PRIMARY ELECTRON-ACCEPTING SITES AND ELECTRON TRANSPORT REACTION IN HUMAN CERULOPLASMIN

Low-temperature radiolysis study

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1. Introduction

The reaction of ceruloplasmin with a reducing substrate is an important primary step in the four-electron reduction of molecular oxygen to water catalysed by the enzyme. The functional properties and the interaction of different copper sites of ceruloplasmin in this reaction have been intensively investigated and the mechanism of electron transfer in the enzyme-catalysed reactions has been rationalized to some extent [1–5]. Specifically, the pathway of reduction equivalent to Type 1 Cu^{2+} in ceruloplasmin has been delineated for reduction in solution by hydrated electron using the method of pulse radiolysis combined with a fast recording spectrophotometric system [6].

In the present work, the reaction of 'dry' electrons, which are the simplest reagent in redox reactions, has been studied at low temperature. The electrons can be produced by high-energy irradiation of aqueous organic solution frozen to a glassy solid at 77 K. It has been shown [7,8] that solutes in such a matrix capture electrons produced upon irradiation yielding the primary electron addition compounds.

The spectral properties of the low-temperature products of ceruloplasmin and their thermal stability investigated by absorption and EPR spectroscopies have been used to identify the primary electron accepting sites and intramolecular electron transport reactions.

2. Materials and methods

Human ceruloplasmin was obtained from Biomed (Poland) and purified before use by preparative polyacrylamide-gel electrophoresis [9]. Only 97% pure (absorbance ratio $A_{610}/A_{280} = 0.044$, copper content 0.27%) and electrophoretically homogeneous preparations were used in our analysis.

Solutions of ceruloplasmin were prepared in aqueous ethylene glycol (usually 1 : 1 or 2 : 3 ethylene glycol–water mixture). The 0.2 cm thick glassy samples were irradiated at 77 K by ^{60}Co γ-rays, at the dose rate of 8 kGy/h, in the dark. Absorption spectra were determined at 77 K with a Beckman DK-2A spectrophotometer, while for measurements at higher temperatures a Beckman Acta MIV spectrophotometer equipped with a thermoregulated assembly was used. For the optical studies, the solvent-trapped electrons were removed by bleaching of the irradiated solution with $\lambda > 450$ nm light. EPR spectra were recorded at 77 K with an X-band, SE/X-20 spectrometer (Poland) with 100 kHz magnetic field modulation. The irradiated samples were annealed in a cold nitrogen gas flow system. 1,1-Diphenyl-2-picryl-hydrazyl radical and Mn^{2+} were used as field markers.

3. Results

γ-Irradiation of ceruloplasmin in ethylene glycol–

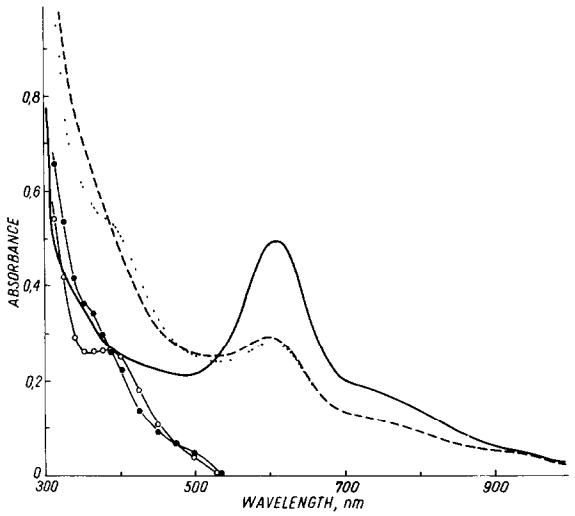

Fig.1. Optical absorption spectra of human ceruloplasmin (~0.2 mM) in ethylene glycol–water glass at 77 K before (solid line) and after (dashed line) irradiation with 34 kGy of ^{60}Co γ-rays at 77 K and after thermal annealing at 150 K for 5 min of the irradiated solution (dotted line). •, Difference between the absorption spectra of the irradiated and unirradiated solutions; ○, difference between the absorption spectra of the annealed and unirradiated solutions.

water glass at 77 K leads both to the decrease of blue copper absorption at around 610 nm and to the appearance of a complex new absorption in the range of 300 to 500 nm (fig.1). The bleaching of Type 1 Cu^{2+} absorption is concomitant with a decrease of the intensity of EPR signal ascribed to Type 1 Cu^{2+} (fig.2). These results indicate a direct reduction of Type 1 Cu^{2+}. Moreover, it can be seen from fig.2B that both kinds of Type 1 Cu^{2+} present in human ceruloplasmin undergo reduction with approximately the same yield (cf. [10]). Type 2 Cu^{2+} is not affected by γ-irradiation at 77 K as shown by invariance of the EPR line at low field arising from this copper.

