Human Ceruloplasmin

INTRAMOLECULAR ELECTRON TRANSFER KINETICS AND EQUILIBRATION*

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Pulse radiolytic reduction of disulfide bridges in ceruloplasmin yielding RSSR$^–$ radicals induces a cascade of intramolecular electron transfer (ET) processes. Based on the three-dimensional structure of ceruloplasmin identification of individual kinetically active disulfide groups and type 1 (T1) copper centers, the following is proposed. The first T1 copper(I) ion to be reduced in ceruloplasmin is the blue copper center of domain 6 (T1A) by ET from RSSR$^–$ of domain 5. The rate constant is $28 \pm 2$ s$^{-1}$ at 279 K and pH 7.0. T1A is in close covalent contact with the type 3 copper pair and indeed electron equilibration between T1A and the trinuclear copper center in the domain 1–6 interface takes place with a rate constant of $2.9 \pm 0.6$ s$^{-1}$. The equilibrium constant is 0.17. Following reduction of T1A Cu(I), another ET process takes place between RSSR$^–$ and T1B copper(II) of domain 4 with a rate constant of $3.9 \pm 0.8$. No reoxidation of T1B Cu(I) could be resolved. It appears that the third T1 center (TIC of domain 2) is not participating in intramolecular ET, as it seems to be in a reduced state in the resting enzyme.

The blue multicopper oxidases are enzymes that catalyze the 4-electron reduction of dioxygen to water by four sequential 1-electron oxidations of substrate (1). These enzymes are widely distributed in nature, from bacteria, fungi, and plants to mammals. All contain at least 4 copper ions of the following types. (i) The blue type 1 site (T1)$^1$ characterized by an intense charge transfer band in the 600-nm region ($\epsilon \sim 5000$ M$^{-1}$ cm$^{-1}$) and a narrow hyperfine coupling constant ($A_{1} < 10^{-3}$ cm$^{-1}$) in the electron paramagnetic resonance spectrum (2). (ii) A “normal” type 2 (T2) copper center characterized by a lack of intense absorption bands and ordinary EPR spectrum. (iii) A copper ion pair, called type 3 (T3), which in the oxidized state is characterized by an intense absorption in the near UV region ($\epsilon \sim 4000$ M$^{-1}$ cm$^{-1}$) and by strong antiferromagnetic coupling. T2 and T3 are proximal and form together a trinuclear cluster, which is the dioxygen reduction site (1). The physiological function of T1 is sequential uptake and transfer of single electrons from substrate molecules to the trinuclear center where dioxygen binds and gets reduced to water. Thus, the enzymatic process takes place by a ping-pong mechanism (1).

Intramolecular electron transfer (ET) between T1 and the trinuclear T2/T3 center is therefore expected to play a crucial role in the molecular mechanism of this class of enzymes. High resolution crystal structures are now available for ascorbate oxidase (3, 4), ceruloplasmin (5, 6), and fungal laccase (7). The laccases are characterized by relatively low substrate specificity toward the reducing substrate, whereas ascorbate oxidase exhibits a high specificity toward l-ascorbate. We have previously studied the intramolecular ET processes that take place in Rhus vernicifera laccase (8) and in ascorbate oxidase (9–11).

The only blue copper oxidase found in humans is ceruloplasmin (hCp) (Fe(II)$^2$O$_2^–$; EC 1.16.3.1). Whereas its physiological role is still debated, a consensus that hCp functions as a plasma ferric oxidase is emerging. Kinetic studies have demonstrated a high affinity of hCp for transferrin bound Fe(II)$^2$ (12), and binding sites for divalent cations have been identified in the recent crystallographic studies (6). Whereas the ferric oxidase activity may be the primary function of ceruloplasmin, a related role in iron transport may also be envisaged. Studies on the genetic disorder aceruloplasminemia have demonstrated extensive deposition of iron in liver and brain in patients suffering from this disease (14). Whether hCp is also involved in active copper transport remains to be proven although the protein could act as copper reservoir as more than 80% of the copper in humans is bound to hCp (1). Early suggestions that hCp contains six copper ions (15) and that its sequence indicates the presence of three putative T1 centers (16) were confirmed by Lindley and co-workers (5, 6) based on the three-dimensional structure. Thus, ceruloplasmin is unique among the blue copper oxidases as it contains additional copper centers beyond the four required for the enzymatic function.

