An Investigation of the Decolorization of Caeruloplasmin by Acid

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1. The acid decolorization of caeruloplasmin was studied at various temperatures and pH values. 2. Two decolorization reactions may be distinguished: (i) an irreversible reaction with the thermodynamic characteristics of a protein denaturation; (ii) the attainment of an equilibrium between blue and colourless forms of caeruloplasmin with apparent pK 3.7 at 25°. 3. The low temperature-dependence of the equilibrium suggests that it does not involve a gross denaturative change. 4. The reversible decolorization occurred both aerobically and anaerobically, indicating that the change does not involve dissociation of a copper-oxygen complex. 5. Spectral changes during the decolorization are described. 6. The changes occurring during acid decolorization are discussed in relation to a formal model of the gross structure of caeruloplasmin.

A number of blue copper proteins, including caeruloplasmin (Holmberg & Laurell, 1948), plant laccase (Nakamura, 1958) and *Pseudomonas* copper protein (Horio, Sezuku, Higashi & Okunuki, 1961), are noteworthy in that the intensities of their colours/g.atom of copper are many times greater than the intensities of the blue colours of copper-amino acid or copper-peptide complexes. Association of the intense colour with a special kind of copper-ligand bond is indicated by electron-spin-resonance data, the cupric signals of the above proteins all having a particularly low hyperfine-structure constant (Malmström & Vänngård, 1960; Mason, 1963). The decolorization of caeruloplasmin that occurs at pH values below 4.75 can be reversed by raising the pH (Curzon, 1963). In addition, an irreversible denaturation occurs in acid solution that also leads to loss of blue colour. A study of the kinetics and thermodynamics of these reactions is presented and an explanation of the results, in terms of changes in intramolecular bonding, is proposed.

EXPERIMENTAL

Caeruloplasmin. For the preliminary stages of this work, human caeruloplasmin was prepared by the method of Curzon & Vallet (1960). In later work, human caeruloplasmin from the American National Red Cross was used (batch nos. 1972 and 1995). On receipt of each batch of refrigerated caeruloplasmin it was divided into 10 ml. portions and stored at -25°. Before use, these were rapidly thawed by gentle agitation in a 45° water bath, dialysed against 0.1 M-NaCl and centrifuged at 35000g with

refrigeration to remove the small white precipitate that formed during storage. The solution was stored at 4° and used within a few days. The E_{605}/E_{280} ratio of such material was 0.043-0.045, and E_{605} after decolorization by KCN was about 3% of the E_{605} value of native caeruloplasmin. These values indicate a high degree of purity (Curzon & Vallet, 1960).

Buffers and pH determination. Unless otherwise stated, 0.5 M-sodium acetate-acetic acid buffers were used. pH values were determined with a model 23A pH-meter (Electronic Instruments Ltd., Richmond, Surrey) by using a micro-volume pipette-type electrode for determination of the pH values of small volumes of caeruloplasmin-buffer mixtures. The effect of the range of temperatures used in the investigation (18-40°) on the pH of acetate buffers is negligible (Harned & Ehlers, 1933). Similarly, the pH values of buffer-caeruloplasmin mixtures were not significantly affected by temperature in the range 20-40°, nor did they alter between 2 and 60 min. of preparation.

Spectrophotometry. The main series of observations were made by using a Unicam SP.500 spectrophotometer with a Thermistor-controlled constant-temperature holder for micro-cells of 1 cm. optical path. This was specially made by A. Adkins Ltd., Albion Hill, Leicester, and was a modification of their constant-temperature holder for standard cells of 1 cm. optical path. Thin copper inserts were placed in the cell compartments to restrict movement of the cells and so to ensure that the light beam passed symmetrically through the solutions in them. As supplied, the holder was attached to its lid by a locking knob that engaged with a hexagonal nut held in a T slot on the top of the housing. The hexagonal nut jammed frequently in use and it was therefore replaced by a T nut made to fit the T slot. Spectrophotometric readings obtained with the assembly were rather variable, owing to a slight instability of the positioning of the holder in the vertical plane that resulted in a

variable fraction of the incident light-beam reaching the photocell. This was avoided by fitting a half-moon stop in front of the beam-correcting lens holder, the latter being part of the standard Unicam micro-cell attachment. The stop blocked off the lower part of the incident beam so that the slight variations in vertical positioning of the holder did not affect the amount of light incident on the photocell. The setting of the dial of the temperature-control unit for any particular temperature drifted slightly from day to day and therefore the temperature of the micro-cells in the assembly was checked each day by using a thermocouple thermometer with a needle-type thermocouple (Electrolaboratoriet, Copenhagen, Denmark).