Fig.3 illustrates the change of the radiation-modified optical spectrum of ceruloplasmin upon the increase of temperature in the range of 90 to 160 K. The change in the absorbance could be detected over the entire spectral range showing that transients absorbing in the range of 300 to 500 nm were formed by irradiation at 77 K, and indicated the additional thermally induced reduction of Type 1 Cu^{2+}. The EPR spectra confirm the latter finding (fig.2C). At least two different transient absorption spectra can be extracted from the overall absorption changes

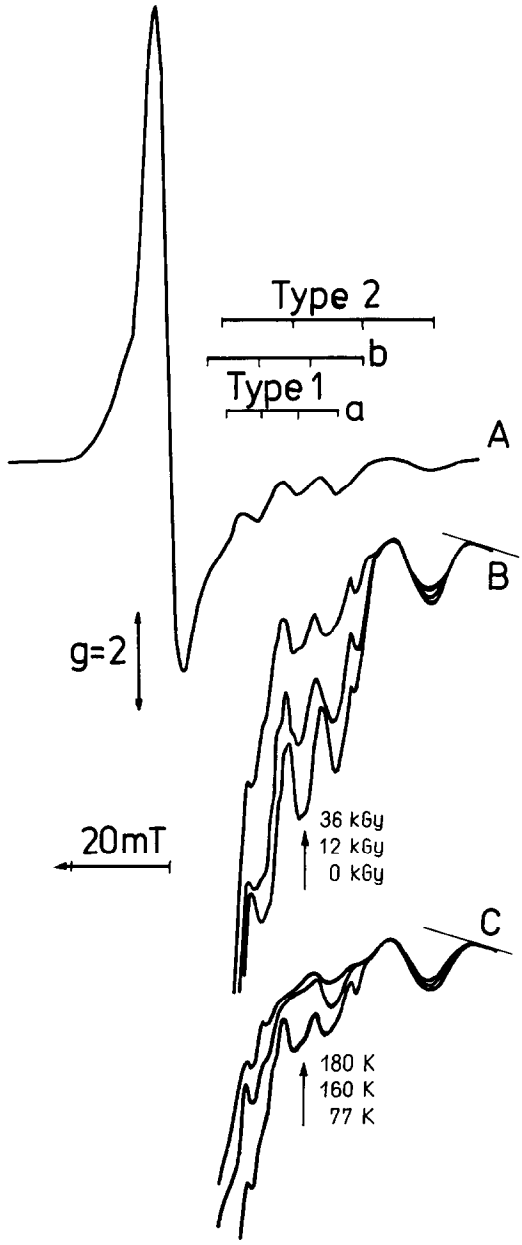

Fig.2. (A) EPR spectrum of human ceruloplasmin (~0.2 mM) in ethylene glycol–water glass at 77 K. (B) Effect of irradiation of human ceruloplasmin in ethylene glycol–water glass on the low-field part of EPR spectra of the enzyme at 77 K. The doses used were 0, 12 and 36 kGy of ^{60}Co γ-rays at 77 K. Gain equals 6 times that in (A). The initial slope of the base line is shown. (C) Effect of annealing temperature on the low-field part of EPR spectra of irradiated human ceruloplasmin in ethylene glycol–water glass at 77 K. The γ-irradiation dose was 36 kGy of ^{60}Co rays. The samples were annealed for 5 min at 160 and 180 K. Gain as in (B). The initial slope of the base line is shown.

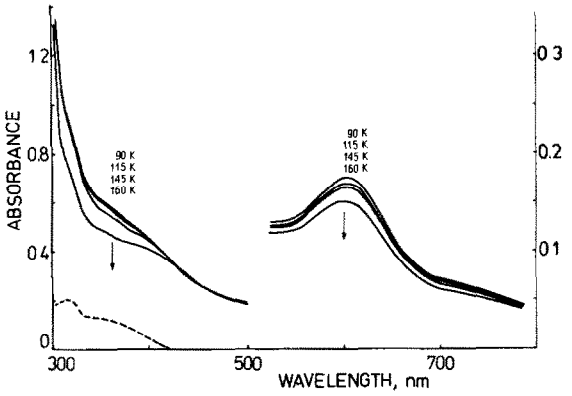

Fig.3. Effect of temperature on the optical absorption spectrum of irradiated human ceruloplasmin (~0.2 mM) in ethylene glycol–water glass. The spectra were taken successively at 90, 115, 145 and 160 K. The γ-irradiation dose was 36 kGy at 77 K. (– – –), Difference between the spectra recorded at 90 and 160 K.

