SPECTROSCOPIC STUDIES ON THE ACTIVE SITE OF *SEPIOTEUTHIS LESSONIANA* HEMOCYANIN

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SUMMARY. The absorption, circular dichroism (CD), and magnetic circular dichroism (MCD) spectra in the visible region have been measured for *Sepioteuthis lessoniana* hemocyanin at 77, 198, and 293K. From the temperature dependence of the CD spectra of oxyhemocyanin, the bands observed at 450, 565, and 700 nm were resolved into those centered at 430, 490, 565, 600, and 700 nm. Since these five peaks are most probably due to the d-d transitions, the two copper ions at the oxygenated active center are inferred to be Cu(II) ions each in a non-equivalent coordination geometry of very low symmetry. The MCD spectral data confirm the view and reasonably explain the diamagnetism of oxyhemocyanin.

Hemocyanin is known to bind one molecule of oxygen per two adjacent copper ions that constitute an active site unit (1-3). The environments and the oxidation states of the copper ions in hemocyanin have been the subjects of many investigations but are still controversial. In deoxyhemocyanin, however, Cu(I) ions have been inferred from the absence of the absorption bands in the visible region (4) and the diamagnetic property (5). Possible modes of oxygen binding by hemocyanin are typically described as follows:

Cu(I) O_2 Cu(I)	Cu(I) O_2^- Cu(II)	Cu(II) O_2^{2-} Cu(II)
a	b	c

The absorption and circular dichroism (CD) spectra in the visible region (4) as well as the weak but detectable ESR signals (1,2) suggest the presence of Cu(II) ions in oxyhemocyanin. On the other hand, the diamagnetism reported for oxyhemocyanin (5) is apparently contradictory to the spectral results.

In order to obtain further information about the states of the copper ions at the active sites, we attempted an examination of the CD and magnetic circular dichroism (MCD) spectra of hemocyanin measured at various temperatures.

MATERIALS AND METHODS

The hemolymph collected from live squid (Sepioteuthis lessoniana) by the method of Omura et al. (6) was centrifuged at 1.8×10^4 g for 20 min, and the supernatant was centrifuged at 10^5 g for 16 hr with a Hitachi 55P-2 ultracentrifuge. The dark blue pellet was redissolved in 0.1 M phosphate buffer (pH 7.4) to make the hemocyanin content approximately equal to that of the hemolymph. The purity of hemocyanin was checked by the ratio of the absorption coefficient at 280 nm to that at 347 nm (6). For spectral measurements at low temperatures, the buffered solution was mixed with an equal volume of ethyleneglycol in order to get a clear frozen solution.

CD and MCD spectra were measured with a JASCO MOE-1 spectropolarimeter. Absorption spectra were recorded on a Shimadzu MPS-50L Multipurpose and a Union Giken SM-401 High-Sensitivity recording spectrophotometer. Fig. 1 shows the cell compartment used for the CD and MCD spectral measurements. The light beam passing through the solution in the cuvette was focused to 1 mm in diameter, and this device proved effective for measuring the frozen solution where it was difficult to find a transparent and homogeneous portion required.

RESULTS

Absorption Spectra. The absorption spectra of oxyhemocyanin measured at room temperature (293K), 198, and 77K are exhibited in Fig. 2a. A broad band was observed at 580 nm at room temperature, whereas at 77K three bands were observed at 450, 575, and 700 nm. The spectra correspond well with those reported for the hemocyanins from molluscs by Van Holde (4). However, a closer look at the spectra suggests a shoulder near 600 nm, which can be detected also in the reported spectra. As is apparent from Fig. 2a, the intensities of the bands increase at lower temperatures partly due to the volume change of the solution. Because the relevant CD, MCD, and absorption spectra were recorded under the same conditions, this intensity difference does not affect the interpretation of the spectra.

CD Spectra. In the visible region the CD spectra at room temperature (Fig. 2c) have peaks at 450, 565, and 700 nm, which are in accordance with the literature (4,7). Lowering the temperature causes the increase of the magnitudes of the peaks at 565 and 700 nm and the shifts of 5-10 nm to shorter wavelengths without affecting the spectral patterns around them. The 450-nm peak, on the other hand, exhibits a complex spectral change with the temperature. In coincidence with the absorption spectral change, the magnitude of the CD curve around 600 nm becomes slightly enhanced at low temperatures, from which an overlapping peak is suspected.

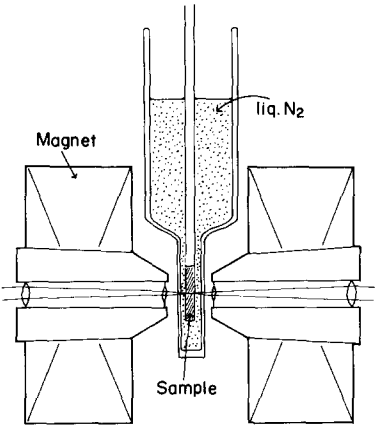

Fig. 1. Schematic view of the cell compartment used for CD and MCD spectral measurements.

MCD Spectra. Since accuracy of the MCD spectral curves of oxyhemocyanin in the visible region was affected by the strong CD bands, repeated measurements were necessary for obtaining the reproducible curves shown in Fig. 2b. Although the MCD magnitudes are dependent on temperature, they are proportional to the intensities of the absorption spectra, so that the C-term in the equation expressing the MCD magnitude (8) may be neglected. The B-term is inferred from the spectral patterns to be dominant in the MCD spectra, while the A-term is almost negligible.