Some observations were also made with a Unicam SP.700 recording spectrophotometer with a micro-cell adapter (temperature uncontrolled).

Assay of caeruloplasmin. Changes in caeruloplasmin concentration were determined either from the relationship mg. of caeruloplasmin copper/100 ml. = $4.9 (\pm 0.1) E_{605}^{1\text{cm}}$ (Curzon & Vallet, 1960) or from measurements of oxidase activity. The latter were carried out as described by Curzon (1960) with *NN*-dimethyl-*p*-phenylenediamine as substrate and incubation for 30 min. at 25°. All experiments were based on determinations of E_{605} , except those of Figs. 4–6, where irreversible denaturation was measured in terms of loss of oxidase activity.

General procedure. For the investigation of the effects of time and pH on the acid decolorization and subsequent recolorization, 0.2 ml. of caeruloplasmin solution (E_{605} 0.60) and 0.5 M-acetate buffer were separately equilibrated in a constant-temperature water bath. Then 0.2 ml. of the buffer was pipetted into the caeruloplasmin during 6–8 sec. with gentle agitation. The mixture was immediately transferred to a micro-cell held in the spectrophotometer at the same temperature as the water bath. The E_{605} was read at intervals starting 1 min. after mixing. Caeruloplasmin and buffer were again mixed as above and after a measured interval 0.4 ml. of sodium acetate, held at the same temperature, was added. This brought the pH above 5. The E_{605} was read 1–2 min. after the addition of the sodium acetate, as turbidity frequently developed on standing. The difference between E_{605} before the addition of any reagents and E_{605} after the reversal of decolorization is proportional to the irreversibly decolorized caeruloplasmin fraction (after correcting for dilution). The difference between E_{605} after reversal and E_{605} after the same period of exposure to the acetate buffer without the addition of sodium acetate is proportional to the reversibly decolorized caeruloplasmin fraction (again after correcting for dilution). The recovery of caeruloplasmin after reversal was also measured by determination of residual oxidase activity.

The main restriction on the spectrophotometric measurement of the various forms of caeruloplasmin is the turbidity, which is most marked after exposure to higher temperatures and more acid pH, as would be expected for a denaturative change. When turbidity developed after the addition of *m*-sodium acetate it was frequently possible to remove it by centrifuging for a few minutes, thus enabling true values of E_{605} to be obtained. Oxidase activity was not significantly affected by the removal of turbidity.

Anaerobic reaction vessel. This was made by Aimer Products Ltd. (London, N.W. 1) and is shown in Fig. 1. The method of use was as follows. Nitrogen was passed through alkaline pyrogallol and water and then bubbled

Fig. 1. Three-chamber anaerobic reaction vessel. Details are given in the text.

individually through the caeruloplasmin, 0.5 M-acetate buffer and *m*-sodium acetate solutions. These were then pipetted into the chambers of the reaction vessel. The double lower chamber was repeatedly evacuated and filled with nitrogen, and finally evacuated. Nitrogen was passed through the *m*-sodium acetate in the upper chamber, which was then closed. The caeruloplasmin and buffer were then mixed with swirling for 1 min. Throughout this period the double reaction chamber was repeatedly evacuated and filled with nitrogen and finally evacuated to remove any gas that might possibly have been evolved during exposure of the caeruloplasmin to the acetate buffer. The three-way tap was then turned, allowing the sodium acetate solution to enter the evacuated lower chamber and bring the caeruloplasmin to a pH above 5 in the absence of oxygen. The decolorization and its reversal were recorded by colour photography under standard conditions and the relative densities of the blue colours on a colour transparency measured with a Zeiss photoelectric exposure-meter. By using this method and averaging triplicate determinations, the densities of standard caeruloplasmin solutions bore a linear relationship to E_{605} within an $E_{605}^{1\text{cm}}$ range 0.2–0.6 (s.d. of a single determination $\pm 13\%$).

