A Circular Dichroism Study of Undegraded Human Ceruloplasmin†

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ABSTRACT: The CD spectrum of human ceruloplasmin (Cp) has been studied between pH 6.90 and 12.00 in the far-ultraviolet, near-ultraviolet, and visible light regions. The spectrum in the far-ultraviolet region showed that undegraded holo and apo single-chain ceruloplasmin and a cleaved ceruloplasmin preparation have a low content of α helix but a high content of β and unordered structure. A conformational transition accompanied by a decrease in β and an increase in unordered structure occurred at pH 11.10 for intact ceruloplasmin. This transition probably involved the ionization of buried tyrosines, as shown by the increase of a near-ultraviolet band at 250 nm. The copper atoms may contribute to the stability of the native structure since the conformational transition occurred at a low pH value (10.50) in the case of apoceruloplasmin. The apo-Cp also presented a more intense CD band at 292 nm, suggesting the presence of tryptophan(s) near the environment of copper(s) in the molecule where no tyrosine residue seems to be involved. The spectrum between 320 and 700 nm of intact and cleaved Cp was resolved into six Gaussian bands which were assigned to type-1 copper atoms. Important changes in only two of these bands upon pH increase (bands III at 541 nm and VII at 322 nm) confirmed the nonequivalence of the two blue coppers in human ceruloplasmin.

Ceruloplasmin [ferroxidase; iron(II):oxygen oxidoreductase, EC 1.16.3.1] (Cp)[1] is the major copper protein present in mammalian serum. It belongs to a group of intensely blue proteins that have been extensively studied by several spectroscopic techniques in order to obtain insight into their copper–ligand structure. The blue color of oxidized Cp that is associated with 610-nm absorption is due to the two paramagnetic type-1 coppers; the paramagnetic type-2 copper has no distinct absorption band in the visible region of the spectrum, whereas the diamagnetic type-3 copper is supposed to be associated with the absorption band at 330 nm (Andréasson et al., 1970; Carrico et al., 1971). The copper atoms of Cp are accessible to external ligands; for example, the interactions of the protein-bound coppers with several inhibitory anions and with nitric oxide have been studied by circular dichroism (Hervé et al., 1976; Van Leuween & Van Gelder, 1978). However, only one CD study of the structure of the protein itself has been reported (Freeman & Daniel, 1978), and the preparation of Cp was the same one for which a multichain structure had earlier been reported (Freeman & Daniel, 1973). Despite the latter and other earlier claims that human Cp has a subunit structure [see review by Poulik & Weiss (1975)], there is now strong evidence that human Cp has single-chain structure and that proteolytic cleavage causes the heterogeneity present in most preparations (Rydén, 1971, 1972; Kingston et al., 1977; Moshkov et al., 1979). This paper describes the effect of pH on the structural changes of the undegraded single-chain protein as shown by circular dichroism in the visible and ultraviolet light regions. The comparison is made with the single-chain apoprotein and with a slightly degraded ceruloplasmin preparation.

The degraded preparation had been cleaved spontaneously by proteolytic enzymes present in plasma (Kingston et al., 1977) and consisted of three major fragments: (1) a 67 000-dalton amino-terminal fragment for which only partial sequence data are available (Dwulet & Putnam, 1980), (2) a 50 000-dalton internal fragment, the sequence of which has just been completed (Dwulet & Putnam, 1981), and (3) the carboxyl-terminal 18 650-dalton fragment for which the sequence has been reported in preliminary form (Kingston et al., 1979) and also has been documented (Kingston et al., 1980a,b). Because of the strong interaction of these three fragments, they could only be separated by use of severe denaturing conditions; this rendered them insoluble, caused loss of the copper, and made them unsuitable for study by circular dichroism.

Experimental Procedures

Preparation of Proteins. Undegraded human ceruloplasmin was isolated as a single-chain protein ($M_r \simeq 135\,000$) by fractionating the cryosupernatant from fresh frozen plasma at 5 °C with poly(ethylene glycol) (PEG 4000), which yielded a Cp-enriched fraction in the 20% PEG supernatant (Hao & Wickerhauser, 1977). Three steps of chromatography on DEAE-Sephacel, hydroxylapatite, and Sephadex G-200 produced a homogeneous protein with a maximal enzymatic activity, an A_{610}/A_{280} ratio of 0.046, and a copper content of 5.85 ± 0.3 atoms of copper per molecule (Noyer et al., 1980). The oxidase activity, tested at 23 °C, with *N,N*-dimethyl-*p*-phenylenediamine as substrate, was $\Delta A_{550}^{1\,min} = 1.45$ per mg of protein, and the K_M value was 0.11 mM. This purified Cp presented a single band on analytical electrophoresis in polyacrylamide gel containing sodium dodecyl sulfate ($M_r \simeq 135\,000$), a single band in the α-globulin region upon analytical zone electrophoresis on cellulose acetate, and an unambiguous NH_2-terminal sequence as determined by automatic sequencing (Noyer et al., 1980). Undegraded Cp preparations were stored at −20 °C in a 0.5 M phosphate buffer, pH 6.90, at a concentration of approximately 2.5%, without noticeable loss of blue color during a few weeks. Protein concentrations were determined spectrophotometrically by using the following extinction coefficients: $\epsilon_{280} = 21.74 \times 10^4\ M^{-1}\ cm^{-1}$ ($A_{280}^{1\%} = 16.1$) and $\epsilon_{610} = 1.00 \times 10^4\ M^{-1}\ cm^{-1}$ ($A_{610}^{1\%} = 0.741$) (Noyer et al., 1980). This preparation was designated Cp 10.

