Spectral Properties of "Non-Blue" Cupric Copper in Proteins. Circular Dichroism and Optical Spectra of Galactose Oxidase†

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ABSTRACT: Galactose oxidase is a protein which contains a single cupric copper atom per molecule. Concentrated solutions of galactose oxidase are a light, murky green. Circular dichroism and optical spectra were obtained for galactose oxidase in order to compare the spectral properties of a "non-blue" Cu^{2+} in a protein to those reported for "blue" Cu^{2+} in proteins. Unusually large copper optical activity of galactose oxidase solutions is exhibited by circular dichroism extrema at 314, 395, 500, and 610 nm with molecular ellipticities equal to $+18.9 \times 10^3$, $+3.0 \times 10^3$, $+1.5 \times 10^3$, and -8.2 (deg cm²)/dmol, respectively. The optical spectrum contains three broad overlapping bands centered at 445, 630, and 775 nm with molar extinction coefficients equal to 1155, 1015, and 905, respectively. An absorbance transition corresponding to the 314-nm circular dichroism extremum was unmasked by recording the direct difference spectrum between the holo- and apoenzymes. Consideration of the optical and circular dichroism spectra suggests that there is ligand orbital and/or charge-transfer character to one or more of the detected transitions since five rather than four maximally allowed d–d transitions are observed. As expected, the molar absorptivity near 600 nm is somewhat lower for "non-blue" Cu^{2+} than for "blue" Cu^{2+} in proteins. In addition, the "non-blue" Cu^{2+} in proteins exhibits an absorption transition below 400 nm that has not been detected in the spectra of "blue" Cu^{2+}. Analysis of the far-ultraviolet circular dichroism spectrum suggests that galactose oxidase contains a small amount of α helix, but that its peptide bonds are principally in unordered conformations. The presence of a positive peak at 292 nm in the apo–holoenzyme optical difference spectrum suggests that a tryptophan residue or residues is in a more hydrophobic environment in the apoenzyme than in the holoenzyme.

The copper atoms in copper proteins which contain cupric copper as evidenced by their electron spin resonance (esr) spectra have been conveniently classified as either "blue" or "non-blue" depending on the color intensities of their protein solutions and their esr spectra characteristics (Malkin and Malmström, 1970). One prerequisite for relating geometry, ligands and function of Cu^{2+} complexes in proteins is to contrast the spectral properties of "blue" and "non-blue" cupric copper. The observation that solutions of D-galactose:O_2-oxidoreductase (EC 1.1.3.9) are either colorless or light blue-green and the nature of the esr spectrum of galactose oxidase establish that its Cu^{2+} atom is of the "non-blue" variety (Blumberg *et al.*, 1965; Kosman *et al.*, 1973). It is uniquely suited for the characterization of the spectral properties of "non-blue" Cu^{2+} since it may be the only copper protein which contains a single, "non-blue" Cu^{2+} atom per molecule and no other prosthetic group (Malkin and Malmström, 1970). The limited spectral data that are available for "non-blue" cupric copper was obtained with superoxide dismutase which contains two "non-blue" Cu^{2+} and two Zn^{2+} atoms (Wood *et al.*, 1971; Weser *et al.*, 1971). It has not been possible to resolve the optical properties of "non-blue" Cu^{2+} in proteins which also contain other types of copper or coenzymes (Malkin and Malmström, 1970).

Galactose oxidase is an extracellular enzyme which is elaborated in cultures of *Polyporus circinatus* or *Polyporus dendroides* (Cooper *et al.*, 1959; Nobles and Madhosingh, 1963). It catalyzes the oxidation by molecular oxygen of the 6-hydroxyl of D-galactose to its aldehyde; hydrogen peroxide is the second product (Avigad *et al.*, 1962). Although reported values for the molecular weight of galactose oxidase range from 42,000 to 75,000 daltons (Amaral *et al.*, 1963; Kelly-Falcoz *et al.*, 1965; Bauer *et al.*, 1967; Yip and Dain, 1968), recent experiments show that it is a single-chain protein with a molecular weight of 68,000 ± 3000 daltons (Kosman *et al.*, 1974). Neutron activation analysis established that it contains 1 g-atom of copper/70,000 g (Kosman *et al.*, 1974) as first suggested by Amaral *et al.* (1963). This copper appears to be present entirely as Cu^{2+} (Blumberg *et al.*, 1965; Kosman *et al.*, 1973). Furthermore, esr spectra of galactose oxidase in the presence of several different combinations of substrates and inhibitors did not result in any evidence for conversion of Cu^{2+} to Cu^{+} over the course of the enzyme reaction (Blumberg *et al.*, 1965; Kosman *et al.*, 1973).

