Isotopic Effect on the Kinetic of Thermal Denaturation of Ceruloplasmin

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The kinetics of thermal denaturation of ceruloplasmin in water and in water with different percentage of D_2O in the temperature range 25–85 °C, following the optical density change of the 610 nm charge transfer band of the protein has been investigated.

A temperature $T_t \simeq 65$ °C above which an irreversible denaturation process of the protein active site takes place has been found.

The kinetics of the denaturation process of the protein are fitted by two first order reactions, which have been assigned to a different thermal denaturation behaviour of the two Cu^{2+} type I sites existing in the protein. Addition of D_2O to the protein solution affects the two kinetics in a different way, *i.e.* the rate of one of them is increased whilst that of the other is decreased.

The different effect of D_2O on the kinetics of disruption of the two copper sites is discussed in terms of different location and degree of hydrophobicity of the two Cu^{2+} type I sites.

Introduction

Ceruloplasmin (CP) is a metal-protein of mammalian blood plasma containing six copper ions. From a magnetic point of view three of them are detectable by electron paramagnetic resonance spectroscopy (EPR), two are the so called type I, or blue copper ions, characterized by an abnormally small EPR hyperfine splitting and a very intense visible band at 610 nm (ε/Cu = 5500 M^{-1} cm^{-1}). The other one is the type II (non blue) copper with an EPR hyperfine splitting in the range considered normal for single tetragonal Cu(II) complexes. From the remaining copper ions, two are of type III, and are thought to form an antiferromagnetic coupled pair, which has an intense near UV (330 nm) absorption band [1, 2]. The sixth copper is known as type IV [3]. The two type I copper in ceruloplasmin have been suggested [4] to have a similar N_2S_2 distorted tetrahedral geometry by comparing their spectral properties with those displayed by plastocyanin, a single type I copper protein, whose structure has been shown by an X-ray diffraction analysis [5]. Nevertheless there are evidence that the two type I sites slightly differ in their redox potential [6], EPR signal [7], circular dichroism behaviour [10]. In view of these findings we have performed a thermal denaturation study of ceruloplasmin at different percentage of D_2O following directly the 610 nm charge transfer band characteristic of the two type I copper.

It was found that exist a threshold temperature above which an irreversible thermal denaturation of the protein takes place. Moreover, the two type I sites show a different kinetics for thermal denaturation suggesting a different location for the two copper centers.

Materials and Methods

Human Ceruloplasmin (CP) was purchased from Serva Feinbiochemica and used without further purification. Deuterium oxide (99.9%) was obtained from Merck, while the water employed was glass distilled after passing through an anion-exchange column.

The pH of the solutions was adjusted to 5.5 with NaOH and HCl both 1 M and was measured with an Orion digital pH-meter Mod. 601 A equipped with a combined electrode Mod. 90/01. The pH indicated is pH-meter readings without correction for the isotopic effect. The kinetics of thermal denaturation were followed measuring the optical density change of the 610 nm ceruloplasmin charge transfer band with a spectrophotometer Cary 118.

Aliquots of the concentrated CP solution were put in 1 cm optical path quartz cuvette and the desired quantity of D_2O were added to obtain solutions with 50 and 96% D_2O in H_2O, respectively. The cuvette

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were accurately sealed to prevent sample evaporation and incubated at 25 °C for 15 min before to start the measurements. The temperature gradient, $\Delta t = 60$ °C, was covered step-by-step in 60 min.

The kinetic studies were started 1 min after the samples were positioned in the thermostatated sample holder at the desired temperature. An Haake thermostatated bath Mod. D3 (±0.1 °C) was used throughout the measurements.

Best fit of the experimental points were performed with a computer Tektronix Mod. 4051.

Results and Discussion

Fig. 1 shows the optical density (OD) change of the 610 nm band of ceruloplasmin (CP) in water as a function of temperature. As can be seen, a threshold temperature $T_t \simeq 65$ °C is evident, at which an abrupt change of the OD takes place. The OD reduction is indicative of the beginning of an irreversible thermal denaturation process. In fact, lowering the temperature just before reaching T_t the original OD of the 610 nm band is restored, whilst for $t > T_t$ the loss of the OD is completely irreversible even after lowering the temperature.

Threshold temperature ranging from 50 to 75 °C in thermal denaturation study have been observed in other proteins usually following the change of the OD of the 280 nm absorption band [11].

In the case of CP the possibility to follow the OD change of the 610 nm charge transfer (CT) band characteristic of the Cu^{2+} type I ions, naturally present into the protein, allows us to obtain direct information on the environment around the two type I copper, which are involved into the catalytic process.