Single-chain apoceruloplasmin (cyanoapo-Cp) was prepared by exhaustively dialyzing a 15 mg/mL solution of holoprotein against 0.05 M KCN in 0.1 M phosphate buffer, pH 7.2. The

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[1] Abbreviations used: Cp, ceruloplasmin; apo-Cp, ceruloplasmin with no copper ions; CD, circular dichroism; M_r, molecular weight; PEG, poly(ethylene glycol); EPR, electron paramagnetic resonance.

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cyanide was subsequently removed by dialysis against buffer. Like the native holoprotein, the cyanoapo-Cp showed a single band on analytical gel electrophoresis but migrated with a higher anodal mobility upon analytical zone electrophoresis on cellulose acetate at pH 8.6. Both samples showed a precipitin line of identity with antihuman ceruloplasmin (Meloy) in Ouchterlony radial immunodiffusion.

Cleaved ceruloplasmin was a commercial preparation obtained from E. R. Squibb and Sons, Inc., through the kindness of Dr. J. J. Hagan. This sample [Cp 1 of Kingston et al. (1977)] was known to be cleaved into at least three major fragments with approximate molecular weights of 18 650, 53 000, and 67 000 as shown by analytical electrophoresis on polyacrylamide gel with sodium dodecyl sulfate (Kingston et al., 1977). The A_{610}/A_{280} ratio was only 0.030; the oxidase activity at 23 °C was $\Delta A_{550}^{1\text{min}} = 0.87$ per mg of protein, and $K_M = 0.20$ mM. The complete amino acid sequence of the 18 650-dalton fragment has been reported (Kingston et al., 1979).

Spectrophotometric Measurements. Absorption measurements were carried out with a Cary 14 or Zeiss (PMQ II) spectrophotometer. CD spectra were recorded with a Durrum-Jasco spectropolarimeter, Model J-10, with a Sproul Scientific SS-10 CD attachment. All recordings were made at 25 °C in an air-conditioned instrument center. Two solutions with different concentrations were routinely prepared for each protein sample. The near-ultraviolet and visible light region usually required a concentration in the range of 2.0 to 5.0 mg/mL and a cell of optical path 0.10 to 1.00 cm, respectively. For the far-ultraviolet region, protein concentrations in the range of 0.5–0.8 mg/mL and an optical path of 0.10 or 0.05 cm were employed, so that the photomultiplier voltage never exceeded 0.7 kV. Slit widths were 0.7 mm or less above 300 nm and 2 mm at 250 nm and below. The spectra were recorded on sensitivity scale settings of (1–2) × 10^{-3} °C/cm and an accumulation time constant of 4 s. The scanning time was 10 to 15 min. Two or three scannings were normally accumulated for each sample. Ellipticities were expressed as mean residue ellipticities, $[\theta]$, in deg cm^2 dmol^{-1} in the far-ultraviolet region and as molecular ellipticities, $[\theta]_M$, in the near-ultraviolet and visible light regions. In the visible region both absorption and CD spectra were resolved manually into a series of six Gaussian bands, retaining the position and width of the corresponding bands, and adjusting the sign and magnitude. The spectra were thus plotted as a function of the wavenumber (cm^{-1}). A mean residue weight of 115 was used in the calculation of $[\theta]$.

For the pH-dependence studies, the protein solutions were dialyzed at 5 °C against buffer of the desired pH for 14 to 15 h. For minimization of any time effect, especially at high pH values, all the samples were always dialyzed during the same period and then maintained in an ice bath until measurement was performed. The buffers used were pH 6.90, 0.05 M phosphate/0.10 M NaCl; pH 9.00–10.60, 0.05 M borates/0.10 M NaCl; pH 11.0–12.00, 0.05 M phosphate/0.10 M NaCl. Tris buffer and the use of azide ions as an antibacterial agent were avoided because of their strong interaction with ceruloplasmin (Kasper & Deutsch, 1963; Hervé et al., 1976). Prior to CD measurements the protein solutions were filtered through a Millipore membrane (type HA, 0.45-μm pore size) to remove particles in suspension. The protein concentration was determined spectrophotometrically by the absorbance at 280 nm immediately after the CD measurement.

For testing the reversibility of the spectra, extra samples were prepared, dialyzed at 5 °C against the desired buffer for 14–15 h, and then immediately dialyzed back for the same time against the 0.05 M phosphate buffer/0.10 M NaCl at pH 6.90.