While optical and esr spectra have been reported for several copper proteins, there has been a paucity of extensive optical activity measurements with these proteins (Malkin and Malmström, 1970). Detailed analysis of the circular dichroic (CD) spectra of azurin which contains a single "blue" copper atom per molecule and laccase which contains both "blue" and "non-blue" copper atoms were given by Tang *et al.* (1968). As shown by their results and those with other metalloenzymes, CD measurements do have considerable potential for providing useful information about the geometry and environment of the copper atom(s) in copper proteins. In this paper we report the optical and CD spectral properties of the galactose oxidase copper atom. The protein CD spectrum of galactose oxidase was also obtained and analyzed in an attempt to gain some insight into possible protein ligands to the copper and as a prerequisite for understanding the relationship between the metal and the protein's conformation in this metalloenzyme.

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Experimental Section

Preparation of Proteins. Galactose oxidase was either purified from commercial sources (Miles) or purified from cultures of *Polyporus circinatus* as previously described (Kosman *et al.*, 1974). The CD spectra of the enzyme from the two sources are identical. The apoenzyme was prepared using diethyldithiocarbamate (Sigma) as reported by Kosman *et al.* (1974).

Protein Solutions. All solutions were prepared in 0.1 M sodium phosphate buffer (pH 7.0) by dialysis of the protein storage solutions containing 1 M $(NH_4)_2SO_4$. The buffer used for the apoenzyme solutions was rendered metal free by passage through a 3 × 45 cm column of a metal-chelating resin (Chelex-100, 100–200 mesh, Bio-Rad).

It was found that dilute (~1.5 mg/ml or less) galactose oxidase solutions were somewhat unstable in that inexplicable changes were observed in the circular dichroism spectra relating to copper optical activity after 2-weeks storage at 4°. Concentrated solutions (5–6 mg/ml or more) were stable for at least 1 month. In any event, for this reason, solutions were usually used within 1 or 2 days after they were obtained from the purification procedure. Protein concentrations were determined from absorbance measurements with a Zeiss PMQII spectrophotometer using the extinction coefficient which was determined by dry weight of $E^{1\%}_{1\,cm} = 15.4$ at 280 nm (Kosman *et al.*, 1974). Protein determinations by the method of Lowry *et al.* (1951) indicated that the extinction coefficient of the apoenzyme is indistinguishable from that of the holoenzyme.

Optical Spectra. The optical absorption properties of the copper atom in galactose oxidase were determined with a 0–0.1 absorbance slide-wire in a Cary 14 spectrophotometer. Absorbance was recorded from 300 to 950 nm by using the visible and infrared number one light sources of the instrument. Cells of 5-cm path length (Hellma) were used which were water jacketed so that the volume of the solution was relatively small. Dynode voltage and sensitivity settings were made to maintain the slit width below 0.5 mm. Ultraviolet difference spectra were obtained with a Cary 15 spectrophotometer on the 0 to 0.1 absorbance scale; cells were used which had a 1-cm path length. For the difference spectra, protein concentrations were matched by absorbancy at 280 nm in the Zeiss spectrophotometer.

CD Spectra. A Cary 60 spectropolarimeter with a Model 6001 CD accessory was used to record spectra from 630 to 185 nm at 27°. Slit widths were programmed to maintain a constant 15-Å bandwidth. The instrument was calibrated at 290 nm with an aqueous solution of *d*-10-camphorsulfonic acid (Matheson, Coleman and Bell) that was recrystallized from ethyl acetate. Overlapping spectra were obtained by using cells of the following path lengths: 0.01, 0.1, 1, and 5 cm. Spectra are reported in terms of mean residue ellipticity, θ, in (deg cm²)/dmol for the optical activity of protein chromophores; and reported as molecular ellipticity, $[\theta]$, in (deg cm²)/dmol for the optical activity originating from the single copper atom per molecule. These ellipticities were calculated from the relationship θ or $[\theta] = \theta_{obsd}(M/100)(1/lc')$. Here, θ_{obsd} is the observed ellipticity in degrees, M the mean residue weight taken as 115 for galactose oxidase or the gram molecular weight = 70,000; l is the path length in decimeters; and c', the protein concentration in g/cm³.