Here we present results of kinetic and equilibrium studies of hCp, which suggest that one of the three T1 copper ions is responsible for the intramolecular ET to the trinuclear center, whereas the other T1 centers do not participate directly in the intramolecular equilibration process.

EXPERIMENTAL PROCEDURES

Isolation and Purification of hCp—Human ceruloplasmin was isolated from freshly withdrawn plasma according to the method of Calabrese et al. (17) with the following modifications. After filtration, 0.5 liter of plasma was added to 6-aminohexanoic acid (20 mmol) and phenylmethylsulfonyl fluoride (100 mmol) and diluted with Milli-Q water to a total volume of 1 liter. This solution was immediately mixed with 100 ml of polyethyleneimine-Sepharose (pre-equilibrated with 50 mM sodium phosphate, pH 7.0). The Sepharose, which was clearly blue, was collected on a glass filter and washed thoroughly with 50 mM phosphate, 20 mM 6-aminohexanoic acid (pH 7.0). The crude ceruloplasmin was then eluted with 300 mM phosphate, 20 mM 6-aminohexanoic acid, pH 7.0, and collected as 50 ml of dark blue eluate. Now 5.5 g of polyethylene glycol 6000 was added, and the solution was left stirring

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2. O. Farver and L. Bendahl, unpublished results.
on an ice bath for 15 min. A white precipitate could hereafter be separated by centrifugation at 3000 g. After another addition of 5.5 g of polyethylene glycol and stirring on an ice bath for 15 min, further precipitate could be discarded. Finally, increasing the polyethylene glycol concentration to 23% by the addition of 5.5 g of the polymer, a dark blue solid precipitated and was isolated by centrifugation. The precipitate was then dissolved in 100 ml of phosphate buffer (100 mM phosphate, 20 mM 6-aminohexanoic acid, pH 7.0). After sterile filtration, hCp was collected on a short (2.5 × 10 cm) Amersham Pharmacia Biotech Source15Q column equilibrated with 100 mM phosphate, 20 mM 6-aminohexanoic acid, pH 7.0. As a 1.5-cm broad band. It was washed first with 150 ml of the same buffer followed by 100 ml of 100 mM phosphate, pH 7.0. Finally the protein was eluted with 150 ml of phosphate buffer, pH 7.0, and collected as 10 ml of dark blue solution.

The enzyme was immediately frozen in liquid nitrogen in small aliquots. After this purification, the A₆₁₀/A₂₈₀ was 0.047, which is equal to or better than reported literature values (17).

*Kinetic Measurements of the ET Reactions—* The kinetic pulse radiolysis experiments were carried out using a Varian V-7715 linear accelerator at the Hebrew University in Jerusalem. 5 MeV electrons were used with pulse lengths in the range from 0.1 to 0.5 μs. All optical measurements were carried out anaerobically in a 4 × 2 × 1-cm Spectrosil cuvette. Three light passes were employed that result in an overall optical path length of 12.3 cm. A 150 watt xenon lamp produced the analyzing light beam, and an appropriate optical filter with a cut-off at 295 nm was used to avoid photochemistry and light scattering. The data acquisition system consisted of a Tektronix 390 A/D digitizer connected to a PC. The temperature of the reaction solutions (kept constant at 5.5 °C) was controlled by a thermostating system and continuously monitored by a thermistor attached to the cuvette (18). In one series of experiments, the buffer solution consisted of 100 mM formate, 10 mM phosphate under purified N₂O (pH 7.0). Under these conditions 0.5–3 mM CO₂ radical ions were produced in each pulse and served as an electron donor. In another series of pulse radiolytic experiments the (uncharged) N-methyl nicotinamide radical was produced and used as reducing agent. These radicals were produced in argon saturated solutions by reacting hydrated electrons directly with 5.0 mM N-methyl nicotinamide. The OH radicals produced by the irradiation process were scavenged with 300 mM tert-butyl alcohol. The pH was kept constant at 7.0 using 10 mM sodium phosphate buffer. Practically all reactions were performed under pseudo-first order conditions, with typically a 10-fold excess of oxidized protein over reductant. The concentration of T[II]Cu(II) was monitored at 610 nm (e₆₁₀ = 5000 M⁻¹ cm⁻¹), and T₃Cu(II) was monitored at 330 nm (Δε₃₃₀ = 2400 M⁻¹ cm⁻¹), whereas formation and decay of the RSSR⁻ radical were followed at 410 nm (e₄₁₀ = 10,000 M⁻¹ cm⁻¹) (18). Enzyme concentrations were usually in the 5–15 μM range.