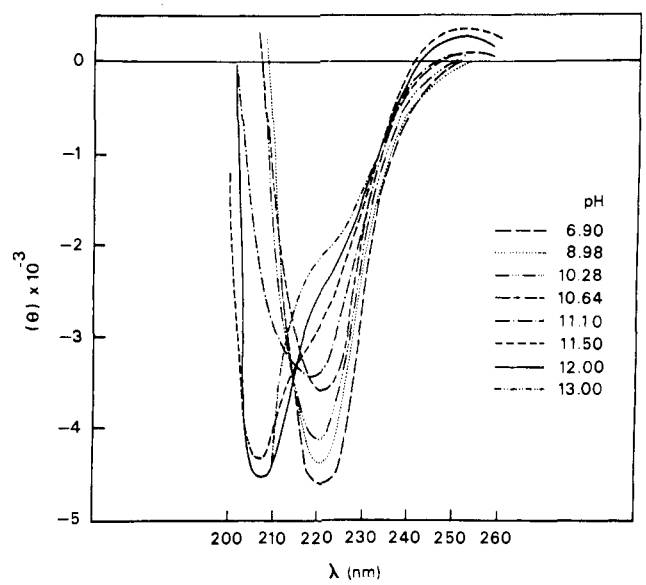

FIGURE 1: Far-ultraviolet CD spectra of undegraded human ceruloplasmin. The ordinate represents the mean residue ellipticity (deg cm^2 dmol^{-1}). Conditions are (---) pH 6.90, 0.05 M phosphate buffer; (···) pH 8.98, 0.05 M borate buffer; (-·-·) pH 10.28, 0.05 M borate buffer; (-·--) pH 10.64, 0.05 M borate buffer; (-·-) pH 11.10, 0.05 M phosphate buffer; (- - -) pH 11.50, 0.05 M phosphate buffer; (—) pH 12.00, 0.05 M phosphate buffer; (·····) pH 13, 0.1 M NaOH. All the buffers contain 0.1 M NaCl and the temperature is 25 °C.

Results

Far-Ultraviolet Region. The CD spectrum between 200 and 250 nm of single-chain human Cp is represented in Figure 1 at neutral pH and at alkaline pHs. The occurrence of a trough at 220 nm similar to the one observed in the β form of poly(L-lysine) is an indication of the presence of β conformation in this protein (Townend et al., 1966). Moreover, the position of an extremum near 200 nm (not shown) suggests that a high percentage of the peptide bands are in unordered conformation 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).

The absence of a trough or shoulder at about 206 nm strongly suggests that very few or no amino acid residues have the α-helical conformation and that the residues not included in the β structure are in an unordered form. Taking the ellipticity values at 208 nm determined by Greenfield & Fasman (1969) for synthetic polypeptides, we have estimated that only about one-tenth of the residues are in the α-helix conformation. This value could not be confirmed, however, by using a computation method derived from Chen et al. (1972) based on the known structure of eight proteins.

The trough present at 220 nm at neutral pH vanishes when the pH is raised, and is displaced to 207 nm at pH 11.50 and above. The transition occurs at about pH 11.10 (Figure 2, lower part). Up to the latter pH, the CD spectra can be reversed by returning the pH to neutrality by dialyzing the sample against the pH 6.90 buffer. Above the pH of transition, the displacement of the trough from 207 nm can not be reversed, and only a small shoulder appears at 220 nm upon lowering the pH to 6.9 (not shown).

The far-ultraviolet CD spectra for the cleaved Cp is very similar over the entire pH range to that of single-chain Cp, and consequently the transition of the trough position appears

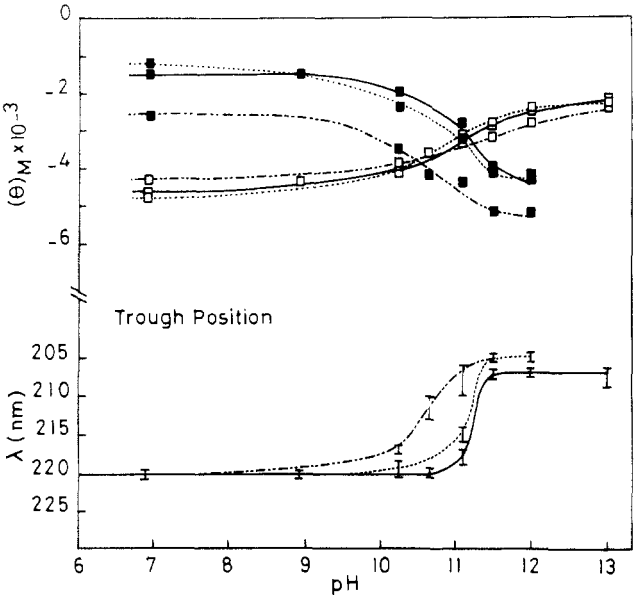

FIGURE 2: (Upper) Mean residue ellipticities (deg cm^2 dmol^{-1}) at 220 nm (□) and 210 nm (■). (Lower) Position of the trough (in nm) at various pH values for undegraded (—), undegraded cyanoapo (----), and commercial cleaved (···) human ceruloplasmin. The conditions are as in Figure 1.

at the same pH value (Figure 2). Above this transition pH, e.g., at pH 11.50 and 12.00, the trough for the cleaved Cp is located at 205 nm with a somewhat higher negative mean-residue-weight ellipticity, but as with the uncleaved protein, the CD spectra can be reversed up to the pH of the transition. The results indicate that the proteolytic fragmentation of the polypeptide chain in this preparation of Cp does not significantly affect the gross structure of the protein and does not alter its refolding ability upon lowering the pH to neutrality.