RESULTS

Reversible decolorization of caeruloplasmin by exposure to low pH. Fig. 2 shows the effect of acid

Fig. 2. Effect of time on E_{605} of caeruloplasmin at various pH values at 25°: ○, pH 5.50; △, pH 4.27; □, pH 4.04; ●, pH 3.80; ▲, pH 3.70; ■, pH 3.59. General conditions are given in the text.

Fig. 3. Partial recolorization of acid-decolorized caeruloplasmin. ○, E_{605} at pH 3.70 and 25° (as % of initial value); △, E_{605} after the subsequent addition of an equal volume of m-sodium acetate at the times shown on the abscissa (as % of initial value) (extinctions corrected for dilution); □, percentage of irreversibly decolorized caeruloplasmin; ●, percentage of reversibly decolorized caeruloplasmin. General conditions are given in the text.

conditions on E_{605} of caeruloplasmin. It is apparent that the decolorization kinetics are not simple, a rapid initial fall being followed by a slower fall. After longer periods of exposure to the more acid pH conditions, the slight turbidity caused an apparent levelling off or slight rise in E_{605} .

Table 1. Effect of recolorization pH on the degree of recolorization

To 0.2 ml. caeruloplasmin samples (E_{605} 1.20) was added 0.2 ml. of 0.5 M-acetate buffer, pH 3.33. After 5 min. incubation at 18° E_{605} was 0.241. The solutions indicated below were added and E_{605} was read again 2 min. later.

Addition	$E_{605}^{1\text{cm.}}$	Final pH
m-Sodium acetate (0.4 ml.)	0.206	5.30
m-Acetate buffer, pH 5.50 (0.4 ml.)	0.203	5.00
m-Acetate buffer, pH 4.97 (0.4 ml.)	0.193	4.70

Fig. 3 shows the partial reversal of the decolorized caeruloplasmin after the pH of the solution has been brought back to a value at which E_{605} remains constant. As the time of exposure to the pH value at which E_{605} is unstable is increased so the reversal becomes less complete. The amount of irreversibly decolorized caeruloplasmin increases progressively with time of exposure to acid pH. The amount of reversibly decolorized caeruloplasmin attains an approximately constant value after short exposure to acid pH.

Effect of the pH at which recolorization occurs on the degree of recolorization and on the determination of oxidase activity. Table 1 indicates that the slight differences in pH of the recolorized caeruloplasmin preparations due to various acetate buffers used in the decolorization are not sufficient to affect the degree of recolorization. The slightly lower E_{605} at pH 4.7 is understandable as this is the lower limit of the pH range in which E_{605} of caeruloplasmin is stable. For the determination of oxidase activity the caeruloplasmin was diluted 15–40-fold and 2 vol. of 0.2 M-acetate buffer, pH 5.50, plus 2 vol. of substrate solution were added. Thus variation of pH or chloride (Curzon, 1960) in the final incubation mixture is not sufficient to affect the activity determination.

Parallelism between irreversible decolorization and loss of oxidase activity. The percentage irreversible decolorization after exposure at 40° or 30° to acid pH is comparable with the percentage loss of oxidase activity. After exposure to acid pH at 25° the percentage irreversible decolorization is about 25% higher than the percentage loss of oxidase activity. There are various possible explanations of the discrepancy:

(i) The reversibly decolorized caeruloplasmin may be altered structurally so that the oxidase activity/ E_{605} ratio is higher after decolorization and reversal than before. If this explanation were correct it could be expected that after short exposure to acid buffer the oxidase activity after recolorization would be higher than that of untreated protein. This has never been observed.

