The Electron Pathway to Cu(II) in Ceruloplasmin

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SUMMARY

The reduction mechanism of type 1 Cu(II) of the human plasma copper protein, ceruloplasmin (EC 1.12.3.1), was investigated using the pulse radiolysis method. The hydrated electron, used as reducing agent, was found to decay by a bimolecular process. At the same rate, a transient absorption band, centered at 410 nm, appeared. It was followed by a slower increase of absorption due to a broad band with a maximum at 305 nm. The first band is assigned to the adduct of e\textsubscript{aq} with the disulfide groups (RSSR\textsuperscript{−} radical ion). The second band is probably due to the adduct of e\textsubscript{aq} with an aromatic amino acid residue (histidyl). Both bands decay in a first order process at the same rate at which type 1 Cu(II) is reduced ((9 \pm 1) \times 10\textsuperscript{5} s\textsuperscript{-1}). The pathway of the reduction equivalent to this oxidation-reduction active center, may therefore be delineated at least for reduction by the very potent e\textsubscript{aq}; it proceeds from the bimolecular encounter step, in which the electron is attached to residues on the protein surface (peptide bonds and disulfide groups) by an internal transfer to an imidazol residue and ultimately to the type 1 Cu(II) center.

EXPERIMENTAL PROCEDURE

Human ceruloplasmin from two sources was used. Most of the experiments were carried out with protein supplied by Miles-Yeda, Rehovot, Israel. A comparison using highly purified protein samples obtained from Dr. R. J. Cariico, Goteborg, yielded similar results. Ceruloplasmin supplied by Miles-Yeda gave one line in an immunoelectrophoresis against anti-human whole serum. The 280 nm/610 nm absorption ratio was 22. Oxidase activity was checked by using p-phenylenediamine as substrate. It was found to be 13 units per 1 mg of ceruloplasmin (11). Solutions were prepared in triply distilled water which had been previously deaerated by continuous bubbling of purified argon (Matheson, ultrapure grade) for at least 30 min. During the bubbling, the pH of the solution was adjusted by addition of either dilute HCl or NaOH solutions. The protein was introduced by adding the appropriate volumes of concentrated stock solutions 1 min before the end of the deaeration procedure. This prevented deauration of the protein by foaming. The residual concentration of oxygen was of the order of \(< 10^{-9}\) M as checked by gas chromatography.

The pulse radiolysis technique was recently applied to the investigation of the mechanism of action of oxidation-reduction proteins (1-4). This method enables production of reactive chemical species of either reducing (hydrated electrons, e\textsubscript{aq}) or oxidizing (hydroxyl radicals) nature in homogeneous aqueous solutions within the nanosecond time range (5). Combined with an appropriate spectrophotometric system, it offers a convenient and effective tool for studying the pathway involved in the electron transfer mechanism of oxidation-reduction proteins. The hydrated electron was found to reduce specifically the ferric ion. The second band is probably due to the adduct of e\textsubscript{aq} with the disulfide groups (RSSR\textsuperscript{−} radical ion). The formntion and decay of the formed transients were followed by optical and EPR oxidation-reduction titrations (10). The study of the reaction of ceruloplasmin with e\textsubscript{aq} has been undertaken in order to identify the first specific electron accepting site and its pathway into and within the oxidase.

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1 The abbreviations used are: e\textsubscript{aq}, hydrated electron; G, number of radicals, atoms, or molecules formed per 100 e.v.
They were composed of at least three stages. The first stage, Fig. 3 shows three typical oscilloscope traces of these transients. On the contrary, transients absorbing in this region were formed. Of ceruloplasmin and dose rate employed, even 45 s after the pulse.

| Table I summarizes the calculated specific rate constants for the reaction between histidine and e\textsuperscript{aq} (1.4 \times 10^6 M\textsuperscript{-1} s\textsuperscript{-1}) compared with the other amino acids (\sim 1.5 \times 10^5 M\textsuperscript{-1} s\textsuperscript{-1}) (18).

The kinetic curves were evaluated by a manual trace follower coupled to an analog to digital converter by which the Polaroid pictures were read and the data were transferred to punched cards. All traces were analyzed for total changes of the optical density and for their fit to either first or second order decay. First or second order rate constants were calculated by the least square method only when a good decay curve plot was obtained for at least two half-lives (linear correlation >99%). At least five traces were analyzed for each rate constant.

RESULTS

When fast electrons are absorbed in liquid water, the effect may be described by the over-all reaction

$$H_2O \rightarrow e_{aq}, H_2O, H_2O_2, H_2, H_2O_3$$ (1)

The ensuing chemical events in a dilute aqueous solution can be accounted for in terms of the reactions of these initial entities (5). The radiochemical yields are: \(G_{e_{aq}} = 2.6\), \(G_{OH} = 2.6\), \(G_{H_2} = 0.45\), \(G_{H_2O_2} = 0.75\), and \(G_{H_2O} = 0.55\). The OH radicals can be removed by reacting them with i-butanol (\(k = 5 \times 10^9 M^{-1} s^{-1}\)) to form a weakly absorbing transient radical (\(\lambda_{max} = 225\), \(\varepsilon_{aq} = 900 M^{-1} cm^{-1}\)) which is relatively unreactive to most reagents (14).