Results

CD Spectrum of the Copper Chromophore in Galactose Oxidase. The copper atom of galactose oxidase exhibits pronounced optical activity as evidenced by at least four distinct CD extrema between 300 and 630 nm (Figure 1). These extrema occur at 314, 395, 500, and 610 nm with molecular ellipticities equal to $+18.9 \times 10^3$, $+3.0 \times 10^3$, $+1.5 \times 10^3$, and -8.2 (deg cm²)/dmol, respectively. There is some indication in the spectra that actually more than four optical activity transitions are detected. Namely, fine structure irregularities are invariably recorded near the 395- and 500-nm extrema. Moreover, although the average ellipticity at 360 nm is statistically indistinguishable from zero, the recorded spectrum is often actually negative at 360 nm. That there is in fact an actual negative contribution at this wavelength is further suggested by the fact that negative values at 360 nm are obtained in the presence of various ligands (Ettinger and Kosman, 1974). The presence of a CD transition above 610 nm is also probable. It was impossible with the instrument used to obtain accurate CD spectra above 630 nm,[1] but an absorbance transition near 775 nm is in the optical spectrum as presented below (Figure 5).

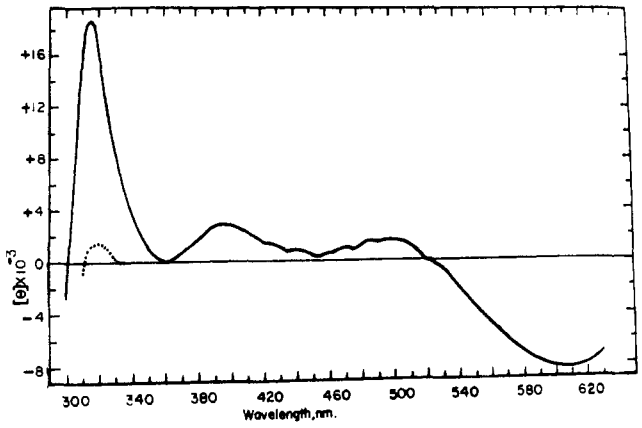

FIGURE 1: CD spectrum from 300 to 630 nm of galactose oxidase (——) and apoenzyme (· · · ·) in 0.1 M sodium phosphate buffer at pH 7.0. Spectra were obtained in a 5-cm path-length cell at a protein concentration of 1.5 mg/ml.

As expected, solutions of the apoenzyme show no optical activity between 310 and 630 nm with the exception of a small band at the wavelength where the CD spectrum of the copper in the holoenzyme reaches its highest value (Figure 1). This apparent anomaly is attributed to residual copper since the enzymatic activity of the apoenzyme corresponded exactly to the relative ellipticities at 314 nm for the apo- and holoenzymes.

Far-Ultraviolet CD Spectra of Galactose Oxidase and Its Apoenzyme. The far-ultraviolet CD spectrum of galactose oxidase is characterized by a positive extremum at 229 nm, negative extrema at 214 and 200 nm, and a positive extremum at 190 nm (Figure 2). The magnitudes of the mean residue ellipticities at 214, 200, and 190 nm are relatively low in comparison to values obtained with polypeptides used as models of protein structure (Greenfield and Fasman, 1969). Although a 229-nm extremum is not usually observed in protein spectra, bands near 230 nm have been observed in the spectra of some other proteins (Fasman *et al.*, 1966; Beychok *et al.*, 1966). Their origin has been attributed to aromatic groups. One certainty about the origin of this 229-nm maximum is that it is not related to any copper interaction since it appears with equal intensity in the spectrum of the apo-

[1] The manufacturer was unable to supply electrooptic plates that could withstand the high voltages needed to yield circularly polarized light at high wavelengths.