Aqueous buffer solutions were deaerated and saturated with either

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**Fig. 1.** Time resolved absorption changes monitored in a 5.3 μM hCp solution following a 0.5-μs pulse of 5-MeV accelerated electrons. Data were collected in the two different time scales indicated. Conditions were: pH 7.0, 100 mM formate, 10 mM phosphate; temperature 5.5 °C. A, T[II]Cu(II) reduction monitored at 610 nm starting from a 0.5-electron equivalent reduced enzyme. B, T[II]Cu(II) reduction measured at 610 nm in a 1.5-electron equivalent reduced enzyme. C, RSSR⁻ formation and reoxidation monitored at 410 nm under the same conditions as in A. D, RSSR⁻ formation and reoxidation monitored at 410 nm under the same conditions as in A.
the number of reduction equivalents introduced by CO₂ reoxidation, and RSSR² reoxidation plotted as a function of the number of reduction equivalents introduced by CO₂ or N-methyl nicotinamide radicals. B, observed amplitudes for T1[Cu(II)] reduction, T1[Cu(I)] reoxidation, and RSSR² reoxidation plotted as a function of the number of reduction equivalents introduced by CO₂ or N-methyl nicotinamide radicals. ● reduction at 610 nm; ○ reoxidation at 610 nm; ■ reoxidation at 410 nm; ▼ slow reduction phase at 610 nm.

N₂O or argon in sterile glass syringes. Afterward, the concentrated protein stock solution was added, and N₂O or argon bubbling was continued for another 5 min. The solutions were then transferred into the pulse radiolysis cuvette under anaerobic conditions. All chemicals were of analytical quality, and Milli-Q water was used throughout the studies.

RESULTS

The steady-state enzymatic activity of the hCp samples used in this study was routinely assayed both before and after pulse radiolysis. The observed oxidase turnover number was typically 2.2 s⁻¹ using Fe(II) as reducing substrate, which demonstrates that the enzyme was highly active. Also, the spectral properties (ΔA₆₁₀/ΔA₂₈₀ = 0.047) showed that the oxidase was in an optimal state.

When N₂O-saturated hCp solutions (100 mM formate, 10 mM phosphate, pH 7.0, N₂O saturated) were subjected to pulses producing CO₂ radicals, absorption changes at 410 nm demonstrated that a disulfide radical anion, RSSR², is formed in a bimolecular process with a rate constant of 2.3 × 10⁹ M⁻¹ s⁻¹ (279 K) i.e. a rate that is essentially diffusion controlled (Fig. 1C). Reduction of T1[Cu(II)] (monitored at 610 nm) in fully oxidized molecules was further observed with a rate constant at 279 K of 28 ± 2 s⁻¹ (Fig. 1A). This process was independent of protein concentration characterizing an intramolecular mechanism. The decay of RSSR² also occurred in a unimolecular process concomitantly with the above reduction of T1[Cu(II)] demonstrating that this process indeed takes place by intramolecular ET. Following this reduction phase partial reoxidation of T1[Cu(I)] was observed (Fig. 1A) with the concomitant reduction of T3[Cu(II)] (monitored at 330 nm). The rate constant of this process (2.9 ± 0.6 s⁻¹ at 279 K) was also independent of the protein concentration, which indicates that an intramolecular ET from T1 to T3 is observed. Identical results were obtained using N-methyl nicotinamide radicals as the reducing agent in argon saturated solutions with tert-butyl alcohol as OH radical scavengers.