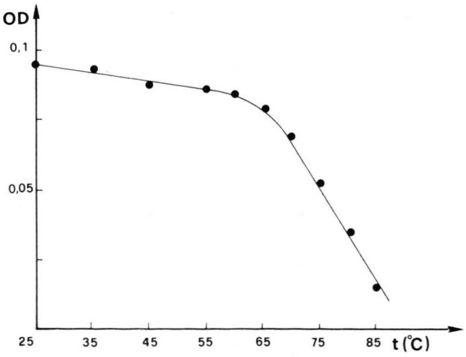


Fig. I. Optical Density change at 610 nm of a 9 μM ceruloplasmin aqueous solution *vs.* temperature.

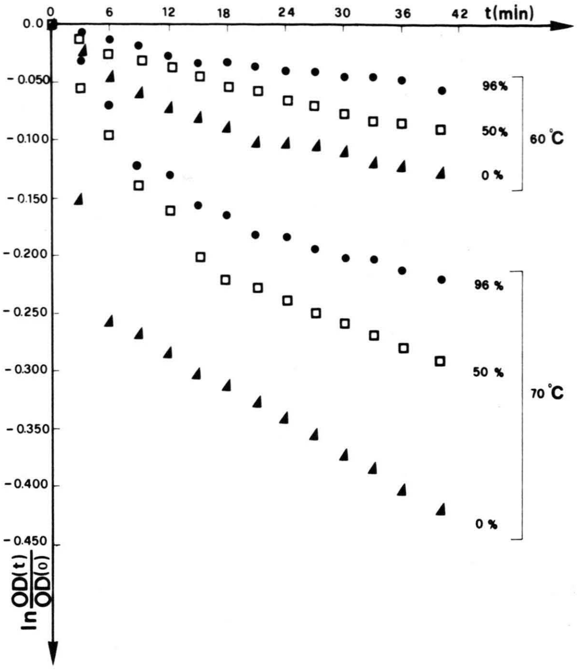
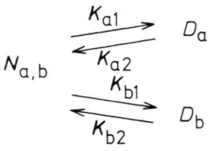


Fig. 2. Kinetics of thermal denaturation of ceruloplasmin 9 μM in water and in solvent containing different percentage of D_2O in H_2O at 5 °C below and upper the transition temperature, $T_t \simeq 65$ °C.
The reaction had been followed at 610 nm.

In Fig. 2 are reported the kinetics of thermal denaturation of 9 μM CP in glass distilled water at pH 5.5 and at different percentage of D_2O 5 °C below and above the threshold temperature, T_t.

At 70 °C the process results clearly biphasic, and can be explained by a different thermal dependence of the microenvironments of the two type I copper existing in the native protein both responsable for the CT-band at 610 nm.

Above T_t, in fact, the two copper sites follow first order decay as easly explained by the following reactions:



$$N_{a,b} \begin{array}{l} \underset{K_{a2}}{\overset{K_{a1}}{\rightleftharpoons}} D_a \\ \underset{K_{b2}}{\overset{K_{b1}}{\rightleftharpoons}} D_b \end{array} \qquad (1)$$

were $N_{a,b}$ and $D_{a,b}$ are the concentrations of the two different type I copper, in the native and denaturated protein, respectively. At $t > T_t$, K_{a2} and $K_{b2} \rightarrow O$ and $\frac{d}{dt} N_{a,b} = K_{a1,b1} \cdot N_{a,b}$ and the reactions respect to time are of the first order. In contrast, at $t < T_t$ it is $K_{a2}, K_{b2} \neq O$ and the expression of $N_{a,b}$ as function of time becomes complicated but surely greater than unity (Fig. 2).

Since the oscillatory strengths of the two copper type I chromophores have been estimated to be equal [6] the experimental data can be fitted for $t > T_t$ by the following equation:

$$OD\,(t) = \frac{OD\,(O) - OD\,(\infty)}{2} \{e^{-K_{a1} \cdot t} + e^{-K_{b1} \cdot t}\} + OD\,(\infty) \qquad (2)$$

where OD (t) and OD (O) are the absorbances at 610 nm at time t and O, respectively; K_{a1} and K_{b1} are the rate constants of disruption of the two type I Cu^{2+} and OD (∞) is the residual absorbance at $t = \infty$ which could be ascribed to light scattering from the denaturated protein plus the contribution of the $d \rightarrow d$ absorption band of the two metal ions chelated to ligands different than in the native protein.

The experimental points have been fitted using Eqn. (2) and the Marquandt's algorithm [12]. The OD (∞), K_{a1}, K_{b1} calculated parameters and the chi-square value for each calculation are given in Table I. Fig. 3 shows, as an example, the comparison between the experimental points with the curve obtained from the computer best fit for the thermal denaturation of CP in water.

As can be seen the quality of fitting being excellent. From the data in Table I it can be observed: i) in water the rate constants, of sites disruption are one order of magnitude different, *i.e.* the microenvironment of one type I copper site is affected ty temperature in a much faster way than the other; ii) deuteration affects differently the two K-values, *i.e.* the faster reaction tends progressively to be stabilized whilst the other to be destabilized by increasing amount of D_2O; iii) the residual absorbance increases as deuteration increases.