In contrast, the far-ultraviolet CD spectrum of the single-chain cyanoapo-Cp shows more important differences from the native holo-Cp than does that of a cleaved Cp (Cp 1). At neutral pH the trough for cyanoapo-Cp at 220 nm is less intense and much broader, which indicates more unordered structure. This larger trough width is maintained at higher pHs until the occurrence of the transition which now appears at pH 10.80 (Figure 2). At pH 11.50 and 12.00, the trough is located at 205 nm as for the cleaved ceruloplasmin but with an ellipticity of -5.9×10^3 and -6.0×10^3 deg cm^2 dmol^{-1}, respectively. All the spectra can be reversed up to pH 10.28. These results confirm the role of the copper atoms in maintaining the native conformation of the molecule. Removal of the copper atoms by the cyanide or by the diethyldithiocarbamate method was reported by Kasper & Deutsch (1963) to cause unfolding of the molecule, as shown by sedimentation studies. The present CD studies show that this unfolding is only partial at neutral pH but in comparison with the holo-Cp is more rapidly enhanced by increasing the net charge of the molecule by raising the pH. The apoprotein also exhibits an increased anodic mobility upon electrophoresis, which we assume to be caused by a large number of carboxyl groups undergoing a pK shift to a lower value (Kasper & Deutsch, 1963; Noyer et al., 1980).

Near-Ultraviolet Region. Ceruloplasmin contains the following amino acid residues which could contribute to its near-ultraviolet CD spectrum: 14 tryptophans, 63 tyrosines, 48 phenylalanines, and 6 disulfide bonds (Noyer et al., 1980; Rydén & Björk, 1976; Kasper & Deutsch, 1963). At neutral pH the spectrum of the single-chain Cp is characterized by a large negative background presenting two distinct extrema:

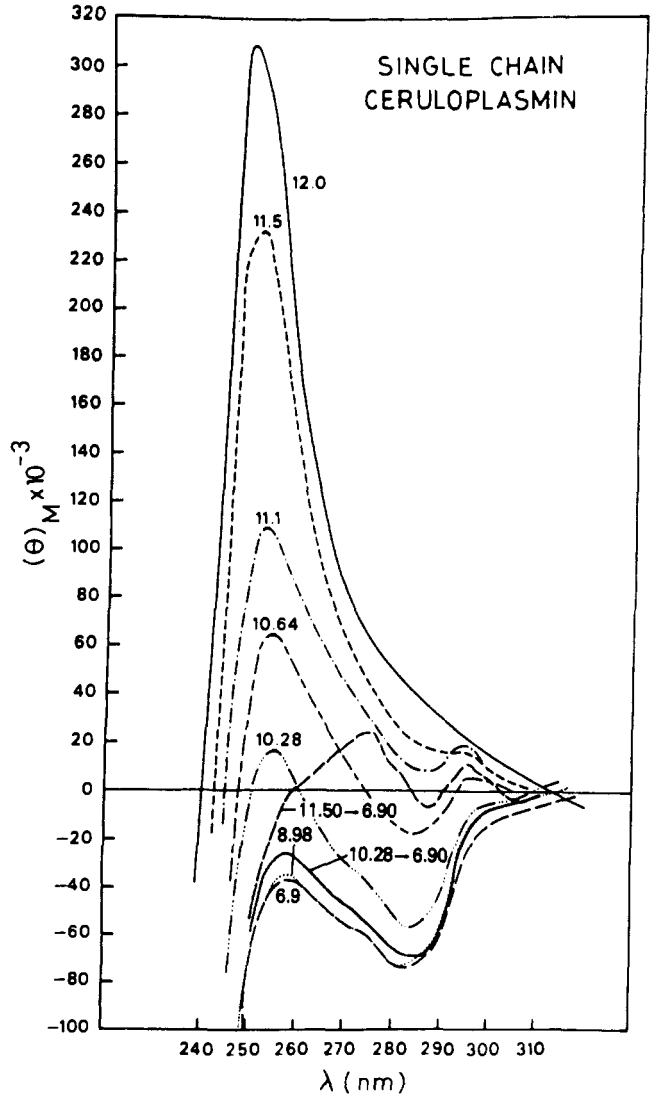

FIGURE 3: Near-ultraviolet CD spectra of undegraded human ceruloplasmin. The ordinate represents molecular ellipticity (deg cm^2 dmol^{-1}). (--) pH 6.90, (···) pH 8.98, (·····) pH 10.28, (----) pH 10.64, (-·-·) pH 11.1, (---) pH 11.50, (—) pH 12.00. Conditions as in Figure 1. In the lower part of the figure the arrows indicate the spectra obtained at pH 6.90 after readjustment from pH 10.28 and 11.50, respectively (see Experimental Procedures).

a minimum located near 282 nm and a maximum at 257 nm (Figure 3). A large shoulder is present at 273 nm and two smaller ones at 277 and 290 nm. Un-ionized tyrosine residues are expected to contribute bands near 273–282 nm.

When the pH is raised, the extremum at 257 nm is considerably enlarged and is shifted to 250 nm while the whole spectrum reaches positive values. The positive peak near 250 nm is similar to the band observed in immunoglobulins during alkaline titration (Finazzi-Agro et al., 1973) and is mainly attributed to ionization of tyrosine chromophores. At a pH above 10.64 a positive band distinctly appears at 292 nm; this is assigned to an "L_b" tryptophanyl band as in the case of azurin and apoazurin (Adman et al., 1978).

The near-ultraviolet spectra of cleaved ceruloplasmin and of single-chain ceruloplasmin under the same conditions are given in Figures A and B of the supplementary material (see paragraph at end of paper regarding supplementary material). The different CD spectra in the aromatic region are given in Figure 4.