The rate constant for RSSR reduction by the pulse radiolytically produced radicals decreases with the extent of reduction of ceruloplasmin. Thus, after introduction of 1.5-electron equivalents (Fig. 1D) the bimolecular rate reduces to 0.5 × 10⁹ M⁻¹ s⁻¹. Intramolecular ET from RSSR² to T1Cu(II) still occurs, but as illustrated in Fig. 1B reoxidation of T1Cu(II) ceases to take place after one reducing equivalent was introduced into the enzyme molecules. The rate constant of T1[Cu(II)] reduction decreased from 28 s⁻¹ to 3.9 ± 0.8 s⁻¹ at 279 K (Fig. 2A), and besides a new reduction phase could be observed at a much slower time scale (Figs. 1B and 2A). The latter seems to be dependent on the reduction state of the enzyme and is probably a slow intermolecular ET process. This reaction pattern was clearly paralleled by the amplitudes of the different reaction phases, which decreased by almost one order of magnitude after introduction of the first electron equivalent (Fig. 2B). It is also evident from the amplitudes that T1[Cu(I)] is only partially reoxidized and that the extent of its reoxidation depends on the redox state of the hCp molecules. At 279 K we determine an equilibrium constant for the intramolecular ET process to be 0.17.

T1[Cu(I)]/T2/T3[Cu(II)] = T1[Cu(II)]/T2/T3[Cu(I)]

SCHHEME I

This corresponds to a difference in reduction potential between T1 and T2/T3 of +43 mV.

Reoxidation of T3[Cu(I)] was not observed during the kinetic experiments irrespective of the time frame employed. This is probably a reflection of the redox potential of this site and confirms the strict anaerobic conditions under which the experiments were performed.

DISCUSSION

hCp is the odd member of the blue copper oxidase family for several reasons. First and foremost, its function as an oxidase is still unresolved, though its capacity to catalyze ferrous ion oxidation by dioxygen has been well established, and its involvement in iron metabolism is strongly supported by considerable physiological and genetic evidence (19). Another exception is its copper content and hence the number and nature of...
sites that have been debated for quite some time. This has now clearly been resolved by the three-dimensional crystallographic structure determination of human ceruloplasmin and shown to contain three distinct T1 sites in addition to the trinuclear cluster (5, 6). Finally, hCp is the only mammalian member of the blue copper protein family. Human ceruloplasmin consists of a single polypeptide with a molecular mass of 132 kDa folded in six domains arranged in a triangular array. A schematic view of hCp is presented in Fig. 3. Each domain comprises a β-barrel constructed in a manner typical for the cupredoxins. Three of the six copper ions are bound to T1 sites present in domains 2, 4, and 6, whereas the other three copper ions form a trinuclear cluster bound at the interface between domains 1 and 6. The spatial relation between the trinuclear center and the nearest T1 site (Fig. 3, A, in domain 6) closely resembles that found in ascorbate oxidase. This was taken to further support that ceruloplasmin has an oxidase function (5, 6). The three T1 sites are separated from each other by a distance of 1.8 nm. This was again related to the ceruloplasmin function because this distance might still allow for internal ET at reasonable rates and could also increase the probability for electron uptake. The coordination sphere of T1 in domain 4 (T1B) is identical to that of domain 6 (T1A). The third type 1 center (T1C), however, contains a nonligating Leu residue instead of the “usual” Met. Deinum and Vännström (20) have earlier, by a combination of redox titrations, EPR, and UV-visible spectroscopy, determined that hCp contains 3 paramagnetic copper(II) ions, two T1 and one T2. The reduction potentials of the two former copper centers were determined to be 580 and 490 mV. However, their experimental accuracy did not allow for a precise determination of whether hCp contains three or four non-paramagnetic copper ions. The x-ray structure of Lindley and co-workers (5, 6) has now unambiguously demonstrated that hCp contains six copper ions, three T1, one T2, and one T3 pair. Studying the reduction potential of the blue T1 copper protein, azurin in which the copper ligating Met was substituted by Leu or Val, it was found that in these mutants the reduction potentials increased from 307 to 412 and 445 mV, respectively, at pH 7.0 (21). Studies of hCp by x-ray absorption spectroscopy suggested that the resting oxidized enzyme contains one permanently reduced T1 center and that this site cannot be involved in the catalytic process (22). The reduction potential of this T1 center was estimated to be at least 1.9 V. So, based on the above electrochemical studies on azurin and the crystal structure we assign this high potential T1 site to T1C (the type 1 center of domain 2). This is also in line with the recent x-ray structure for fungal laccase from Coprinus cinereus (7), which also has a leucine residue replacing methionine in the strongly oxidizing T1 copper(II) center.

