Intramolecular Electron Transport in Human Ferroxidase (Caeruloplasmin)

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The oxidation of reduced human ferroxidase by molecular O₂ was studied in a stopped-flow spectrophotometer. It was shown that the two type 1 copper atoms behave differently in the absence or iron. The effect of iron on the kinetic parameters was investigated. A working model for intramolecular electron transport in the enzyme is proposed.

Ferroxidase (caeruloplasmin, EC 1.16.3.1), a blue plasma protein, contains 50% diamagnetic and 50% paramagnetic copper. The latter is further divided into type 1 or 'blue' copper, characterized by a strong absorption at 610nm and an e.p.r. (electron-paramagnetic-resonance) spectrum with an unusually small hyperfine splitting constant, and type 2 or 'non-blue' copper, with no visible absorption and an e.p.r. signal comparable with that of inorganic Cu(II) (Malmström *et al.*, 1970). The ratio of type 1/type 2 copper is a subject of discussion (Andréasson & Vänngård, 1970; Veldsema & Van Gelder, 1973; Wever *et al.*, 1973; Deinum & Vänngård, 1973). Nernst plots of titration data show that there is an equilibrium between type 1 and type 2 copper that have the same redox potential at pH7 (Veldsema & Van Gelder, 1973). Azide causes a difference in this redox potential. Type 2 copper has been suggested as the site of inhibition by azide and fluoride (Andréasson & Vänngård, 1970). By measuring the spin-relaxation times of different copper proteins, it was shown that only type 2 copper is accessible to exchangeable water molecules, in contrast with type 1 copper (Boden *et al.*, 1974).

Carrico *et al.* (1971a,b) have called attention to a diamagnetic electron acceptor in the protein, absorbing at 330nm and participating in the oxidase mechanism. The oxidation of reduced ferroxidase by O₂ has been followed by stopped-flow measurements at 610, 420 and 330nm (Carrico *et al.*, 1971b; Osaki & Walaas, 1967; Manebe *et al.*, 1972, 1973). During this reoxidation, a transient intermediate absorbing at 420nm has been observed. A similar absorption band centred at 410nm has been noticed during pulse radiolysis of ferroxidase (Faraggi & Pecht, 1973).

The kinetics of ferrous iron oxidation by ferroxidase at pH6.5 have been described in terms of two K_m values, $K_{m1} = 0.6\mu\text{M}$ and $K_{m2} = 50\mu\text{M}$ (Osaki,

1966). Besides that, ferroxidase exhibits true ascorbate oxidase activity in the presence of EDTA with a K_m of 4.70mM and a V_{max} of 3.19 electron transfers/min per Cu atom (Curzon & Young, 1972). It was shown that iron plays an important role in the oxidase activity of the enzyme (McDermott *et al.*, 1968).

Materials and Methods

Ferroxidase

Ferroxidase was prepared from Cohn-IV fraction of human plasma, as described earlier (Osaki *et al.*, 1964). It was twice recrystallized and stored at -80°C . The crystalline material was dissolved in Chelex-100-treated 0.1M-sodium acetate buffer (pH6.0) just before the experiment. The enzyme concentration was calculated from the absorbance at 610nm ($E_{1cm}^{1\text{mg}} = 10.8$).

Ascorbic acid

Ascorbic acid solutions in Chelex-100-treated 0.1M-acetate buffer (pH6.0) were prepared daily.

Ferrous iron

Crystalline ferrous ammonium sulphate hexahydrate $[\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}]$; J. T. Baker Chemical Co., Phillipsburg, N.J., U.S.A.] was dissolved in doubly deionized distilled water and used as the Fe(II) source.

Stopped-flow measurements

Rapid kinetic measurements were carried out in a Durrum stopped-flow spectrophotometer, equipped with a 2mm cuvette. The oxidations were performed by mixing equal volumes of air-saturated buffer (0.1M-acetate buffer, pH6.0, with or without EDTA or Fe) and reduced enzyme solution in the same buffer equilibrated with pre-purified nitrogen. The solutions for anaerobic experiments were deoxygenated by repeated flushing with pre-purified

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nitrogen. Anaerobic manipulations were carried out in a glove bag filled with constantly flowing pre-purified nitrogen.

