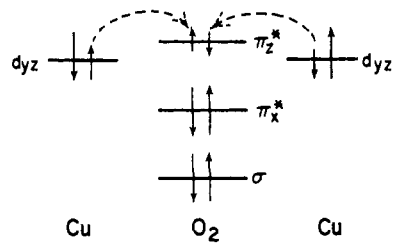

FIGURE 4: Mechanism for stabilization of antiferromagnetic copper spin coupling through electron delocalization to a bridging oxygen molecule.

covalency in the O_2–Cu bonds, but it will certainly be antiferromagnetic. Stabilization of spins in a particular orientation *via* interaction with a bridging ligand is one of several mechanisms proposed for "superexchange" coupling by Anderson (1950) and discussed in some chemical detail by Goodenough (1963).

The concept of two copper(II) atoms bridged by O_2 and coupled through it gains additional support from the observation of a $g = 4$ signal attributed to dipole coupled spins in the epr spectrum of NO-treated hemocyanin (Schoot-Uiterkamp, 1972). Presumably with NO treatment, the copper atoms are fully oxidized and not bridged by a single ligand (2NO molecules probably bind per two copper atoms (Schoot-Uiterkamp, 1972)), removing the possibility of superexchange. Observable dipole coupling of the two Cu spins in the disrupted complex locates them within 6 Å of each other (Price *et al.*, 1970; Schoot-Uiterkamp, 1972), however, and gives credence to the idea that there could be exchange coupling when they are bridged. It should be noted that dipole coupling alone cannot account for the diamagnetism we observed in oxyhemocyanin. Dipole coupling can lead to a nonmagnetic ground state, but split by less than 0.1°K from the magnetic excited state even at the shortest possible non-binding distance.

Considering the O_2–hemocyanin complex in terms of a bond between copper atoms and an empty Π_{Z^*} of O_2, the oxidation state of the Cu becomes a question of the relative energy of the orbitals involved and resulting ligand or metal character of the bonding and antibonding orbitals formed. There is a parallel with the case of oxygenated hemoglobin, where discussions of the oxidation state of iron with O_2 bound have also had to proceed in the face of the fact that the iron remains ferrous when O_2 is removed. In that case, the O_2 bonding is such that the orbitals of the iron electrons acquire substantial O_2^- character on O_2 complex formation (Lang and Marshall, 1968); that is, the expected oxidation does occur. The *apparent* stability of the ferrous iron is a manifestation of the fact that electrons are "returned" to the iron as molecular oxygen dissociates from the complex, presumably due to the hydrophobic environment of the heme pocket (Wang, 1961). Similarly, even if deoxyhemocyanin contains Cu(I) ions, they may be reversibly oxidized upon O_2 binding in the sense that considerable electron density is donated to molecular orbitals of primarily O_2 character. The resulting partly or totally cupric ions are left with any unpaired spins coupled antiparallel.

Hemocyanin may be compared with other members of the rapidly growing class of biologically important polynuclear metal compounds in which association among the metals is so close that exchange coupling is verified or probable. In some members of this class, paramagnetism has been detected at high temperatures so that the strength of the exchange coupling could be measured (Palmer *et al.*, 1971; Moss *et al.*, 1972; Dawson *et al.*, 1972); in others only a lower limit has been reported (Moss *et al.*, 1969, 1971; Moleski *et al.*, 1970). The very strong couplings (>200°K) seen in some iron–sulfur proteins have led to suggestions that there must be a direct metal–metal bond as well as superexchange through bridging sulfides (Lewis *et al.*, 1972; Moleski *et al.*, 1970). In a complex with reversibly associated molecular O_2 as the bridging ligand it is difficult to imagine a direct metal–metal bond existing, yet our accurate low temperature measurements for oxyhemocyanin put a definite lower limit of $J = 5$°K on the exchange coupling, and the room temperature measurement suggests this to be greater than $J = 100$°K. This indicates that superexchange alone may result in very strong magnetic coupling of metal ions in binuclear complexes.

The binuclear metal complex has previously been shown to be an important functional unit for electron-transfer proteins (Moss *et al.*, 1969; Palmer *et al.*, 1971; Moleski *et al.*, 1970), and with the inclusion of hemerythrin (Dawson *et al.*, 1972; Moss *et al.*, 1971), tyrosinase (Schoot-Uiterkamp and Mason, 1973), and hemocyanin in the binuclear class, it is apparent that this important general mode of metalloprotein construction is useful for oxygen carriers and oxygen transferases as well.

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Fatty Acid Synthetase Activity in *Euglena gracilis* Variety *bacillarius*. Characterization of an Acyl Carrier Protein Dependent System†

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ABSTRACT: *Euglena gracilis* variety *bacillarius* has been shown to have two fatty acid synthetase systems which differ in organizational complexity. One enzyme system is independent of added acyl carrier protein (ACP) for activity *in vitro* and is a complex of molecular weight greater than 1,000,000. The second synthetase is dependent on added ACP for activity. Bio-Gel chromatography of the latter system suggests that it is associated loosely in an active complex with a molecular weight of about 360,000. Studies of the kinetics of the ACP-dependent system gave parameters which are similar to those reported for multienzyme complex fatty acid synthetases which contain tightly bound ACP. Investigation of ACP substrate dependency showed that when ACP and the enzymes are incubated together prior to initiation of reaction a sigmoidal relationship between ACP and initial reaction velocity and the highest V_{max} was obtained. The degree of expression of each of the two fatty acid synthetases in comprising total enzyme activity is dependent on the stage of chloroplast development.

Studies on fatty acid biosynthesis with preparations from a variety of organisms have indicated that there are two types of synthetase systems which differ in organizational complexity. In yeast (Lynen, 1961) and animals (Burton *et al.*, 1968; Hsu and Yun, 1970; Larrabee *et al.*, 1965; Smith and Abraham, 1970) the steps of fatty acid biosynthesis from malonyl-CoA and acetyl-CoA are catalyzed by a multienzyme complex containing tightly bound ACP.[1] In plants (Overath and Stumpf, 1964; Brooks and Stumpf, 1966; Simoni *et al.*, 1967) and bacteria (Alberts *et al.*, 1963; Goldman *et al.*, 1963; Lennarz *et al.*, 1962) except mycobacteria (Brindley *et al.*, 1969), a notable exception which represents the more advanced procaryotes, the overall reaction of fatty acid biosynthesis is carried out by a series of reactions catalyzed by discrete enzymes which are not isolated as multienzyme complexes. In the latter systems, ACP is easily separated from the other proteins involved in the reactions.

Euglena gracilis is particularly interesting because it exhibits characteristics of both plants and animals. Strain Z of this organism was found by Delo *et al.* (1971) to possess both types of fatty acid synthetase systems. Further study (Ernst-Fonberg and Bloch, 1971) indicated that the ACP-dependent fatty acid synthetase present in the organism is linked with chloroplast development and the chloroplast protein biosynthetic apparatus. These studies have been extended to *Euglena gracilis* variety *bacillarius* with the hope of using several chloroplast mutants of this variety to define better the role of the organelle in the appearance of the ACP-dependent fatty acid synthetase. The results of this work will be reported shortly. Here is presented a description of the fatty acid syn-

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[1] Abbreviations used are: Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; ACP, acyl carrier protein; NADPH, reduced nicotine adenine dinucleotide phosphate.