The trinuclear coordination site consists of four histidine pairs, two pairs from domain 1 and the other two from domain 6. Like in ascorbate oxidase, two of the copper ions are bound to six histidines and assigned as T3, whereas the third copper (most distant from T1) is coordinated to two histidines only and is designated as bound to the T2 site. Histidine pairs bridge two copper ions, and by further analogy to the ascorbate oxidase structure, an oxygen atom connects the two T3 coppers, whereas another one is bound to the T2 copper ion (5, 6). An additional relevant structural feature is the domain 6 cysteine providing a thiolate ligand to T1. This residue is placed between the two sequential His residues that are coordinated to the T3 copper pair. This structural element has first been observed in ascorbate oxidase and was proposed to provide the ET path between the single T1 and the trinuclear center (3, 4).

A considerable body of results from activity studies of hCp has accumulated during earlier decades and is now awaiting a more meaningful analysis and interpretation using the available three-dimensional structure. Catalysis of amine oxidation by hCp, in particular biogenic ones present in plasma, cerebral, spinal, and intestinal fluids as well as of ferrous ions is probably physiologically relevant and has been studied extensively (23). The mechanism of dioxygen reduction by hCp at the trinuclear center is of particular interest as the presence of three distinct T1 sites raises the question of which sites are involved in the internal ET to the single O2 reduction site.

Pulse radiolysis was first applied to studying ET within this multicentered blue oxidase 25 years ago where internal ET to the T1 Cu(II) was observed from the disulfide radical ions produced upon hCp reaction with hydrated electrons (24). Both the transient absorption changes because of the disulfide radicals and the T1(Cu(II)) site were found to decay in unimolecular processes at identical rates of ~900 s⁻¹ at room temperature. The results reported here show that both CO3⁻ and N-methyl nicotinamide radicals react with disulfide groups of hCp at diffusion controlled rates to produce the RSSR⁻ radicals. From here the electrons are transferred intramolecularly to T1(Cu(II)) with a rate constant at 279 K of 28 ± 2 s⁻¹. Following this step, in molecules with an oxidized T2T3 center, an intramolecular electron equilibration (Scheme I) is observed with an equilibrium constant of 0.17 at the same temperature. After introduction of one electron equivalent into this particular T1 center, intramolecular ET to the trinuclear site ceases to take place. At the same step the rate constant for intramolecular ET from the disulfide radical to a T1(Cu(II)) is slowed down by almost a factor of 10 to be 3.9 ± 0.8 s⁻¹. This is interpreted as ET to one of the other T1 centers; the first T1 center to be reduced is most probably the one closest to the trinuclear site (T1A), because reoxidation only takes place as long as less than one reducing equivalent is added to the hCp molecules. This could be the T1(Cu(II)) with the highest reduction potential (580 mV) because in reductive titrations the first 50% of the total absorption at 610 nm decays within 3 min, whereas the further reduction proceeds much slower (20). From the equilibrium constant for the internal ET equilibration process (K, 0.17 at 279 K), we calculate a difference in reduction potential between T1A and T3 Cu(II)/Cu(I) of 43 mV. Now, a reduction potential for T3(Cu(II)/Cu(I)) of 537 mV can be calculated. The potential for the second (lower) T1B was reported to be 490 mV (20). This places the reduction potential of the trinuclear site midway between those of the two redox active T1 centers. Identification of T1A as the primary electron acceptor among the T1 centers is further substantiated by the analysis of the relative solvent exposure of the disulfide groups (see below) and their distance from the T1 centers.