(cf. difference spectra in figs.1 and 3). The spectrum with maxima at about 300 and 360 nm is probably due to the adduct of electron to the histidyl residue [11,12], while the band centered at 410 nm can be assigned to the adduct of electron to the disulphide group [13,14].

4. Discussion

The present results demonstrate that the reaction of quasi-free, 'dry', electrons with ceruloplasmin in the low-temperature glassy matrix leads to a direct reduction of Type 1 Cu^{2+}, as well as to the formation of radical species (intermediates). Pulse radiolysis investigations in solution have shown [6] that this copper site in ceruloplasmin can only be reduced indirectly by hydrated electron in a multistep, intramolecular electron transfer process from primary electron adducts. The studies on model compounds indicated [11–13] that several solvent accessible groups, like carbonyls, disulphides and imidazoles, can act as the primary and/or secondary electron accepting sites producing the transient ion-radicals. During the steady-state reaction of electrons with ceruloplasmin in glassy solution at 77 K both Type 1 Cu^{2+} is reduced and radical intermediates are stabilized. Hence the question arises how reducing equivalents are transported from the surface of the molecule to Type 1 copper. At present two hypothetical mechanisms can be proposed: (i) Type 1 Cu^{2+} is reduced by a quantum mechanical process, i.e. electron tunneling. (ii) Electron transfer to Type 1 Cu^{2+} proceeds by an electron exchange equilibrium state of the Type 1 Cu^{2+} with a solvent accessible redox site.

The first hypothesis seems to explain the temperature independence of Type 1 Cu^{2+} reducibility. It has been observed [7,8,14] that only the first reaction step occurs in a number of redox processes initiated at a suitable low temperature, there being insufficient energy available for overcoming the energy barrier to the subsequent intermediate or the final product. Reduction of Type 1 Cu^{2+} in ceruloplasmin irrespective of temperature points to a small or zero apparent activation energy of this process, consistent with the tunneling model. On the other hand, the first-order time course of Type 1 Cu^{2+} reduction in solution [6] is inconsistent with an appropriate tunneling model used to explain the reduction of ferricytochrome *c* by H and OH radicals [15].

The second hypothesis is attractive for several reasons. In the reduction reactions the transient absorption centered at about 410 nm, due to $RSSR^{\cdot-}$ ion-radical [6,13,14] is formed (cf. fig.1). Spectroscopic evidence for a similar intermediate in the reaction of the reduced blue copper oxidase with oxygen has been presented [16]. Since the decay of this species was closely related to reoxidation of Type 1 Cu^{2+} in reduced enzyme [17] and reduction of Type 1 Cu^{2+} in oxidized enzyme [6], respectively, it can be concluded that the same species is involved in intramolecular electron distribution among Type 1 Cu^{2+} and other copper sites.

Let us consider the electron transfer properties of the disulphide group. It is a two-electron system which has stabilized intermediate radical:

$$RSSR + e^- \rightleftharpoons RSSR^{\cdot-} \quad RSSR^{\cdot-} + e^- \overset{+2H^+}{\rightleftharpoons} 2RSH$$

The potentials of the two steps are closely matched so that reactions backwards and forwards are possible [18]. A strong coupling between the disulphide system and Type 3 Cu^{2+} has been proposed [19]. It is also accepted that there exist intramolecular redox equilibria between the different copper sites in blue copper oxidases. Because of this, it can be assumed that the known electron exchange between Type 1 Cu^{2+} and Type 3 Cu^{2+} sites is mediated by the following one-electron reaction:

$$\left\{\begin{matrix}\text{Type 1 } Cu^{2+}\\ RSSR^{\cdot-}\end{matrix}\right\} \rightleftharpoons \left\{\begin{matrix}\text{Type 1 } Cu^{1+}\\ RSSR\end{matrix}\right\}$$

If the equilibrium is forced to the left by lowering the temperature of the redox reaction the $RSSR^{\cdot-}$ can be stabilized. On the other hand, the temperature increase should cause the reduction of copper, as it has been observed by us. On the basis of our qualitative optical data it can be assumed that histidine radical is the electron-donating group to the copper-disulphide system (fig.1, closed and open circles, and fig.3).

It has been suggested that among different copper sites Type 1 Cu^{2+} is a primary electron acceptor. The reduction of Type 1 Cu^{2+} is a prerequisite for the reduction of Type 2 Cu^{2+} [20]. However, it is unclear why Type 2 Cu^{2+} is not available for very potent mobile electrons produced on γ-irradiation at 77 K (fig.2B), in spite of its high oxidation-reduction potential [1]. Presumably, this site in oxidized ceruloplasmin is shielded by a negatively charged electric field [21] in addition to the above requirement.

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