(ii) In general, E_{605} after recolorization was measured within 2 min. of the addition of the M -sodium acetate, whereas oxidase activity was determined up to 120 min. later. Therefore it appeared possible that the discrepancy was due to the reversal reaction being incomplete at the time when E_{605} was measured. However, the discrepancy persisted when E_{605} was measured after instead of before determination of oxidase activity.

(iii) After measurement of E_{605} of the recolorized caeruloplasmin considerable dilution is necessary before the oxidase determination may be made. This could result in a greater degree of reversibility. Samejima & Yang (1963) have observed this phenomenon during the reconstitution of acid-denatured catalase and suggested that the lower recoveries at higher concentrations were due to the entangling of unfolded denatured polypeptide chains. This would imply that, in the present work, results derived from percentage loss of oxidase activity are more suitable for kinetic evaluation than those derived from E_{605} , as equilibrium would perhaps be more closely approached under the conditions of the oxidase-activity determinations. Therefore the degree of irreversible destruction of caeruloplasmin was determined from oxidase-activity measurements unless specifically stated otherwise.

Kinetics and thermodynamics of irreversible acid denaturation. The decline in the sum of the apparently unchanged and the reversibly decolorized fractions of caeruloplasmin with time is a first-order reaction for a short time of exposure to acid pH (Fig. 4). This suggests that the velocity constants for irreversible denaturation of both the unchanged and reversibly decolorized caeruloplasmin are the same. If the rate constants were significantly different a linear relationship would not occur in

Fig. 4. Effect of time on the irreversible acid denaturation of caeruloplasmin determined by loss of oxidase activity. $\circ$, pH 3.80 and 25°; Δ , pH 3.80 and 40°; $\square$, pH 3.70 and 40°. General conditions are given in the text.

Fig. 4: the slope would change as the proportion of the above components changed. Velocity constants for the irreversible denaturation (calculated from the first-order plots) show a linear relationship to pH (Fig. 5). The slopes of the lines are 2.1, 2.3 and 2.1 at 25°, 30° and 40° respectively. According to Levy & Benaglia (1950), this means that at temperatures between 25° and 40° two critical prototropic changes occur in the formation of the particular ionic species of caeruloplasmin that is susceptible to irreversible denaturation in the pH range 3.59–4.27.

The linear relationship between the velocity constants and temperature is shown in Fig. 6. From this, ΔH and ΔS for the activation reaction

Fig. 5. Effect of pH on the velocity constant of the irreversible acid denaturation of caeruloplasmin determined by loss of oxidase activity. $\circ$, 40°; Δ , 30°; $\square$, 25°.

Fig. 6. Effect of temperature on the velocity constant of the irreversible acid denaturation of caeruloplasmin determined by loss of oxidase activity. Velocity constants at pH 3.75 were obtained from Fig. 5.

involved in the irreversible denaturation are calculated to be 27 kcal./mole and 66 cal./mole/degree respectively. These values are typical for a denaturative change.

Kinetics of the reversible decolorization. As the rate constants for irreversible denaturation of the unchanged caeruloplasmin and the reversibly decolorized caeruloplasmin are the same, formation of denatured caeruloplasmin would not displace an equilibrium between unchanged and reversibly decolorized protein and therefore this reaction may

be treated as shown in Fig. 7. This suggests that a pH-dependent equilibrium between unchanged and reversibly decolorized caeruloplasmin is attained within a few minutes of exposure to acid pH. The relationship between the equilibrium and pH is shown in Fig. 8. This is in shape like part of a typical ionic dissociation curve, the groups involved having pK about 3.7 that does not vary appreciably with temperature.