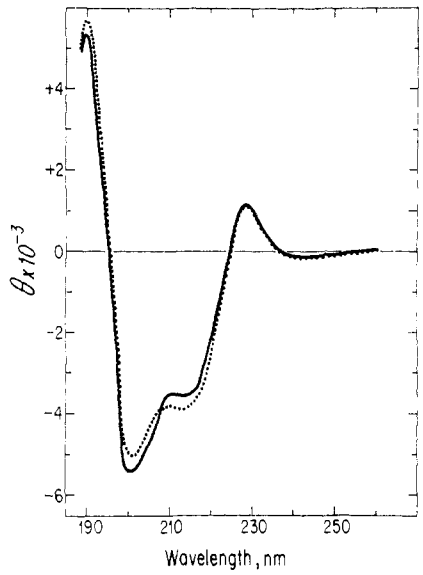

FIGURE 2: Far-ultraviolet CD spectra of galactose oxidase (——) and apoenzyme (· · · ·) in 0.1 M sodium phosphate buffer at pH 7.0. Spectra were obtained at a protein concentration of 1.5 mg/ml with the use of cells of 0.1- and 0.01-cm path lengths.

enzyme (Figure 2). The small but significant differences between the far-ultraviolet spectra of the holo- and apoenzymes suggests that there are small but significant differences in their conformations.

Near-Ultraviolet CD Spectra of Galactose Oxidase and Its Apoenzyme. Galactose oxidase contains the following amino acid residues which could contribute to its near-ultraviolet CD spectrum: 18 tryptophans, 26 tyrosines, 16 phenylalanines, and 2 disulfide bonds (Kosman *et al.*, 1974). Perhaps the most striking characteristics of the near-ultraviolet CD spectrum of galactose oxidase below 300 nm are the sharp bands with extrema at 295 (−) and 290 (+) nm (Figure 3). These bands most probably originate from tryptophan residues in galactose oxidase; this is the wavelength region where absorption and CD maxima occur in the spectra of model tryptophan compounds (Strickland *et al.*, 1969). The remainder of the near-ultraviolet CD spectrum of galactose oxidase is characterized by a broad asymmetrical band with a positive extremum near 268 nm and a shoulder near 285 nm (Figure 3). This probably represents unresolved contributions from several aromatic side chains and disulfide bonds.

The spectrum for the apoenzyme revealed a small, but

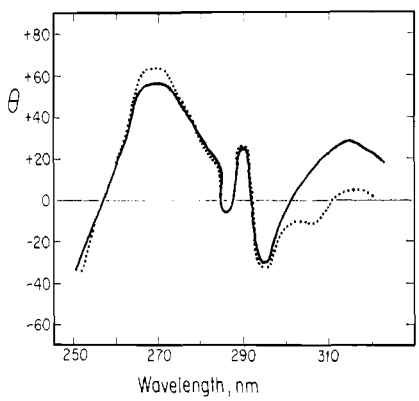

FIGURE 3: Near-ultraviolet CD spectra of galactose oxidase (——) and apoenzyme (· · · ·) in 0.1 M sodium phosphate buffer at pH 7.0. Spectra were obtained in a 1-cm path-length cell at a protein concentration of 1.5 mg/ml.