As shown in Figure 5, a transition in the ellipticities at 250 nm (assigned to ionized tyrosyls) occurs at pH 11.10, i.e., the pH at which the secondary structure of the protein undergoes

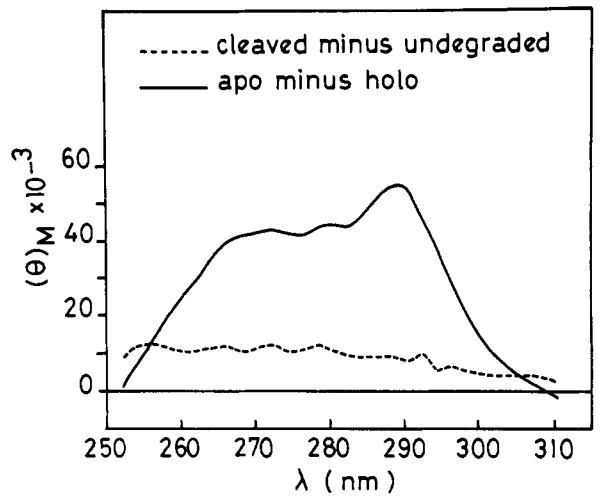

FIGURE 4: Difference CD spectra in the aromatic region of single-chain cyanoapoceruloplasmin (—) and cleaved ceruloplasmin (---) minus that of native ceruloplasmin at pH 6.90.

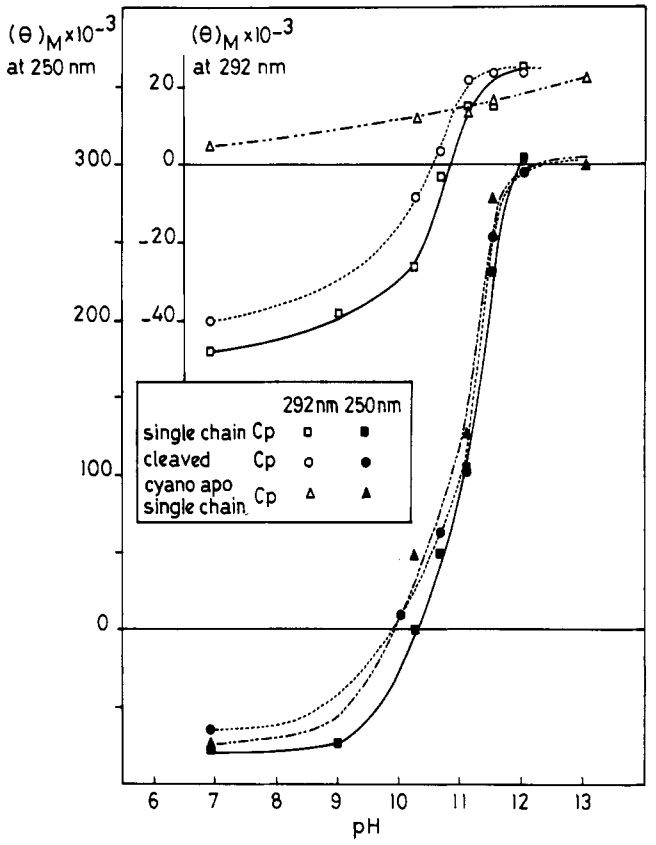

FIGURE 5: Molar ellipticities (deg cm^2 dmol^{-1}) at 292 nm (open symbols) and at 250 nm (filled symbols) at various pH values for undegraded single-chain (—), cleaved (···), and undegraded apoceruloplasmin (·····). Conditions as in Figure 1.

a radical but almost completely reversible conformational change. It is compatible with the behavior of buried tyrosine residues and with the apparent pK value near 11.0 for tyrosine in native and apo-Cp found by acid–base titration (Kasper & Deutsch, 1963). It is interesting to note that at 250 nm the titration curves for the cleaved protein (Cp 1) and for the undegraded apocyanoprotein are the same as for the holoprotein (Figure 5). In other words, neither the proteolytic cleavage of the polypeptide chain nor the removal of the copper atoms from the undegraded protein affect the interactions of the tyrosyl residues with their environment and more particularly with the solvent upon alkaline titration. This strongly supports the idea that the tyrosine residues are not involved

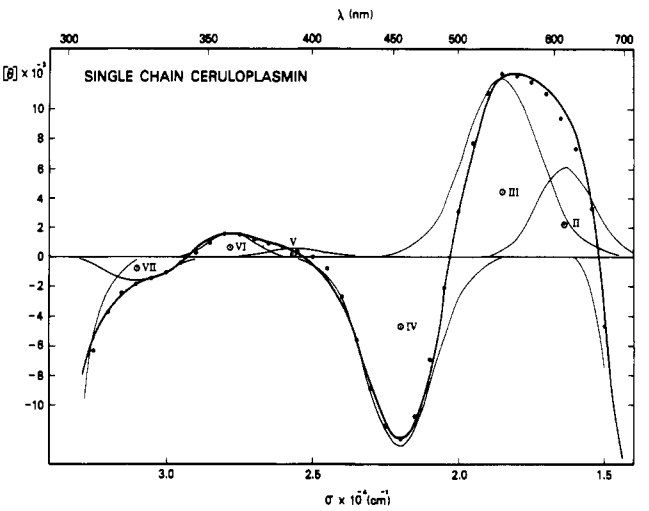

FIGURE 6: CD spectrum of undegraded ceruloplasmin at pH 6.90 in the visible region. The spectrum is resolved into six Gaussian functions (fine line). (···) Calculated spectrum from the resolved Gaussian components.