Results

The reoxidation of anaerobically reduced ferroxidase by molecular O_2 was observed in a stopped-flow cuvette (2mm optical path) at 610nm and plotted against time on a stepwise-contracting time-scale (Fig. 1). Only 75% of the E_{610} was recovered in the presence of $70\mu M$ -EDTA. There was no absorbance change during the next 20h (at $4^\circ C$), until $80\mu M$ -Fe(II) was introduced in the enzyme solution to over-ride EDTA. The introduction of Fe(II) induced a rapid recovery of the E_{610} to the original value, 0.032. Reduced enzyme solutions containing no EDTA or various amounts of iron originally introduced as Fe(II), on the other hand, regained their original E_{610} values within 30min. Generally, the more iron the reaction mixture contained, the faster the recovery of E_{610} occurred. Fig. 1 represents the overall behaviour of the E_{610} in the presence of chelator or various amounts of iron. A close examination of the kinetic data plotted on a semi-logarithmic scale suggests that there are at least two parallel reactions in the presence of EDTA, and three in the absence of chelator: a fast one, and one or two slow ones, depending on the conditions.

Fig. 1. Time-course of the absorbance change at 610nm during the reoxidation of $15.2\mu M$ -reduced ferroxidase

The oxidation of the enzyme was performed by mixing equal volumes of air-saturated buffer ($0.1 M$ -acetate buffer, pH 6.0, with or without EDTA or Fe) and reduced enzyme solution. Approx. 55% of the first rapid absorbance change was not recorded, as the dead time of the 2mm cuvette rose to 2.4ms. ●, $70\mu M$ -EDTA; ■, no EDTA or Fe; □, $5\mu M$ -Fe; ▲, $13\mu M$ -Fe; △, $25\mu M$ -Fe; ○, $56\mu M$ -Fe.

The rate of the initial rapid appearance of E_{610} was not affected by chelators (EDTA, desferral), nor by various amounts of iron, as shown in a first-order plot (Fig. 2). The average first-order rate constant and the half-life period for the reaction were $439 \pm 34 s^{-1}$ and $1.6 \pm 0.05 ms$ respectively. The reaction constants for each separate experiment are summarized in Table 1. The high iron concentration in the enzyme solution may have a slight effect on the k_{11} value. In the presence of EDTA or desferral there was no reaction represented by k_{13} , which is iron-dependent. The values of k_{13} were obtained mathematically (Frost & Pearson, 1961), assuming k_{12} to be constant (iron-independent). By raising the iron concentration from 4.8 to $56\mu M$, there is a hundredfold increase in k_{13} value. Osaki & Walaas (1967) reported that the E_{610} recovered to the original value within minutes after an initial rapid reduction, when the oxidized form of the enzyme was mixed with Fe(II). Carrico *et al.* (1971b) confirmed the above fact, but at the same time they found that the enzyme, after complete reduction with ascorbate, was reoxidized in two distinct phases: 'About 55% of the absorbance was recovered in the first 20ms and the remainder of the reaction was not completed for several hours.' Our present observation strongly

Fig. 2. First-order plot of the initial absorbance change at 610nm during the reoxidation of reduced ferroxidase in $0.1 M$ -acetate buffer (pH 6.0)

○, no Fe added; □, $4.8\mu M$ -Fe; △, $12.5\mu M$ -Fe; ▲, $25\mu M$ -Fe; ■, $56\mu M$ -Fe; ●, $72\mu M$ -EDTA; ○, $45\mu M$ -desferral. E_t , E_0 and E_∞ are the E_{610} values at time t , zero and infinity respectively. The average value for the first-order rate constant and half-life period are $439 \pm 34 s^{-1}$ and $1.6 \pm 0.05 ms$ respectively.

Table 1. First-order rate constants and half-life periods for the reoxidation of type 1 copper in ferroxidase (EC 1.16.3.1)

These values were derived from the experiments shown in Figs. 1 and 2, by using standard kinetic methods (Frost & Pearson, 1961).