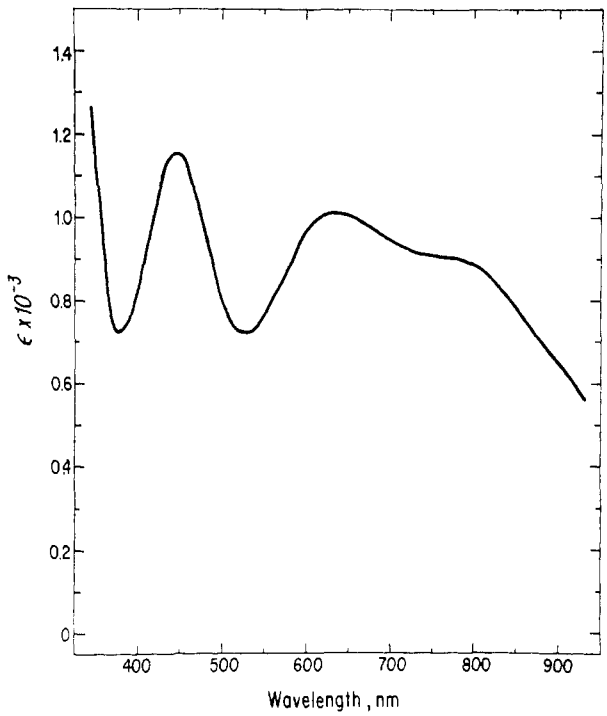

FIGURE 4: Visible optical spectrum of galactose oxidase in 0.1 M sodium phosphate buffer at pH 7.0. Spectrum was obtained at a protein concentration of 1.5 mg/ml with a 5-cm path-length cell. A molecular weight of 70,000 daltons was assumed to calculate molar extinction values, ε.

probably significant, difference from the holoenzyme at the 268-nm maximum (Figure 3). It is impossible to deduce from this observation alone whether this difference is due to removing an amino acid–copper interaction or to an indirect effect on protein conformation. A negative CD band with a maximum at 305 nm is also observed in the spectrum of the apoenzyme (Figure 3). This transition(s) is masked by the 314-nm copper band in the holoenzyme spectrum. It is also present in the spectra recorded for the holoenzyme in the presence of some substrates and products which reduce the 314-nm band (Ettinger and Kosman, 1974). Tryptophan or disulfide bond optical activity could give rise to a CD transition at this wavelength (Strickland *et al.*, 1969; Beychok, 1967).

Optical Spectrum of the Copper Chromophore in the Galactose Oxidase. The optical spectrum of galactose oxidase is characterized by broad overlapping absorption bands centered at 630 and near 775 nm with a sharper band centered at 445 nm (Figure 4). Molar extinction values at 445, 630, and 775 nm were 1155, 1015, and 905, respectively. The values at 445 and 630 nm are in agreement with those previously noted by Bauer *et al.* (1967). The apoenzyme does not absorb in this region.

Difference Absorbance Spectrum of Apo- vs. Holoenzyme. Since the optical spectrum was obtained with a 5-cm cell, the possibility of resolving any absorbance transition corresponding to the 314-nm CD transition was precluded by the appearance of relatively large absorbancy from aromatic chromophores. To eliminate this obstacle, an ultraviolet difference absorbance spectrum was recorded directly with the apoenzyme as the sample and galactose oxidase as the reference. As seen in Figure 5, a negative peak at 314 nm ($\Delta\epsilon = -1370$) is observed in the difference spectrum, *i.e.*, a 314-nm absorption transition is associated with the copper atom of galactose oxidase.

A positive peak at 292 nm ($\Delta\epsilon = +950$) is also observed in the apo–holoenzyme difference spectrum (Figure 5). This

suggests that at least one tryptophan side chain is in a somewhat different environment in the apoenzyme than the holoenzyme. The positive peak near 260 nm suggests that other aromatic residues may be in different environments as well. While the measured difference magnitudes at 314 and 292 nm were highly reproducible, the magnitude at the 278-nm difference peak was extremely variable. Therefore, the difference at 278 nm reflects at least in part some experimental error in matching the concentrations of the apo- and holoenzymes. Note that the molar difference at 278 nm is approximately 1% of the molar extinction coefficient of galactose oxidase or the apoenzyme.