Table I: Band Characteristics of Resolved Gaussian Components of Visible CD Spectrum of Single-Chain Cp at pH 6.90

band	band position λ (nm)	band position $\sigma \times 10^{-4}$ (cm^{-1})	band width $\Delta\sigma \times 10^{-3}$ (cm^{-1})	molecular ellipticity $[\Theta]_M \times 10^{-3}$ (deg cm^2 dmol^{-1})	rotatory strength[a] $R \times 10^{38}$ (cgs units)
II	613.5	1.630	1.32	+7.28	+7.27
III	541.0	1.850	1.80	+14.09	+16.00
IV	454.5	2.200	1.65	−15.03	−13.90
V	392.2	2.250	1.50	+0.70	+0.51
VI	359.7	2.780	1.10	+1.88	+0.92
VII	322.6	3.100	1.30	−2.11	−1.09

[a] R is about $1.234 \times [\theta]_{max} (\Delta\sigma/\sigma_{max}) \times 10^{-42}$.

in the binding of copper by ceruloplasmin. On the contrary, the titration curve at 292 nm shows important differences between the apo- and the holoprotein whether the latter is degraded or intact. In the apoprotein the prominence of the shoulder at 292 nm is greater and may reflect a more hydrophobic environment for the tryptophan residues. In the holoproteins (cleaved or intact) this environment is relatively sensitive to the pH. The midpoint of the titration curve at 292 nm is somewhat less than pH 11.00, and the ellipticity almost reaches its maximum value at pH 11.10, i.e., before the complete unfolding of the secondary structure and the complete ionization of the tyrosine residues.

Up to pH 11.10 the return of the single-chain protein to neutral pH entirely regenerates the original native spectrum (Figure 3). This is not the case for solutions above pH 11.10; for example, at pH 11.50 the positive extremum at 250 nm is shifted to the red to form a shoulder, and a new maximum appears at 273 nm, while the band at 292 nm remains positive as in the case of the apo-Cp (not shown). Hence, a permanent transformation of the tryptophan environment occurs which could parallel the loss of copper atoms. However, the reversibility of the spectra of cleaved Cp below pH 11.10 is not as good. At pH 10.28, for example, although the general profile of the native spectrum is restored upon decreasing the pH to neutrality, the negative extremum at 282 nm appears less intense and the maximum at 292 nm is not reversed at all.

Visible Region. Figure 6 gives the CD spectrum between 320 and 700 nm of uncleaved human ceruloplasmin at neutral pH; it shows the resolution into six Gaussian bands with the use of the parameters in Table I. There are two major positive

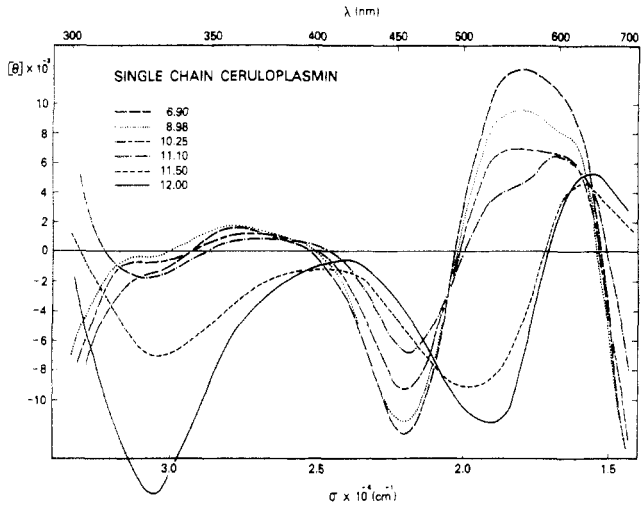

FIGURE 7: CD spectra of undegraded ceruloplasmin in the visible region. Conditions as in Figure 3.

bands at 613 (II) and 541 nm (III), two minor positive bands at 392 (V) and 360 nm (VI), a large negative band at 455 (IV) and above 700 nm (I) (where no CD data could be obtained), and finally a minor negative one at 323 nm (VII). The resolved bands have the same band positions and width as their counterparts in the resolved absorption spectrum (not shown) but have variable magnitudes and signs.

Figure 7 illustrates the pH dependence of the CD spectrum in the visible range under the conditions defined under Experimental Procedures. When the pH is raised, a large decrease is found in the ellipticity at 541 nm (band III) so that its sign is changed above pH 11.10. This band and band VII present a sharp transition between pH 11.10 and 11.50 where the secondary structure of the protein drastically changes. Interestingly, the pH increase only slightly affects band II, while, as is well-known, the absorbance around 600 nm is strongly pH and ligand dependent for Cp and other blue copper proteins (Hervé et al., 1976; Van Leeuwen & Van Gelder, 1978; Falk & Reinhammar, 1972; Rotilio et al., 1975; Sjöholm & Stigbrand, 1974).

The CD spectra are fully reversible at pH values lower than 11.10. When brought back to neutral pH, more alkaline solutions show a flat CD spectrum, even after addition of equivalent amounts of an oxidizing agent such as ferricyanide. This suggests a loss of copper atoms. Above pH 11.50 the reduction of Cu^{2+} can be very fast, and the protein can adopt a nonreversible conformation which favors the loss of some of the copper atoms.

In spite of its lower absorbance at 610 nm (A_{610}/A_{280} = 0.030), the cleaved protein (Cp 1) shows a CD spectrum very similar to that of the intact protein at neutral pH. However, bands III and IV are more pH dependent for the degraded protein (not shown). As previously shown for the near-ultraviolet region, the reversibility of the visible CD spectra of the cleaved Cp was not as complete as that of the undegraded protein pH values below 11.10.