In the three-dimensional structure of hCp (5, 6) five disulfide bridges are found distributed evenly throughout the protein in domains 1–5 (cf. Fig. 3). All five disulfides are found near the bottom of a β-barrel, and in two domains the T1 copper centers (T1B and T1C) are placed at the opposite end of the barrels. The only domain lacking a disulfide is domain 6, which contains T1A and the trinuclear copper center. All three T1 centers are well protected from the solvent by the protein matrix. Thus, it is not surprising that only indirect copper reduction via the exposed disulfide groups is observed. In fact, this behavior is quite reminiscent of results of our studies of intramolecular ET in azurins (25, 26), where long range electron transfer takes place from the disulfide radical to T1(Cu(II)) over a distance of 2.45 nm. The main difference between azurin and hCp is that no direct Cu(II) reduction is observed in hCp, whereas in azurin approximately 50% of the reducing radicals react directly with the blue copper center (25, 26). Rate constants for ET between the disulfide radical and Cu(II) in azurin have been determined.
as a function of driving force ($-\Delta G^0$), separation distance ($r-r_0$), electronic coupling ($H_{DA}$) and reorganization energies ($\lambda$), cf. Equations 2 and 3 (27).

$$k = \frac{2\pi}{\hbar} \frac{H_{DA}}{(4\pi\hbar RT)^{1/2}} \exp\left(-\frac{\Delta G^0 + \lambda}{RT}\right)$$ \hspace{1cm} (Eq. 2)

$$H_{DA} = H_{DA0} \exp\left(-\frac{\lambda}{RT}\right)$$ \hspace{1cm} (Eq. 3)

For hCp we may analogously calculate a driving force for intramolecular ET between disulfide radicals and the first and second T1(Cu(II)) of 0.99 and 0.90 eV, respectively. Pathway calculations, as developed by Beratan et al. (28), identifying the optimal electronic couplings between the three T1 centers and disulfide radicals show that the optimal ET route connecting a disulfide with a T1 center occurs between the highly solvent exposed disulfide Cys-855/Cys-881 of domain 5 and the T1A center of domain 6, which also happens to be the T1 center adjacent to the trinuclear T2/T3 copper center. Furthermore, the domain 5 disulfide bridge is surrounded by a number of arginines, the positive charges of which make this site even more attractive for the negatively charged CO$_2^-$ radicals. This pathway, shown in Fig. 4, consists of 20 covalent bonds, 3 hydrogen bonds, and one van der Waals contact connecting the disulfide group with the T1A-ligating His-975. This gives rise to a coupling decay factor of $P = 1.0 \times 10^{-8}$. Assuming similar reorganization energies as in azurin, a theoretically calculated rate constant of 10 $s^{-1}$ is obtained, in good agreement with the experimentally observed rate constant of the first phase of intramolecular T1 reduction.

Electronic coupling between the three different T1 centers has also been calculated (cf. Fig. 5). These centers are connected pairwise by 13 covalent and 2 hydrogen bonds, giving rise to an electronic decay coupling factor of $1.7 \times 10^{-4}$. However, despite this relatively short distance, intramolecular ET between these sites are not expected. The high potential T1C is, as mentioned above, most likely in the reduced state even in the resting enzyme and thus not involved in ET at all. The driving force for T1A to T1B ET is very low or even negative. Indeed, no kinetic evidence for intramolecular T1 to T1 ET was observed during the pulse radiolytic experiments. As is also mentioned above and illustrated in Fig. 5, the T1A center is in close covalent connection to the T3 site; the Cys thiolate-coordinating T1[Cu] is placed between two His residues, which are both coordinated to the T3 pair. These two pathways consist of nine covalent bonds, yielding a total separation distance of 1.34 nm. An alternative pathway is provided by the carbonyl oxygen of Cys, which is hydrogen bonded to the N$_\alpha$ of one of the His imidazoles. We may now calculate the relative electronic coupling between electron donor and acceptor and find a value of 0.01, i.e. a very effective coupling. Therefore, the T1A copper ion is by all probability the one engaged in ET equilibrium with the trinuclear site (Fig. 5, right side).