Reversible acid decolorization under anaerobic conditions. Orgel (1958) has suggested that the intense blue colour of caeruloplasmin is due to the formation of a complex between copper and oxygen. If this is correct then it might be that reversible decolorization by acid was associated with reversible dissociation of oxygen from caeruloplasmin. To test this hypothesis the apparatus shown in Fig. 1 was used. A 0.4 ml. sample of caeruloplasmin solution was brought to pH 3.60 by the addition of 0.2 ml. of M -acetate buffer, pH 3.3, anaerobically. A decline in extinction from 0.108 unit (correcting for dilution) to 0.046 unit occurred at 1 min. at room temperature, during which time the reaction vessel was repeatedly evacuated and swept with nitrogen. When the pH was brought to 5.3 by the immediate addition of 0.6 ml. of M -sodium acetate under aerobic conditions the extinction rose to 0.086 unit (corrected for dilution). When the sodium acetate

Fig. 7. Effect of time on the reversible acid decolorization of caeruloplasmin at 25°. $\circ$, pH 3.59; Δ , pH 3.70; $\square$, pH 3.80; $\bullet$, pH 4.04; $\blacktriangle$, pH 4.27.

Fig. 8. Effect of pH on the reversible decolorization of caeruloplasmin. $\circ$, 18°; Δ , 25°; $\square$, 30°.

Fig. 9. Comparison of the spectra of caeruloplasmin in pH 5.5 (—) and pH 3.6 (---) buffer. Scanning began 1 min. after the addition of an equal volume of 0.5 M -acetate buffer to the caeruloplasmin. Spectra were scanned at 23–24°. The rate of scanning was 10000 wave numbers/min.

was added anaerobically the extinction rose to 0.102 unit. Since reversible decolorization occurs under both aerobic and anaerobic conditions it is suggested that native caeruloplasmin is not an oxygenated form.

Effects of acid pH on the absorption spectrum of caeruloplasmin. When caeruloplasmin is exposed to pH 3.6, not only does the 605m μ peak decline but a new absorption band appears at about 425m μ (Fig. 9). Similar results were observed at other pH values between 3.5 and 4.2. The peak at 425m μ was most prominent after only 16sec. exposure to pH 3.6, this being the shortest time after the addition of acid buffer at which measurements could be made. The peak disappeared on the addition of sodium acetate.

DISCUSSION

Various treatments have been reported to alter the absorption spectrum of caeruloplasmin. Thus dithionite (Ehrenberg, Malmström, Broman & Mosbach, 1962) and ascorbic acid (Blumberg, Eisinger, Aisen, Morell & Scheinberg, 1963; Kasper, Deutsch & Beinert, 1963) cause decolorization associated with reduction of the cupric copper of caeruloplasmin to the cuprous state. Decolorization by urea (Broman, Malmström, Aasa & Vänngård, 1962) and heat (Holmberg & Laurell, 1948) indicates that the tertiary structure of the protein plays a critical part in the binding of the copper atoms in caeruloplasmin (Curzon, 1959, 1962). A third group of agents, including azide and thiocyanate, causes decline in the 605m μ peak with the appearance of new peaks at lower wavelengths. These substances probably act by binding the caeruloplasmin copper (Laurell, 1960). However, the powerful chelating agent EDTA only causes the blue colour to fade very slightly, except when denaturing conditions (Curzon, 1962) or prolonged periods of exposure (Kasper & Deutsch, 1963) are used. This resistance is probably related to steric factors, the EDTA ion being much larger than the azide ion though the stability constant of its copper complex is much greater (Bjerrum, Schwarzenbach & Sillen, 1957, 1958). The decolorization of caeruloplasmin by H⁺ ions is of some interest because here (if decolorization is due to a direct reaction of H⁺ ion with copper) the minute size of the decolorizing agent suggests that complications due to non-accessibility of the copper atoms are likely to be negligible except perhaps if those copper atoms are in the hydrophobic inner part of the protein structure.