Discussion

The present CD study was undertaken to characterize some structural properties of single-chain Cp in comparison with a degraded but still enzymatically active protein and to evaluate the role of the copper atoms in the structure and in the conformational changes of the undegraded molecule.

Inspection of the far-ultraviolet CD spectra demonstrates that single-chain Cp and degraded Cp exhibit the same general secondary structure at neutral pH, i.e., a rather small α-helix content (~10%) and approximately equal amounts of β and random structure. The computation of the α, β, and random contents by the method of Chen et al. (1972) was not applicable here, perhaps because of the high β content. Whereas the current CD analyses give reasonably good estimates of the helical content, the estimated β content is often uncertain.

The presence of a high β structure content in human Cp has been demonstrated by infrared absorption measurements (Freeman & Daniel, 1978). Various estimates reported in the literature for the β-structure content of Cp as deduced from CD data vary from 0.2 (Moshkov et al., 1972) to 0.46 (Freeman & Daniel, 1978), but neither the degree of cleavage nor the enzymatic activity of the protein studied was stipulated in these cases. It is remarkable that removal of the copper takes place without any important conformational change of the secondary structure of the protein. However, the behavior of cyanoapo-Cp in sedimentation, electrophoresis, and immunoelectrophoresis has been reported to indicate some molecular modifications (Kasper & Deutsch, 1963). For undegraded cyanoapo-Cp (Cp 10), a higher anodal mobility in analytical zone electrophoresis has been confirmed by us (Noyer et al., 1980). These modifications probably involve some conformational changes in the tertiary structure without any important modification of the secondary structure. Although the copper atoms do not seem to be obligatory to maintain the native secondary structure, they do exhibit a stabilizing effect upon the pH-dependent folding and unfolding of the protein. This is demonstrated by the blue shift of the trough at 220 nm showing a transition at pH 11.10 and by the lower reversibility of the far-ultraviolet CD spectra of the apoprotein. A similar finding reported for galactose oxidase indicates that the apoenzyme is less stable than the holoenzyme to thermal denaturation and to denaturation at both high and low pH (Ettinger, 1974). Our data suggest that the stabilizing effect of the copper is more important than the integrity of the polypeptide chain, as shown by the CD spectra of the cleaved protein.

The optical activity of apo-Cp in the aromatic region and particularly near 290 nm clearly demonstrates that the removal of the copper induces some local structural transformations. When X-ray data for azurin (Adman et al., 1978) and plastocyanin (Colman et al., 1978) are taken into consideration, it might be thought that the copper atoms of Cp (or some of them) are also buried in an apolar environment. Our CD measurements suggest that the tryptophan residues responsible for the CD band at 292 nm might present strong hydrophobic interactions with a ligand that binds a metal atom. The interactions might be enhanced by the removal of the copper or by structural changes resulting from the alkaline titration and leading to a higher hydrophobicity of the environment and a higher optical activity. The idea of a possible copper–tryptophan neighborhood is reinforced by the comparison of holo-Cp with its apoprotein, which reveals that the presence of copper brings about a decrease of the tryptophanyl fluorescence (Freeman & Daniel, 1978). Moreover, X-ray studies have shown that the single tryptophan residue in azurin is in close contact with the side chain of phenylalanine-110 which, together with its ligands, shields the Cu atom from the solvent (Adman et al., 1978).

As is shown in Figure 6, the visible CD spectrum of Cp between 320 and 700 nm can be analyzed as a composite of six Gaussian bands. Comparison of the CD spectrum of Cp with that of three proteins that contain only a single type-1 copper, namely, stellacyanin (Falk & Reinhammar, 1972), umecyanin (Sjöholm & Stigbrand, 1974), and azurin (Tang

& Coleman, 1968), with that of laccase (Tang & Coleman, 1968), which contains one type-1, one type-2, and two type-3 coppers, reveals that the six transitions between 320 and 700 nm are associated with the type-1 copper chromophores. Because of their absorption near 320–330 nm, a CD band at this wavelength has been assigned to the diamagnetic type-3 coppers (Van Leeuwen & Van Gelder, 1978; Freeman & Daniel, 1978); however, in this region the contribution of the aromatic amino acid residues to the CD spectra of apo-Cp and holo-Cp is large compared to the contribution of the copper chromophores, and the determination of the CD difference becomes uncertain due to the large error involved. Indeed, the assignment of this band to type-1 or type-3 copper is not unambiguous. This CD band is present in the single type-1 copper protein umecyanin (Sjöholm & Stigbrand, 1974) but only at alkaline pH. Moreover, it is interesting to note that cupric complexes of polypeptides exhibit a CD band near 300–315 nm where no discernible shoulder or maximum appears in the absorption spectra (Hartzell & Gurd, 1969; Tsangaris et al., 1969). This optically active transition of low absorption intensity was assigned to an $n \rightarrow \pi^*$ transition moved to longer wavelengths in the cupric chelates of peptides. Such an interaction might be involved in Cp. At the same time, the bands near 320 nm and at 525 nm indicate a pronounced transition at pH 11.10 where the protein completely unfolds. Thus, it is plausible to assign both transitions to the disruption of the environment of the same type-1 copper. However, an assignment of this 320-nm band to type-3 copper might not be rejected, because from structural models for type-3 copper multicopper oxidases it is conceivable that the same cysteine residue, in addition to its function as a bridge between the two metals of the type-3 copper unit, serves as a further ligand bridge and as a mediator of intramolecular electron transfer between type-3 and type-1 coppers (Lane et al., 1978). In accord with the different models for cupric complexes, the various CD bands in the visible light spectrum of the blue proteins are generally assigned to d–d transitions in a coordination sphere with distorted tetrahedral symmetry and also to a ligand-to-metal charge-transfer transition (Thompson et al., 1979). The most intense absorption bands at 610 and 520 nm probably arise from a charge-transfer transition from a cysteine sulfur ligand to a Cu(II) atom. This deduction is supported by EPR studies on *p*-(chloromercuri)benzoate-treated plastocyanin showing the involvement of S^- in the binding of type-1 copper and by resonance Raman spectra determined on plastocyanin, azurin, and Cp in which the spectra arise from Cu–S stretching vibrations (Graziani et al., 1974; Miskowski et al., 1975).