Finally, it is of interest to compare intramolecular ET in hCp with the corresponding processes in laccase and in ascorbate
oxidase, which were both studied previously (8–11, 13, 29, 30). As in hCp, laccase pulse radiolytically produced RSSR− radicals deliver the reduction equivalents to the T1[Cu(II)] center in an intramolecular process. The rate of T1[Cu(I)] reoxidation by intramolecular ET to T2/T3 takes place unimolecularly with a rate constant of 2 s−1 at room temperature (8), similar to that observed in hCp (2.9 ± 0.6 s−1 at 279 K). This is not surprising; however, because the structural arrangements of the T1/T2/3 sites in these two proteins are quite similar. Also the driving forces are comparable. In ascorbate oxidase the situation is more complex, however. Ascorbate oxidase is a homodimer consisting of two subunits both containing the four copper arrangement. Here, two intramolecular ET processes are observed between T1[Cu(I)] and T3[Cu(II)], one with a rate constant of 200 s−1 and a slower one with a rate constant of 2.3 s−1 (9). The difference between the two rates could be explained by differences in activation entropies, which were attributed to differences in electronic coupling between the electron donor and acceptor in the two ascorbate oxidase monomers. Intramolecular ET in hCp is thus reminiscent of the similar process in laccase and in one of the ascorbate oxidase subunits.

An underlying assumption in our studies of long range electron transfer in hCp is that the structure of the blue oxides has most probably been evolutionary selected for intramolecular ET in the catalytic dioxygen reduction. Therefore hCp represents a relevant and very interesting system for investigating the parameters that control physiological intraprotein ET. Several important questions then arise. Is there a control of the intramolecular ET rate in hCp during the multielectron reduction and oxidation? In other words, does the internal ET rate depend on the number of reduction equivalents taken up by the molecule? How does the rate of electron transfer relate to the conformational changes of the enzyme upon reduction resolved by the three-dimensional structure determination? Does the presence of reducing substrates or dioxygen affect the internal ET rates (i.e. by an allosteric mechanism and changes in driving force)? It is noteworthy that steady-state kinetic measurements of hCp activity with Fe(II) as reducing substrate yield turnover numbers of 2.2 s−1. This value is similar to the rate constant observed for intramolecular T1 to T3 ET. Intramolecular ET thus seems to be the rate-limiting factor in the catalytic cycle of this enzyme.

One important point is that all experiments reported here were performed under strict anaerobic conditions, i.e. in the absence of an oxidizing substrate. This together with a reduction potential of T1A, which is more positive than that of T3, is probably the reason why only slightly more than two electrons are taken up by hCp. Coordination of dioxygen to the trinuclear site, which occurs under physiological conditions, will undoubtedly increase the reduction potential and thus the driving force for intramolecular T1[Cu(I)] to T2/T3[Cu(II)] ET. Under these conditions, further ET from reduced T1 copper to the oxygen-coordinated trinuclear center must take place to fulfill the requirement for a 4-electron reduction of dioxygen to water. Finally, the question arises why does hCp contain two extra T1 centers, which apparently play a minor (if any) role in the enzymatic processess? Most likely the physiological form of hCp is a 4-electron oxidized molecule, consistent with the 4-electron reduction of dioxygen to water. The role of the two additional T1 copper sites remains to be clarified. Ceruloplasmin was once termed the "enigmatic blue oxidase." Some enzymes still remain to be resolved!