Discussion

Multiple ligand orbital and/or charge-transfer contributions have been suggested to account for the intensity and multiplicity of transitions in cobalt and copper systems (McCaffery *et al.*, 1965; Tang *et al.*, 1968). Although four high-intensity pure d–d transitions can be accounted for in terms of just distortions from square planar geometry of copper complexes (Blumberg, 1966), at least five copper transitions are detected in the galactose oxidase spectra. The 630-nm absorbance band has its CD counterpart at 610 nm; the 314-nm difference absorbance peak corresponds to the 314-nm CD band. It would appear that the 395-nm CD band corresponds to the 445-nm absorbance envelope but it is displaced due to overlaps in CD transitions. At least one transition near 500 nm is indicated by the positive ellipticity in this region. The fifth transition is the 775-nm band in the optical spectrum which could not be recorded with the CD instrument used here. When an instrument is used which is accurate out to 1000 nm, the CD spectra of the copper proteins laccase, stellacyanin, and ceruloplasmin contain an extremum near 800 nm (Falk and Reinhammer, 1972). Since the 314-nm transition is at the highest energy of the five transitions and the optical activity at 314 nm is unusually large, it is tempting to assume that this is a charge-transfer transition while the remaining four transitions are the allowed d–d transitions. However, charge-transfer transitions are usually of higher intensity than d–d transitions while the molar absorptivity of the 314-nm band is not larger than that recorded for any of the other transitions. Moreover, pure d–d transitions at this energy are quite possible. There is no *a priori* reason, then, for assigning charge-transfer character exclusively to the 314-nm band. Furthermore, although fine structure in a pure d–d transition is possible, the fine structure which is consistently detected in the CD spectra near the 395-nm and 500-nm extrema and the possible negative ellipticity near 360 nm suggest that actually more than five transitions might contribute to the CD spectrum above 300 nm. Thus, while square-planar distortions may well be the principal determinants of one or more of the transition intensities, ligand contributions must also be invoked to account for the multiplicity of detectable transitions. More than four transitions are also evident in the CD spectrum of the "blue" Cu^{2+} atom in azurin (Tang *et al.*, 1968).

Two possible generalities emerge from this work about the optical properties of "non-blue" Cu^{2+} in contrast to the "blue" Cu^{2+} in proteins. Although the molar extinction coefficient at the 630-nm maximum in the galactose oxidase optical spectrum is approximately five times greater than observed in model copper–amino acid complexes, it is between one-third and one-sixth the absorptivity reported per mole of Cu^{2+} for "blue" proteins (Malkin and Malmström, 1970; Brill *et al.*, 1964; Nakamura and Ogura, 1966). Hence, as

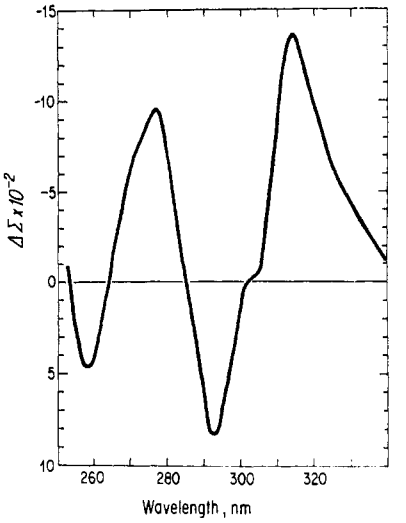

FIGURE 5: Difference optical spectrum with apoenzyme as the sample and galactose oxidase as the reference. Protein concentrations were 1.5 mg/ml in 0.1 sodium phosphate buffer at pH 7.0. 1-cm path-length cells were used. A molecular weight of 70,000 daltons was assumed to calculate difference molar extinction values, $\Delta\epsilon$.

expected, the absorptivity in the 600-nm region is greater for "blue" than "non-blue" cupric copper in proteins. However, that there is a relatively wide range of absorptivities for "non-blue" cupric copper is indicated by the molar absorptivity of superoxide dismutase at 680 nm which is only one-fourth that of galactose oxidase at 625 nm though this enzyme contains two "non-blue" copper atoms (Bannister *et al.*, 1971; Weser *et al.*, 1971).

In addition to the relative extinctions near 600 nm, there is a second apparent common spectral characteristic of galactose oxidase, superoxide dismutase, and those proteins which contain at least 1 "non-blue" Cu^{2+} atom/mol (laccase, ceruloplasmin, ascorbate oxidase). The recorded optical spectrum of each protein with "non-blue" Cu^{2+} is characterized by the appearance of a band between 310 and 340 nm. Moreover, what is particularly striking is the fact that the recorded optical spectra of copper proteins containing only "blue" Cu^{2+} invariably do not contain an absorbance peak or shoulder in this wavelength region. This difference may reflect a greater resolution between the energies of the individual d orbitals or between ligand–copper orbital hybrids in the "non-blue" Cu^{2+} systems. Alternatively, a near-ultraviolet band may be masked by protein chromophores in the spectra of "blue" Cu^{2+}. It may be possible to distinguish between these possibilities by applying the difference spectra method reported here to a protein such as azurin that contains only "blue" Cu^{2+}. It should be noted that although laccase contains one "non-blue" Cu^{2+}, the 330-nm peak in its optical spectrum was assigned to a postulated Cu^{2+}–Cu^{2+} couple (Fee *et al.*, 1969). On the other hand, fluoride has recently been shown to cause changes near 320 nm in the optical spectra of laccase and ceruloplasmin which have been attributed to binding at the "non-blue" copper of these proteins (Brändén *et al.*, 1973).