The different visible CD bands of Cp are very differently affected by an increase in pH. This suggests that the two type-1 copper atoms in Cp are nonequivalent and that the environment of one of them is more easily affected by the conformational and/or electrical changes caused by an increase in pH. Bands III (545 nm), VII (325 nm), and probably I (>700 nm) are the most pH sensitive in Cp, and it is assumed that they arise for the most part from the same chromophore. The other arguments to support the nonequivalence of the two type-1 coppers in Cp are the difference in their redox potentials (Deinum & Vänngard, 1973), their oxidation rates (Carrico et al., 1971), their EPR signals (Gunnarsson et al., 1973), and their resonance Raman patterns (Tosi et al., 1975). Moreover, those bands respond differently to anion binding and to treatment with ntiric oxide. Upon incubation with nitric oxide for 5 min, the CD spectrum of Cp shows a slight decrease in the ellipticity around 600 nm and a marked decrease at 456 nm (Van Leeuwen & Van Gelder, 1978) while the bands in the 500–700-nm region are strongly perturbed by the effect of anions such as N_3^-, OCN^-, and SCN^- and the band at 450 nm is not (Hervé et al., 1976). The differences observed in the sensitivity of these CD bands after treatment by these reactants are not surprising because they are known to produce conformational changes in the protein. Moreover, it is conceivable that the same CD band can be the result of the optical activity of both type-1 coppers. For example, upon pH increase band IV (450 nm) is slightly affected at the beginning but remains unchanged even after increase to high pH values; the two type-1 copper atoms might be involved in this band. The change at high pH of band III (540 nm) suggests a drastic change in the environment of the copper atom involved. Because of the fading of the blue color in this wavelength region, a disruption of a Cu–S bond and/or a spontaneous reduction of the copper chromophore to Cu^+ are possible.

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Supplementary Material Available

Figures A and B giving the near-ultraviolet CD spectra of cleaved ceruloplasmin and of single-chain apoceruloplasmin, Figure C giving the CD spectra of cleaved ceruloplasmin in the visible region, and Figure D giving pH change vs. rotatory strength of different bands of Cp in visible light (4 pages). Ordering information is given on any current masthead page.

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Native Deoxyribonucleic Acid Transcription by Yeast RNA Polymerase-P_{37} Complex†

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ABSTRACT: The specific activity of yeast RNA polymerases A or B, when complexed with P_{37} cofactor, compares favorably with that of *E. coli* RNA polymerase. The stimulation is observed only with double-stranded DNA but does not result from DNase action. The K_m for nucleotide substrates and the optimal conditions of transcription are not modified. P_{37} stimulates RNA synthesis by ternary transcription complexes in the presence of poly(rI) which prevents reinitiations. The RNA chain length, estimated by 5′ end labeling or sedimentation, is increased in the presence of P_{37}. On the other hand, the trinucleotide synthesis, which reflects the chain initiation reaction, is not affected. Therefore, the cofactor appears to act at the elongation step of RNA synthesis.

Purified eucaryotic RNA polymerases display a very low specific activity on double-stranded DNA templates which do not contain nicks or gaps (Gniadowski et al., 1970; Nohara et al., 1973; Flint et al., 1974; Gissinger et al., 1974; Dreyer & Hausen, 1976). Yeast RNA polymerase B in particular was shown to require single-stranded regions to transcribe native DNAs (Dezélée et al., 1974a). Several reports mention proteins which increase, to various extents, the activity of eucaryotic RNA polymerases. Most of them allow the synthesis of larger RNA transcripts on native templates (Stein & Hausen, 1970; Seifart et al., 1973; Lee & Dahmus, 1973; Sekimizu et al., 1976; Ernst & Sauer, 1977; Spindler, 1979; Revie & Dahmus, 1979). However, the functional significance of these proteins has not been clearly established.

We have recently described a yeast protein, called P_{37}, which stimulates transcription of double-stranded DNA by binding to the homologous RNA polymerases A and B, a fact that strongly suggests the in vivo involvement of P_{37} in RNA synthesis (Sawadogo et al., 1980a,b). Here, we show that the activity of the yeast RNA polymerases, in the presence of P_{37}, compares favorably with that of *E. coli* RNA polymerase even with intact DNA duplex. The mode of action of P_{37} was studied in the case of RNA polymerase B. The stimulation occurs mainly at the elongation step.

Material and Methods

RNA Polymerases and Other Enzymes. Yeast RNA polymerase B was prepared as previously described (Sawadogo et al., 1980a). Standard reaction mixtures (0.1 mL) contained

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