REFERENCES

1. Farver, O., and Pecht, I. (1997) Electron Transfer Reactions in Multi-Copper Oxidases (Messerschmidt, A., ed) pp. 355–389, World Scientific Publications, Singapore
2. Boss, J. F. (1984) in Copper Proteins and Copper Enzymes (Lantie, R., ed) Vol. 5–6, CRC Press, Inc., Boca Raton, FL
3. Saitzev, V., Saitzeva, I., Card, G., Ralph, A., Bax, B., and Lindley, P. (1995) J. Inorg. Biochem. 59, 719
4. Saitzeva, I., Saitzev, V., Card, G., Moshkov, K., Bax, B., Ralph, A., and Lindley, P. (1996) J. Biol. Inorg. Chem. 1, 15–23
5. Ducros, V., Brzozowski, A. M., Wilson, K. S., Brown, S. H., Ostergaard, P., Schneider, P., Yaver, D. S., Pedersen, A. H., and Davies, G. J. (1998) Nat. Struct. Biol. 5, 310–316
6. Farver, O., and Pecht, I. (1991) Mol. Cryst. Liq. Cryst. 194, 215–224
7. Rusche, T., Karlsson, B. G., Nordling, M., Malmstrom, B. G., and Vannagard, T. (1993) Eur. J. Biochem. 212, 289–296
8. Machonkin, T. E., Zhang, H. H., Hedman, B., Hodgson, K. O., and Solomon, E. I. (1998) Biochemistry 37, 9570–9578
9. Avigliano, L., and Finazzi-Agro, A. (1997) Neurology 37, 761–767
10. Farver, O., and Pecht, I. (1991) J. Inorg. Biochem. 45, 194–206
11. Farver, O., and Pecht, I. (1993) Eur. J. Biochem. 212, 289–296
12. Fee, J. A. (1975) Struct. Bond. 22, 1–59
13. Calabrese, L., Carbonaro, M., and Musci, G. (1989) J. Biol. Chem. 264, 6135–6137
14. Miyajima, H., Nishimura, Y., Mizoguchi, K., Sakamoto, M., Shimizu, T., and Honda, N. (1987) Neurology 37, 761–767
15. Hazzard, J. T., Martin, S. J., Tolin, G., and Marchesini, A. (1997) Arch. Biochem. Biophys. 339, 24–32
16. Pecht, I., and Farver, O. (1998) in Photochemistry and Radiation Chemistry: Complementary Methods for the Study of Electron Transfer (Wishart, J., and Noera, D., eds) pp. 65–86., Boca Raton, FL
17. Harris, E. D. (1996) J. Am. Chem. Soc. 118, 9570–9578
18. Deinum, J., and Va˚nngård, T. (1973) Biochim. Biophys. Acta 310, 321–330
19. Paschke, T., Karlsson, B. G., Nordling, M., Malmstrom, B. G., and Vannagard, T. (1993) Eur. J. Biochem. 212, 289–296
20. Machonkin, T. E., Zhang, H. H., Hedman, B., Hodgson, K. O., and Solomon, E. I. (1998) Biochemistry 37, 9570–9578
21. Avigliano, L., and Finazzi-Agro, A. (1997) Electron Transfer Reactions in Multi-Copper Oxidases (Messerschmidt, A., ed) pp. 251–264, World Scientific Publications, Singapore
22. Farver, O., and Pecht, I. (1977) J. Biol. Chem. 252, 4619–4623
23. Farver, O., and Pecht, I. (1995) J. Biol. Chem. 270, 15–23
24. Marcus, R. A., and Sutin, N. L. (1985) Biochim. Biophys. Acta 811, 265–292
25. Beratan, D. N., Betts, J. N., and Onuchic, J. N. (1991) J. Am. Chem. Soc. 113, 5453–5454
26. Farver, O., and Pecht, I. (1995) J. Biol. Chem. 270, 387–392
27. Marcus, R. A., and Sutin, N. L. (1985) Biochim. Biophys. Acta 811, 265–292
28. Beratan, D. N., Betts, J. N., and Onuchic, J. N. (1991) Science 252, 1285–1288
29. Meyer, T. E., Marchesini, A., Cusanovich, M. A., and Tollin, G. (1991) Biochemistry 30, 4619–4623
30. Tollin, M., Meyer, T. E., Cusanovich, M. A., Curir, P., and Marchesini, A. (1993) Biochim. Biophys. Acta 1183, 309–314

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