When neutral deaerated solutions containing ceruloplasmin in the presence of i-butanol are subjected to a single pulse, the transient absorption due to the hydrated electron is produced (15) (\(\lambda_{max} = 720 nm\), \(\lambda_{720} = 18,500 M^{-1} cm^{-1}\)). The decay of the \(\varepsilon_{aq}\) follows at 550 nm (\(\varepsilon_{550} = 10,000 M^{-1} cm^{-1}\)) was found to be a bimolecular process (\(k = (9 \pm 1) \times 10^9 M^{-1} s^{-1}\)). The reduction of the 605 nm band due to the Cu(II) designated as type 1 of ceruloplasmin (10, 16) was found to be a first order process, slower by two orders of magnitude. Such a kinetic behavior has also been observed in the reaction of \(e_{aq}\) with lactase (3). Fig. 1 shows the decay curve of the \(e_{aq}\) absorption. Fig. 2 presents the decay of the 605 nm band due to the type 1 Cu(II) ion. Table I summarizes the calculated specific rate constants for the decay of the 605 nm band at different protein concentrations and dose rates. These results indicate that the reduction step of type 1 Cu(II) is a first order process, where the electron is transferred by an intramolecular process from the initial reduction site on the protein surface.

No net decrease in the absorbance in the near ultraviolet region (330 nm) could be detected over the entire concentration range of ceruloplasmin and dose rate employed, even 45 s after the pulse. On the contrary, transients absorbing in this region were formed. Fig. 3 shows three typical oscilloscope traces of these transients. They were composed of at least three stages. The first stage (3A) is a very fast process, where the transient was formed at a rate concomitant with that of the disappearance of the \(e_{aq}\) (\(k ~ 1 \times 10^{10} M^{-1} s^{-1}\)). This was followed by a slower second step in which the absorption further increases (\(k = 7 \times 10^9 s^{-1}\)) (Fig. 3B). Fig. 3C shows the last stage where the transient absorption decays (\(k = (9 \pm 1) \times 10^8 s^{-1}\)). In Fig. 4 the constructed spectra of the transients produced in the ultraviolet region are shown (Fig. 4, A and B, respectively). Fig. 4C shows their difference spectrum. The spectrum of the first transient with a maximum at 410 nm (Fig. 4A) is identical to that of the radical anion, RSSR\textsuperscript{-}, formed on reduction of simple disulfides such as cysteine (17).

In order to establish the nature of the second transient (Fig. 4C), aqueous solutions of aromatic amino acids known to react rapidly with \(e_{aq}\) (10^9 to 10^{8} s^{-1}) (18) were pulse-radioabeled and the spectra of the produced transients were recorded. These included the following amino acids: phenylalanine, histidine, tyrosine, and tryptophan. Peptides containing several peptide bonds (\(\text{glycyl, Ala}, \text{Ala}\)) also react with \(e_{aq}\) at rates of the order of 10^{9} M^{-1} s^{-1} to form, however, transients absorbing in the far ultraviolet region. The transient spectra observed are shown in Fig. 5. Comparison between these transient spectra and that observed in ceruloplasmin solutions seems to indicate that all the electron adduct of histidyl residues are responsible for the observed transient. This is also in agreement with the high rate constant obtained for the reaction between histidine and \(e_{aq}\) (1.4 \times 10^6 M^{-1} s^{-1}) compared with the other amino acids (\sim 1.5 \times 10^5 M^{-1} s^{-1}) (18).

DISCUSSION

The reduction of type 1 Cu(II) in ceruloplasmin by the hydrated electrons is a multistep process. In the first phase, the \(e_{aq}\) decays by reacting with the protein molecules at a rate close to that of the diffusion control. In this step, the \(e_{aq}\) is attached to several groups on the surface of the macromolecule. Protein residues known to be most reactive towards the hydrated electrons are the disulfide (17) and peptide groups (18)\textsuperscript{2} (\(k > 10^9 M^{-1} s^{-1}\)). In fact, the formation of the transient absorption centered at 410 nm, due to the RSSR\textsuperscript{-} radical ion, is concomitant to the decay of \(e_{aq}\). Since the yield of RSSR\textsuperscript{-} radical ion amounts to only \sim 20% of the \(e_{aq}\) produced by the pulse, it is plausible that the remaining \(e_{aq}\) reacts with the peptide groups. The absorption spectrum of the electron adduct of the peptide group lies below 300 nm and could not be followed by our system.

The second phase of the reduction process is the monomolecular formation of a transient, absorbing in the range below 500 nm. It has a well defined maximum at about 300 nm (Fig. 5). This transient is assigned to an electron adduct of histidine. It is most probably formed by electron migration from the primary electron adduct of the peptide bond. The exclusion of the RSSR\textsuperscript{-} as electron donor is based on the following. The extinction coefficient of the RSSR\textsuperscript{-} (1.3 \times 10^9 M^{-1} cm^{-1}) is much higher than that of the electron adduct of the histidyl residue (\sim 6 \times 10^8 M^{-1} cm^{-1}). Therefore, any electron transfer from the RSSR\textsuperscript{-} should lead to a net decrease in absorbance at 410 nm. The fact that a net increase is observed implies that the electron donors to the histidyl are the electron adducts of the peptide groups.