Reoxidation in the presence of	Fast		Slow-1		Slow-2* (iron-dependent)	
	k_{11} (s ⁻¹)	$t_{\frac{1}{2}}$ (ms)	k_{12} (s ⁻¹)	$t_{\frac{1}{2}}$ (s)	k_{13} (s ⁻¹)	$t_{\frac{1}{2}}$ (s)
70 μ M-EDTA	430	1.6	0.037	19	—	—
45 μ M-desferral	425	1.6	0.034	20	—	—
No Fe†	430	1.6	‡	‡	0.0009	770
4.8 μ M-Fe	415	1.7	‡	‡	0.006	110
13 μ M-Fe	400	1.7	‡	‡	0.034	20
25 μ M-Fe	480	1.4	‡	‡	0.21	3.3
56 μ M-Fe	490	1.4	‡	‡	0.63	1.1
Average $k_{11} = 439 \pm 34 \text{ s}^{-1}$ $t_{\frac{1}{2}} = 1.6 \pm 0.05 \text{ ms}$						

* Graphically estimated.

† Sub-micromolar contamination by iron is inevitable.

‡ Assumed to be equal to the values obtained for EDTA or desferral.

Table 2. Reoxidation of type 1 copper

This table summarizes the relative amounts of Cu(II)_{1,1} and Cu(II)_{1,2} as measured in different experiments. All measurements were made after the indicated time by using a Cary 15 spectrophotometer. For the stopped-flow experiments the solutions were collected after mixing equal volumes of anaerobically reduced ferroxidase and air-saturated 0.1 M-acetate buffer, pH 6.0.

Percentage determined by:	Reoxidation depends on ...	Cu(II) _{1,2} Nothing (%)	Cu(II) _{1,1} Fe (%)
Reoxidation in the presence of 70 μ M-EDTA (20h)		65	35
Stopped-flow in the presence of 72 μ M-EDTA (20h)		75	25
Stopped-flow in the presence of 45 μ M-desferral (20h)		77	23
Reduction with 203 μ M-ascorbate in the presence of 70 μ M-EDTA (7h)		63	37
$1/t = k(E_t - E_r - E_i) = k\Delta E_a$		59	41
		56	44

suggests that (i) recovery of the E_{610} is never completed in the presence of 70 μ M-EDTA or 45 μ M-desferral (Table 2), (ii) the slow reoxidation can be accelerated by the addition of iron, and (iii) complete reoxidation of all type 1 copper atoms may require the electron transfer shuttled by a trace amount of iron.

The reduction of type 1 copper with ascorbate and the reoxidation by atmospheric O_2 at 30°C in the presence of 70 μ M-EDTA were carried out in an anaerobic cuvette and measured with a Cary 15 spectrophotometer. The time-course of the absorbance change is shown in Fig. 3. The conditions were much closer to the steady-state kinetics than in stopped-flow experiments. The enzyme was kept reduced for 1 h before it was allowed to be re-oxidized by atmospheric O_2 . The E_{610} recovered to 0.208 (65% of total) and remained unchanged for the next 19 h, until the introduction of 80 μ M-Fe(II)

caused an additional 0.112 E_{610} change to 100% recovery, i.e. complete reoxidation of type 1 copper.

Ferroxidase oxidizes various compounds, such as ascorbate, *p*-phenylenediamine and *NN*-dimethyl-*p*-phenylenediamine, even in the presence of EDTA or desferral (Curzon, 1961; Osaki, 1966; Curzon & Young, 1972). A very high K_m value of 4.7 mM for ascorbate has been found (Curzon & Young, 1972). This fact explains why ascorbate oxidation by ferroxidase was not observed when low ascorbate concentrations of 50–100 μ M were used for kinetic studies (Osaki *et al.*, 1966; McDermott *et al.*, 1968). The presence of trace amounts of iron in the reaction mixture enhances the oxidase activity of ferroxidase towards those compounds (Curzon, 1961; Osaki, 1966; Osaki *et al.*, 1966; McDermott *et al.*, 1968; Curzon & Young, 1972). These facts together with the present observations led us to the hypothesis that ferroxidase may contain two different type 1 copper

Fig. 3. Time-course of the absorbance change at 610nm during reduction and reoxidation of 29.6 μM -ferroxidase

The enzyme was reduced anaerobically with 60 μM -ascorbate in 0.1 M-acetate buffer (pH 6.0), followed by the introduction of 70 μM -EDTA. After being left for 1 h the enzyme was allowed to reoxidize by exposure to air. The complete recovery of the E_{610} was only obtained after adding 80 μM -Fe(II) to the solution. $E_t = 0.320$, $E_i = 0.112$ (=35%), $E_a = 0.208$ (=65%). The experiment was carried out at 30°C in a 1 cm cuvette, by using a Cary 15 spectrophotometer.

atoms: one is autoxidizable and enzymically active, whereas the other is not autoxidizable, and hence not active in the absence of iron.