DISCUSSION

Out of the three possible oxidation states of the copper ions in oxyhemocyanin, the mode with two Cu(I) ions may be excluded on the basis of the absorption and CD spectra in the visible region. The temperature dependence of the CD spectra suggests that the band around 450 nm consists of the peaks at 430 and 490 nm, the latter disappearing nearly completely at 77K. This interpretation gains support from the fact that an approximately Gaussian curve centered at 490 nm is obtained by subtracting the peak at 430 nm from the spectrum on the assumption that the intensities at 450, 565, and 700 nm are enhanced

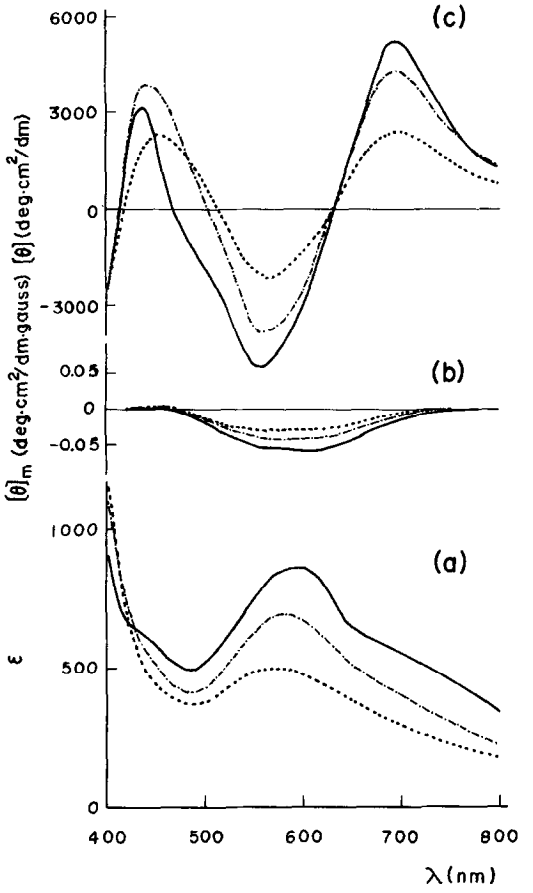

Fig. 2. Absorption, CD, and MCD spectra of oxyhemocyanin at various temperatures (a) Absorption spectra; (b) MCD spectra; (c) CD spectra. Temperatures: ————, 77K; -·-·-·-, 198K; ·····, 293K. The intensities are expressed relatively to the value of the 575-nm absorption band at 293K (ε=500) (ref. 4).

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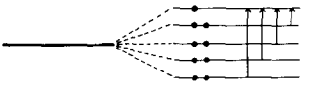
equally at 77K. This spectral resolution gives five CD spectral bands for oxyhemocyanin, i.e. four at 430, 490, 565, and 700 nm, and one at 600 nm. In the ligand field of low symmetry, up to four d-d transitions are theoretically expected for Cu(II) with a d^9 configuration (Fig. 3). Actually, however, observation of four separate peaks have seldom been reported because of their broadness (9). Considering that the above five peaks in the visible region are most probably due to the d-d transitions, we may infer that the both copper ions at the oxygenated active center are cupric (mode c) and that they are non-equivalent



low symmetry field

Fig. 3. d-d Transitions for a d^9 configuration in the low symmetry field.

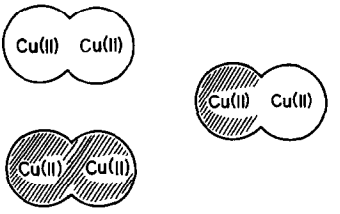


Fig. 4. Schematic representations of the environments of Cu(II) ions in oxyhemocyanin. (i) illustrates the existence of two types of active centers each with equivalent ligand fields, and (ii) the existence of a single type of active centers with non-equivalent ligand fields.

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to each other with respect to the ligand field. Formation of a peroxide ion in this binding mode has also been concluded by Loehr et al. from the resonance Raman spectra (10).

For the Cu(II) environments to be different from each other, the active center is supposed to be either one of the two possibilities illustrated in Fig. 4. Reactions of thiourea, thiocyanate, and several other reagents with oxyhemocyanin (1-3,11) seem to indicate that all the active sites are equally accessible to and attacked by these reagents. Therefore, it is more reasonable to assume a single type of active centers with two non-equivalent Cu(II) sites ((ii) in Fig. 4) rather than two types of active centers each with equivalent environments ((i)). This conclusion is in agreement with the infrared spectral finding about the CO-hemocyanin (12,13), where the carbon monoxide molecule has been inferred to be bound to one of the two copper ions.

It was suggested from the resonance Raman studies (10) that the 565-nm

band could be a charge-transfer band. Even if this is taken into account, the remaining four bands still point to the existence of a different environment for each of the Cu(II) ions, for the reason mentioned above.

Dependence of the MCD spectra only on the B-term indicates that the energy levels of the Cu(II) ions are not degenerate regarding the orbitals and accordingly that the molecular environments of the Cu(II) ions are of very low symmetry. Spin degeneracy at the ground state of the Cu(II) is considered to be absent because the C-term has no effect on the spectra. This reasonably explains the diamagnetism of oxyhemocyanin and the absence of definite ESR signals.

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