The final stage of the reduction process is the decay of the absorption bands of both the RSSR\textsuperscript{-} (410 nm) and the electron adduct of the histidyl residue (300 nm), which is concomitant with the reduction of type 1 Cu(II) (610 nm). This is borne out

\* M. Faraggi and Y. Tendler, submitted for publication.
Fig. 1 (top left). Oscilloscope trace of the decay of $e_{\alpha\alpha}$ followed at 605 nm in ceruloplasmin = 1.0 \times 10^{-6} M. Dose $\sim$ 200 rads. Vertical displacement corresponds to a decrease in transmission due to the $e_{\alpha\alpha}$ formation during the pulse followed by an increase of transmission due to the decay of $e_{\alpha\alpha}$ (one large division = 2.7%), horizontal displacement corresponds to time (one large division = 1 ps).

Fig. 2 (bottom left). Oscilloscope trace of the type 1 Cu(II) reduction observed at 610 nm. Ceruloplasmin = 1 \times 10^{-4} M. Dose $\sim$ 200 rads. Vertical displacement corresponds to an increase in transmission (one large division = 2.5%), horizontal displacement corresponds to time (one large division = 1 ms).

Fig. 3 (right). Absorption changes at 340 nm. Ceruloplasmin = 1.0 \times 10^{-4} M; pH $= 6$; dose $= 200$ rads. Vertical displacement corresponds to changes in transmission, horizontal to time: A, decrease of transmission (one large division = 2.3%), time 2 ms per division; B, decrease of transmission (one large division = 1.67%), time 100 ms per division; C, increase of transmission (one large division = 1.81%), time = 1 ms per division.

**TABLE I**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Pulse length</th>
<th>$k \times 10^2$ s$^{-1}$</th>
</tr>
</thead>
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<tr>
<td>$1.8 \times 10^{-6}$</td>
<td>0.5</td>
<td>10.0</td>
</tr>
<tr>
<td>$4.4 \times 10^{-6}$</td>
<td>0.1</td>
<td>8.2</td>
</tr>
<tr>
<td>$4.4 \times 10^{-6}$</td>
<td>0.5</td>
<td>8.4</td>
</tr>
<tr>
<td>$4.4 \times 10^{-6}$</td>
<td>0.75</td>
<td>8.4</td>
</tr>
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<td>1.0</td>
<td>8.2</td>
</tr>
<tr>
<td>$1.0 \times 10^{-5}$</td>
<td>0.5</td>
<td>9.6</td>
</tr>
</tbody>
</table>

Reduction was followed at 605 nm. Solutions contained human ceruloplasmin without any added salts, the pH was adjusted to 7.0, and the temperature was 20°C. Standard error of the specific rates, ±10%.

Table 1 summarizes the specific rates of reduction of type 1 Cu(II) of human ceruloplasmin. The table includes the concentration of ceruloplasmin, the pulse length, and the specific rate constant ($k$). The specific rates were determined by fitting the experimental data to a monoexponential decay function.

The proposed mechanism, Scheme 1, shows the reaction between $HCP$ and $RSSR^{-}$ to form $R'C-\text{NHR}^{-}$, which then undergoes a second reaction to form $RSSR^{-}$ and $\text{Cu}^{+}$.

By the fact that all three decay processes are monomolecular and have the same specific rates, Scheme 1 summarizes the proposed mechanism.

Reduction was followed at 605 nm. Solutions contained human ceruloplasmin without any added salts, the pH was adjusted to 7.0, and the temperature was 20°C. Standard error of the specific rates, ±10%.
azurin (a bacterial electron-mediating protein) is reduced by the hydrated electron in a direct reaction. Thus, the reduction of Cu(II) in this type of site may depend on the function which the protein is performing.

The suggested pathway of the electron to type 1 Cu(II) in ceruloplasmin is based on the use of a highly reactive reducing agent. It is, therefore, conceivable that a different electron pathway may be followed with milder reductants. Preliminary data from studies in which O₂⁻ is used as reductant have shown that type 1 Cu(II) is reduced. However, no intermediates could be resolved during the time interval between the decay of the O₂⁻ radical and the reduction of the 610 nm band.

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Fig. 4. Spectra of the transients formed from ceruloplasmin in the near ultraviolet region: a, absorption changes recorded immediately after the pulse; b, absorption changes recorded 500 μs after the pulse; c, difference spectrum.

Fig. 5. Transient absorption spectra formed by the reaction of e⁻ with some aromatic amino acids.

The reduction of type 1 Cu(II) in lactase, the other copper oxidase investigated by the pulse radiolysis method, is similar in its general pattern to that observed for ceruloplasmin. Namely, the metal ion is reduced indirectly by an intramolecular electron transfer process from the primary electron adducts. This indirect reduction mechanism of type 1 Cu(II) ions in this oxidase constitutes a further illustration of the cryptic nature of these copper binding sites. It is of interest that the type 1 Cu(II) in
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