The copper optical activity of galactose oxidase solutions is unusually large. The molecular ellipticity at 314 nm is approximately five times that of the resolved band at 350 nm in the spectrum of superoxide dismutase (Weser *et al.*, 1971). Moreover, the magnitude at the 314-nm extremum is twice that of any of the resolved extrema from the CD spectra of *Pseudomonas azurin* or *Polyporus laccase* (Tang *et al.*, 1968). Its

position is also at a higher energy than any copper protein band reported thus far. What is perhaps even more striking is the observation that the molecular ellipticity at 610 nm of galactose oxidase solutions is equal to or greater than that reported for azurin or laccase in this spectral region (Tang *et al.*, 1968). Thus, it has been seen that while the absorbance of "non-blue" Cu^{2+} proteins is lower than the "blue" type near 600 nm, the optical activity in this wavelength region can be greater for "non-blue" Cu^{2+} in proteins. This higher optical activity at 610 nm may arise from distortions from square-planar geometry of the copper complex that give rise to transitions characterized by relatively high magnetic dipole moments and low electric dipole moments. Alternatively, the high optical activity may reflect a highly asymmetric protein environment at the copper locus rather than higher asymmetry of the copper *per se*. A third possibility is that the high optical activity bears a direct relation to the nature of, or the contributions from, the protein ligands to the copper. Most likely, even though one cannot as yet quantitatively assess the individual contributions, each of these factors has an effect on the optical activity. Given the high copper optical activity of galactose oxidase, one can assert that to equate "non-blue" copper with simply less distortion than "blue" copper is not valid.

Mere inspection of the far-ultraviolet CD spectrum of galactose oxidase reveals that it does not contain large amounts of either α helix or β structure since the spectrum does not contain the distinguishing characteristics obtained with models for these structures. The position of the 200-nm extremum suggests that a high percentage of the peptide bonds in galactose oxidase are in unordered conformations since this is near the position of the extremum in the CD spectra of proteins such as α-casein which are purported to be largely unordered (Fasman *et al.*, 1970). Although the absence of a distinct extremum at 220 nm and the relatively low ellipticity at this wavelength preclude the possibility that there is a large amount of α helix in galactose oxidase, the presence of the positive 190-nm maximum suggests that some α helix is present. The fact that there is a negative extremum near 217 nm (214 nm) suggests that a small amount of antiparallel β structure may also be present (Townend *et al.*, 1966), but a shoulder near 217 nm is also present in the spectra of unordered proteins (Fasman *et al.*, 1970). It is increasingly apparent that unless a protein contains a preponderance of its peptide bonds in α helix or antiparallel β structure, its far-ultraviolet CD spectrum cannot be interpreted unambiguously (Greenfield and Fasman, 1969; Chen *et al.*, 1972; Madison and Schellman, 1972).

Since the differences between the far-ultraviolet CD spectra of apo- and holoenzyme are small, it can be concluded that any possible contributions to the holoenzyme's spectrum from copper–peptide or copper–aromatic group interactions must be rather small.

From both the near- and far-ultraviolet CD spectra of apoenzyme it can be inferred that there are small, but significant, differences between the conformations of the apo- and holoenzymes. In agreement with this finding are recent results in our laboratories that indicate that the apoenzyme is less stable to thermal denaturation and to denaturation at both high and low pH than the holoenzyme (Ettinger, 1973).

The difference absorbance peak at 292 nm was the most definitive spectral difference observed between the apo- and holoenzymes below 300 nm. Since this difference peak had a positive sign, a tryptophan residue or residues must be somewhat less exposed to solvent water in the apoenzyme than the holoenzyme (Yanari and Bovey, 1960). However, there are no significant differences between 290 and 295 nm in the CD spectra of the two enzyme forms. This implies that either the CD spectra are insensitive to this difference in tryptophan environment or that the absorbance difference is accompanied by a difference in magnetic dipole moment(s) of a tryptophan(s) transition(s) which results in no net change in optical activity.