Table I. Kinetic constants K_{a1} and K_{b1} and OD (∞) value of the CP denaturation reaction at different isotopic composition of the solvent as obtained by computer best fit of the experimental points. The chi-square value for each fit is also reported. T = 70 °C.

D_2O [%]	OD (∞)	K_{a1}x10^3 [sec^{-1}]	K_{b1}x10^3 [sec^{-1}]	χ^2x10^5
0	0.57	5.7	0.33	5.9
50	0.71	4.8	0.53	17.0
96	0.79	1.7	0.81	9.1

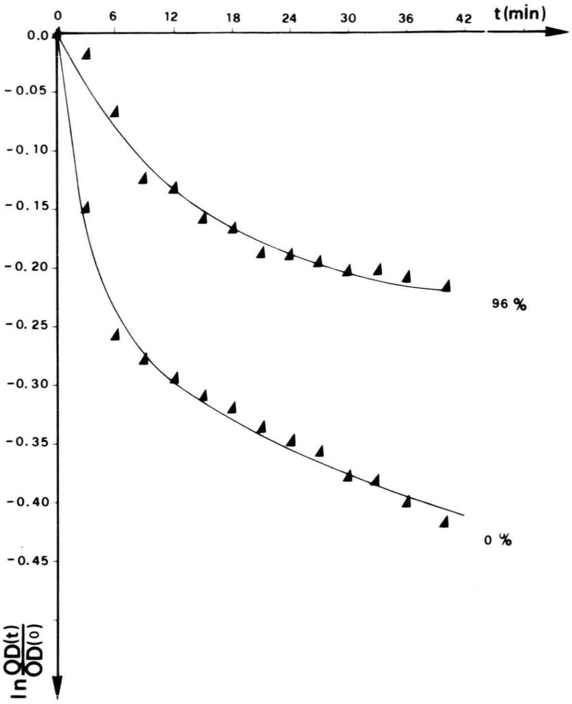


Fig. 3. Comparison between the experimental points (▲) and calculated curve for the kinetics of thermal denaturation of CP in water and in 96% D_2O at 70 °C.
The comparison was done by substituting into Eqn. (2) the parameters reported in the first and third row of Table I, respectively.

That deuteration has a different effect on the thermal stability of the two type I copper it is clearly shown by the change of the free energy of transfer from H_2O to D_2O for the two sites. In fact, following the transition state theory [13, 14] it results that:

$$\ln \frac{K_{Ha}}{K_{Da}} = \frac{\triangle \triangle G_a}{KT} \qquad (3a)$$

$$\ln \frac{K_{Hb}}{K_{Db}} = \frac{\triangle \triangle G_b}{KT} \qquad (3b)$$

where K_H and K_D are the rate constants of thermal denaturation in H_2O and D_2O respectively, $\triangle \triangle G = \triangle G(D_2O) - \triangle G(H_2O)$ is the isotopic effect on the activation free energy of the denaturation reaction and the sub-labels a and b refer to the two different copper sites. Making use of the K-values reported in Table I we obtain a $\triangle\triangle G_a = 0.82$ Kcal/mol for the faster copper type I disrupting site and a $\triangle\triangle G_b = -0.67$ Kcal/mol for the slower one. The most striking fact is that the free energy of transfer $\triangle\triangle G_b$ for the slow site disruption is negative. As far as we know thermal denaturation studies on protein have shown that D_2O has stabilizing effect with respect to H_2O [15, 16].

On the other hand, similar studies performed on amino acids, hydrocarbons and α-helix containing polymers have suggested that D_2O can be either a stabilizing or destabilizing agent. In fact, Kresheck *et al.* [17] have reported nagative values for the free energy of transfer for hydrocarbons and positive ones for the non polar portion of the amino acids.

The negative and positive $\triangle\triangle G$ values found in our case strongly indicate that the microenvironment of the two copper type I sites must be very different. In particular the microenvironment of the Cu^{2+} type I ion characterized by a negative $\triangle\triangle G$ value must have a more hydrophobic character. This implies a location of this Cu^{2+} ion in a deep buried hydrophobic environment. On the other hand, the positive free energy of transfer value found for the second type I copper ion suggests its location into a more polar environment. This different variation in the degree of polarity of the microenvironment and therefore in the degree to which the two copper centers are buried into the protein is in agreement with a previous work where it was noticed that addition of anions like N_3^- and SCN^- to ceruloplasmin resulted in a 50% decrease of the OD of the 610 nm CT-band bringing to the proposal that only one of the two type I copper, *i.e.* the more exposed, is affected by the anion binding [18]. From these findings it can be concluded that the two type I Cu^{2+} metal ions present in the ceruloplasmin are characterized by a considerably different microenvironment. To elucidate whether the contributions to the Gibbs free energy of transfer are entropic and/or entalpic works are in progress.

Acknowledgement

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