The following experiments were performed to test this hypothesis.

(1) A small volume of a concentrated ascorbate solution, more than enough to reduce all the O_2 in solution, was added in an air-tight cuvette to an enzyme solution which had previously been equilibrated to atmospheric O_2 . The general time-course of the absorbance change at 610nm, and the symbols used in the following theoretical derivation, are summarized in Fig. 4. The steady-state value of the E_{610} (E_r) can be expressed by the following equation:

$$E_r = E_t - E_i - \Delta E_a$$

where E_t is the total absorbance at 610nm, E_i is a hypothetical absorbance at 610nm due to non-autoxidizable type 1 copper, and ΔE_a is the steady-state reduction of absorbance due to autoxidizable type 1 copper. The value of ΔE_a , i.e. ($E_t - E_r - E_i$), should be proportional to the enzyme activity, or inversely proportional to the total time required for depletion of O_2 by the enzyme in an air-tight cuvette. Therefore

$$1/t = k(E_t - E_r - E_i) = k\Delta E_a$$

When substrate concentration approaches zero:

$$E_a \rightarrow 0, t \rightarrow \infty \text{ or } 1/t \rightarrow 0$$

Fig. 4. Schematic representation of the time-course of the absorbance change at 610nm of the enzyme mixed with ascorbate in an air-tight cuvette

The amount of ascorbate was chosen such as to reduce all the O_2 in the cuvette, resulting in the complete decolorization of the enzyme.

$$E_t = E_r + E_i + \Delta E_a$$

E_r , steady-state level of E_{610} ; E_i , non-autoxidizable type 1 Cu(II); ΔE_a , autoxidizable type 1 Cu(II); S, substrate.

Fig. 5. Reduction of feroxidase by different amounts of ascorbate at 30°C in 0.1 M-acetate buffer, pH 6.0 (+70 μM -EDTA) in an air-tight cuvette

After the addition of ascorbate (1–8 mM) to a feroxidase solution (43 μM) E_{610} was recorded in a Cary 15 spectrophotometer. The values of $E_t - E_r$ were plotted against $1/t$, t being the time needed for complete loss of E_{610} . Extrapolation to $1/t = 0$ yields the value of E_i .

By plotting ($E_t - E_r$) values against $1/t$ and extrapolating to $1/t = 0$, one can estimate the value of E_i . If there were no type 1 copper which is non-autoxidizable, $E_t - E_r$ should approach zero. Fig. 5 shows that there is a substantial amount (43.7%) of non-autoxidizable 'blue' copper (type 1) present in the enzyme. This apparently non-active type 1 copper [$\text{Cu(II)}_{1,1}$] was estimated under different experimental

conditions, the results of which are summarized in Table 2. $\text{Cu(II)}_{1,1}$ can only be reoxidized when a trace amount of iron is present in the reaction mixture. Determination of $\text{Cu(II)}_{1,1}$ from stopped-flow experiments in the presence of either EDTA or desferral resulted in lower values than other methods. This was possibly due to iron contamination in the stopped-flow apparatus, a large part of which was made from stainless steel, or to a difference in enzyme preparation.

(2) A small amount of ascorbate ($203\text{ }\mu\text{M}$), enough to reduce the blue colour of the enzyme, but not enough to deplete all the O_2 in the reaction mixture, was added to an enzyme solution equilibrated with air. After a slight recovery at the beginning, the E_{610} remained stationary. After 7 h only 63% of the E_{610} was regained. This fact strongly suggested that there was non-autoxidizable type 1 copper present in the preparation.