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Circular Dichroism Spectra of the Copper Enzyme, Galactose Oxidase, in the Presence of Its Substrates and Products†

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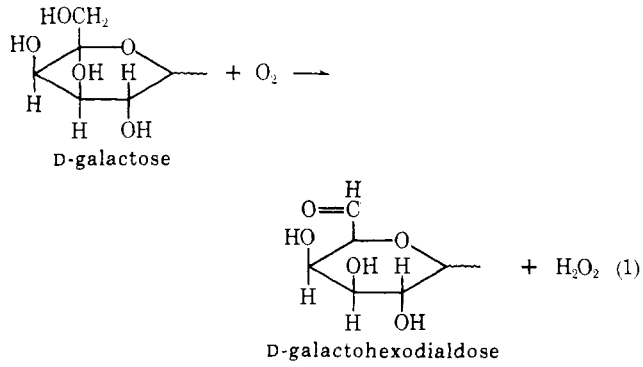
ABSTRACT: Circular dichroism spectra were recorded for galactose oxidase in the presence of its substrates and products to determine which of these ligands interacts with the copper atom of the enzyme. Neither galactose nor oxygen has any detectable effect on protein conformation as evidenced by near- and far-ultraviolet circular dichroism spectra. In the absence of oxygen, D-galactose at concentrations an order of magnitude higher than its K_m causes a marked reduction of galactose oxidase copper optical activity. At the 314- and 610-nm copper extrema, the ellipticity is reduced to less than half of its value for the enzyme alone. On the other hand, removal or addition of oxygen to galactose oxidase solutions results in only small, barely significant, changes in copper optical activity which are in the same direction. In view of circular dichroism, kinetic, and electron spin resonance results, it is inferred that during the course of the enzyme reaction oxygen binds after galactose and interacts directly with reducing equivalents on the substrate rather than within the inner coordination sphere of the copper atom. In a manner similar to galactose, the aldehyde product of the enzyme reaction also interacts with the copper atom as evidenced by large changes in optical activity. Experiments with the other product, hydrogen peroxide, were somewhat ambiguous since this compound has effects on protein conformation in addition to its effects on copper optical activity. In addition to these effects on copper optical activity, large changes in near-ultraviolet circular dichroism spectra are observed with dihydroxyacetone and galactohexodialdose. These changes indicate that a tryptophan(s) residue(s) is at or near the active site of galactose oxidase.

This paper reports the effects of the substrates and products of the reaction catalyzed by D-galactose:O_2 oxidoreductase (EC 1.1.3.9) on the enzyme's pronounced copper and tryptophan optical activity (Ettinger, 1974a). Galactose oxidase catalyzes reaction 1. Michaelis constants which have been determined are: K_m = 20 mM for galactose at saturating oxygen concentrations; K_m = 0.6 mM for oxygen at 20 mM galactose; K_I = 13 mM for hydrogen peroxide at saturating concentrations of both galactose and oxygen; K_I is undetermined for galactohexodialdose (Kwiatkowski and Kosman, 1974). Although galactose oxidase is relatively specific for D-galactose when its activity with other hexoses is tested (Cooper *et al.*, 1959; Avigad *et al.*, 1962; Schlegel *et al.*, 1968), it also catalyzes the oxidation of nearly all aliphatic or aromatic primary alcohols (Hamilton *et al.*, 1973; Kwiatkowski and Kosman, 1974). In particular, dihydroxyacetone was found to be a better substrate for the enzyme than D-galactose in that k_{cat}/K_m for this substrate is fourfold greater than for D-galactose itself (Hamilton *et al.*, 1973; Zancan and Amaral, 1970). Both sequential and Ping-Pong kinetic schemes have been proposed for the galactose oxidase reaction (Hamilton *et al.*, 1973; Kwiatkowski and Kosman, 1974; Hamilton, 1969).



The objectives of this work were to demonstrate the utility of copper circular dichroism (CD) spectra as a probe of ligand interactions with copper proteins, to compare the spectral properties of the liganded and unliganded copper complex in galactose oxidase, to determine if the order of binding in the galactose oxidase bisubstrate reaction could be discerned from spectral measurements which were independent of

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