During the acid decolorization of caeruloplasmin at least two reactions are involved. The partial return of blue colour on the addition of sodium acetate shows that at least one of the reactions is

reversible. The formation of the irreversibly decolorized and inactivated form is interpretable as a protein denaturation, according to the views of Levy & Benaglia (1950), the assumption being made that both the native blue form of caeruloplasmin and the reversibly decolorized form are converted into the irreversibly denatured form at the same rate. By using this assumption, values of ΔH and ΔS for the inactivation process are obtained that are typical of a denaturation reaction (Laidler, 1958). The finding that the velocity constant of the irreversible denaturation varies in a linear manner with pH is strong evidence that the assumption is correct; otherwise the velocity constant would vary with pH in a manner related to the ratio of native to reversibly decolorized caeruloplasmin and a non-linear result would almost certainly be obtained. The lower resistance of apocaeeruloplasmin than of intact caeruloplasmin to denaturation by heat or by organic solvents (A. G. Morell, personal communication) indicates that the copper atoms stabilize the tertiary structure of the protein (Curzon, 1959), and thus significant weakening of copper-protein binding should result in easier denaturation. Therefore, as reversibly decolorized caeruloplasmin apparently denatures at the same rate as native caeruloplasmin, it is suggested that the copper atoms of the reversibly decolorized form are still strongly bound to the protein. These concepts are illustrated in a formalized manner in Scheme 1. In the native state (I) the conformation of the backbone depends on intrachain bonds in the usual way and also on the bonds between the copper atoms and the protein. Such a structure would explain why the rate of decrease of the blue colour of caeruloplasmin caused by EDTA together with urea is greater than the sum of the rates of decrease in the presence of these substances separately (Curzon, 1962). The irreversible decolori-

Scheme 1. Model for the effects of acid pH on caeruloplasmin. (I) Native caeruloplasmin; (II) reversibly decolorized caeruloplasmin; (III) the two forms of the unstable ionic species of caeruloplasmin derived from (I) and (II) (these are shown superimposed to represent their identity with respect to irreversible denaturation); (IV), irreversibly denatured caeruloplasmin; ●, copper atom.

zation may be considered to occur via unstable species such as (III) in which certain critical hydrogen bonds are broken, to give the disoriented structure (IV).

The reversible decolorization apparently involves a dissociation with pK 3.7. The temperature-independence of the equilibrium indicates that the dissociation involved is of largely electrostatic nature. The pK value is suggestive of a carboxyl group. However, metal binding may cause a considerable shift in the pK values of protein side-chain groups. For example, Tanford & Epstein (1954) found that two imidazole groups, which titrated at pH 6.5–7 in zinc-free insulin, titrated at pH 4 in zinc-insulin.

Experiments carried out anaerobically provided no evidence to suggest that the blue form of caeruloplasmin is a copper-oxygen complex. This is in agreement with Morell, Aisen, Blumberg & Scheinberg (1964), who were able to reconstitute caeruloplasmin from the apoprotein and copper under anaerobic conditions.

The principal spectral changes observed during the reversible acid decolorization, i.e. decline of the peak at $604m\mu$ and the appearance of a peak at approx. $425m\mu$, are different from the changes observed during reductive decolorization when absorption at all parts of the caeruloplasmin spectrum above $300m\mu$ declines proportionately (Blumberg *et al.* 1963). This finding is consistent with the observation that the strength of the electron-spin-resonance cupric signal remains unchanged during acid decolorization (Curzon, 1963). The relationship of the $425m\mu$ peak to the changes of the type indicated in Scheme 1 remain obscure; peaks in the region frequently occur as minor components of this spectra of copper-containing proteins. Thus caeruloplasmin itself in the pH range at which it is stable shows a plateau in the 400 – $500m\mu$ region that Blumberg *et al.* (1963) have calculated to represent a minor peak at about $459m\mu$. The copper-containing protein of the mung-bean seedling (Shichi & Hackett, 1963) and the copper-containing proteins of lac-latex pigment (Omura, 1961), spinach plastocyanin (Katoh, Shiratori & Takamiya, 1962) and laccase (Nakamura, 1958) all exhibit a minor peak, plateau or shoulder in the 440 – $460m\mu$ region together with a larger peak in the $600m\mu$ region. Also, the β -mercaptopyruvate-cleaving enzyme (Kun & Fanshier, 1959), which contains copper, exhibits a peak at $415m\mu$, and the copper-containing monoamine oxidase of plasma (Yamada & Yasunobu, 1962) exhibits a shoulder at $410m\mu$ and a peak at $480m\mu$.

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