(3) A ferroxidase solution with an original E_{610} of 1.49 was reduced with ascorbate in the presence of $54\text{ }\mu\text{M}$ -EDTA. Reoxidation resulted in the recovery of E_{610} to 1.28 (=85.6%). This partially oxidized EDTA-containing sample was then again subjected to reduction and reoxidation. The final absorbance after the second reoxidation was 1.28, indicating a 100% recovery. These observations suggested that the reoxidizable type 1 copper was not altered by EDTA during the reduction and reoxidation process. Comparing the e.p.r. spectra of an original sample with $54\text{ }\mu\text{M}$ -EDTA and a reoxidized one showed 83.4% recovery of the Cu(II) signal (measured by integration), with no apparent difference in the hyperfine structure. The degree of E_{610} recovery after reoxidation in the presence of EDTA ($54\text{--}70\text{ }\mu\text{M}$) was the same regardless of the concentration of ferroxidase ($76\text{--}278\text{ }\mu\text{M}$ -type 1 copper), prepared from the

same stock preparation. This also suggests that EDTA prevented the reoxidation of a specific type 1 copper, rather than reacting in a non-discriminating manner with all type 1 copper atoms of ferroxidase.

Discussion

The intramolecular electron-transport system of ferroxidase is complex. The interpretation of data has been very difficult, mainly because of the variety of copper atoms in the molecule. If the molecular weight of crystalline ferroxidase is assumed to be 160000, there are seven copper atoms in the molecule. Approximately half of this copper is paramagnetic, the remainder diamagnetic. Paramagnetic copper atoms are divided into type 1 Cu(II) and type 2 Cu(II) (Malmström *et al.*, 1970). Diamagnetic copper atoms may form a cluster of highly coupled Cu(II) . The latter have a broad absorption band at 330–340 nm, disappearing on reduction of the enzyme (Carrico *et al.*, 1971a). For convenience we shall call this copper 'type 3 Cu'.

The data from the present study, as well as those published elsewhere (Osaki & Walaas, 1967; Andréasson & Vännegård, 1970; Malmström *et al.*, 1970; Carrico *et al.*, 1971a,b; Manebe *et al.*, 1971; Curzon & Young, 1972) provide the following information on types 1, 2 and 3 copper.

(a) There are two kinds of type 1 copper atoms in the enzyme. They are the first copper to be reduced by substrate: the one is autoxidizable through an intramolecular electron-transport system, but the other one is not, unless there is at least a trace amount of iron present in the reaction mixture. In either case type 1 copper cannot transfer its electron directly to oxygen.

(b) There may be only one type 2 Cu(II) atom, to

Fig. 6. Schematic representation of the proposed intramolecular electron-transport system in ferroxidase

The assumption is made that the two different type 1 copper atoms belong to the same enzyme molecule, which is not necessarily the case for all the ferroxidase molecules. The ratio of $\text{Cu(II)}_{1,1}/\text{Cu(II)}_{1,a}$ may depend on the enzyme preparation. DF, desferral; p, paramagnetic; d, diamagnetic.

which inhibitors such as N_3^- or F^- bind. In a kinetic study, similar to the one shown in Fig. 5, the presence of $7\text{ }\mu\text{M-N}_3^-$ decreased the E_r value and increased the time for complete reduction of all O_2 , without changing the 330–340 nm absorbance, indicating that type 2 copper is located in the electron-transport chain between type 1 and type 3 copper.

(c) The remainder of the copper belongs to type 3. It could be a cluster of electronically interacting and hence diamagnetic copper atoms. If the total number of copper atoms in ferroxidase is seven, there should be four type 3 copper atoms.

The working model for intramolecular electron transport shown in Fig. 6 is based on the above information, with the assumption that the two different type 1 copper atoms are in the same molecule. The presence of iron enables the non-oxidizable type 1 copper to transfer its electron slowly, either directly or through type 3 copper to O_2 . The substrate activation observed by Huber & Frieden (1970) and the two K_m values for Fe(II) oxidation reported by Osaki (1966) can be explained by the effect of iron on the non-oxidizable type 1 copper, the latter becoming oxidizable in the presence of iron. In the absence of iron there should be only one K_m value, as observed by Curzon & Young (1972) in the ascorbate oxidation by ferroxidase in the presence of EDTA.

The first-order rate constants k_{11} and k_{12} are too large and too small respectively to explain the rate of enzymic catalysis. The rate-determining step of the oxidation still could be a conformational change after the enzyme is reduced, but before the re-oxidation, as has been discussed elsewhere (Osaki & Walaas, 1967). The question whether or not the two different type 1 copper atoms are located on the same molecule still remains to be answered. The relative proportion of type 1 copper atoms may depend on the enzyme preparation and we are now investigating the electron transport in ferroxidase isolated from fresh sera instead of Cohn-IV fraction.

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