

# Formation and decay of cytochrome *c* peroxidase compound ES during aerobic reduction with dithionite

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Received 25 April 1990

Stopped-flow and rapid scanning studies have clearly demonstrated that mixing of an oxygen-saturated solution of yeast cytochrome *c* peroxidase with sodium dithionite yields compound ES, indicating generation of H<sub>2</sub>O<sub>2</sub>. The formation of compound ES was most pronounced when [Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>]/[O<sub>2</sub>] ≈ 1, and it reverted to the ferric form while standing. Even in the presence of an excess of dithionite ([Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>]/[O<sub>2</sub>] = 3.4) compound ES was formed immediately, but was soon replaced by the ferric form, followed by its final reduction to the ferrous state. The apparent first order rate constant for the decay of compound ES to the ferric form increased linearly with the square root of the dithionite concentration, thus involvement of SO<sub>2</sub><sup>-</sup> in that process being suggested.

Cytochrome *c* peroxidase, Stopped-flow, Rapid scanning; Sodium dithionite; Compound ES; Hydrogen peroxide

## 1. INTRODUCTION

Sodium dithionite is a powerful reagent which is used widely to reduce several components in biological oxidation-reduction systems. Since dithionite also reduces molecular oxygen to water, we usually add an excess of dithionite to aerobic solutions to prepare reduced components, depleting oxygen at the same time [1,2]. However, the reaction processes are often complicated because side reactions other than the direct reduction of the biological components proceed [3–6]. Most probable is participation of H<sub>2</sub>O<sub>2</sub> which is generated by dismutation of superoxide anion, a one-electron reduction product of molecular oxygen by a monomeric species of dithionite ion SO<sub>2</sub><sup>-</sup> [7]. Therefore, it is quite possible that prior to the initiation of anaerobic reduction by dithionite some redox components react with the peroxide thus formed, although this possibility has not been confirmed unequivocally [8,9]. Cytochrome *c* peroxidase (CcP) reacts with H<sub>2</sub>O<sub>2</sub> forming a rather stable intermediate, compound ES [10], and accordingly can be used as a probe of the peroxide. In this report, we present, based on rapid scanning and stopped-flow studies, evidence that CcP transiently forms compound ES by reaction with H<sub>2</sub>O<sub>2</sub> produced during the aerobic reduction with dithionite. Its unique transformation into ferric CcP followed by reduction to the ferrous form is also analyzed.

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*Abbreviations:* CcP, cytochrome *c* peroxidase; Mes, 2-(*N*-morpholino)ethanesulfonic acid

## 2. MATERIALS AND METHODS

CcP was isolated from baker's yeast and purified to a crystalline state according to the published procedure [11] with some modifications. It was recrystallized twice from distilled water, dissolved in 0.1 M potassium phosphate buffer, pH 7 and stored at temperatures below –80°C until used. The concentration of CcP was determined spectrophotometrically by using a millimolar extinction coefficient of 98 at 408 nm [12]. Throughout the present study 0.1 M Mes/KOH buffer, pH 6.0 was used for preparation of sample solutions, and the reactions were carried out at 20°C. A weighed amount of solid sodium dithionite was dissolved in the same buffer solution deaerated by bubbling oxygen-free nitrogen gas, and the concentration was estimated with an assumption of 90% purity [7]. The absorption spectra were recorded on a single beam spectrophotometer [13]; stopped-flow and rapid scanning recordings were carried out on an apparatus described previously [14,15]. Depletion of oxygen from solutions in the reservoir syringes of the stopped-flow apparatus was achieved by bubbling oxygen-free nitrogen gas for at least 15 min at 20°C. Usually rapid-scanning recording of a spectrum took 12 ms/200 nm and 100 spectra were measured with a minimal time interval of 30 ms (including the recording time of 12 ms) after initiation of the reaction. Computer programs for a spectral display in two- and three-dimensions were developed by one of us (Y.O.) by using Turbo C 2.0 (Borland International Inc.) and GraphiC 5.0 (Scientific Endeavors Corp.).

## 3. RESULTS AND DISCUSSION

Fig. 1A illustrates spectral changes recorded upon mixing of an aerobic solution of ferric CcP with an equal volume of 1.6 mM sodium dithionite on the stopped-flow, rapid scanning apparatus. The initial spectrum for the ferric enzyme was replaced gradually by that of the ferrous form. Clear isosbestic points were located at 543.5 and 609 nm on the two-dimensional display (data not shown), indicating involvement of only the ferric and ferrous forms of CcP. The absorbance at 559 nm increased exponentially giving an apparent

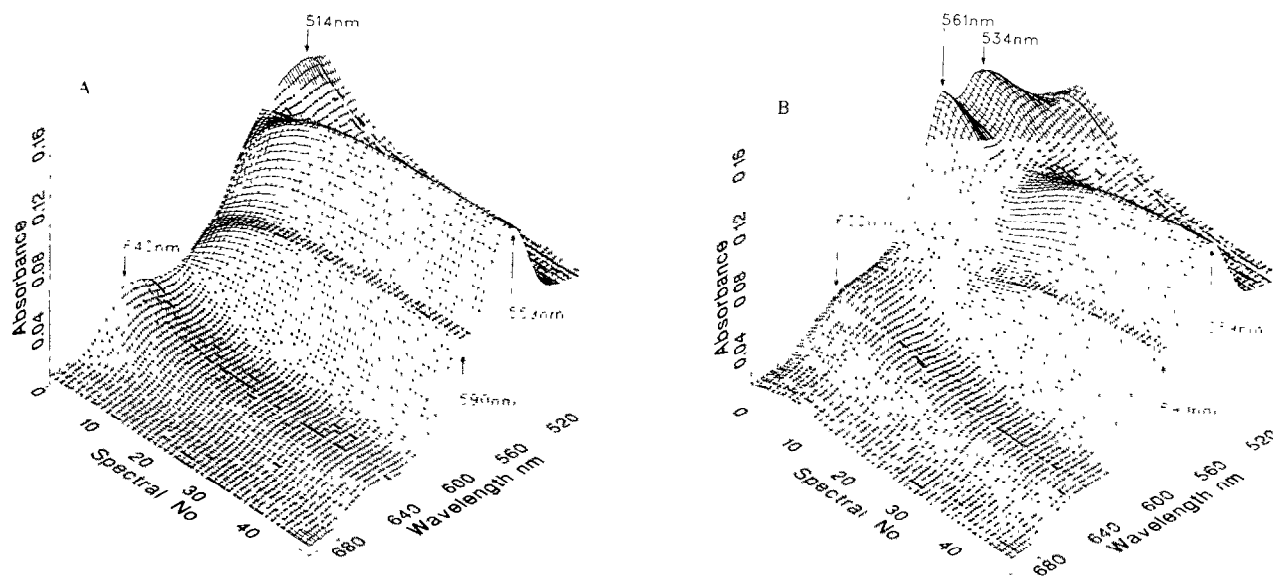


Fig. 1. Rapid-scanning recording of spectral changes of CcP during reaction with dithionite. (A) Anaerobic reduction of 11  $\mu\text{M}$  ferric CcP with 0.8 mM dithionite. (B) The reaction of 11  $\mu\text{M}$  CcP with 2.4 mM dithionite was initiated in the presence of 0.7 mM oxygen. Initial 50 spectra with 50-ms spacing are displayed

first order rate constant of  $2.4 \text{ s}^{-1}$  (Fig. 2A) for the formation of ferrous CcP.

When a ferric CcP solution saturated with 100% oxygen was mixed with the same dithionite solution as above (0.7 mM  $\text{O}_2$ /0.8 mM  $\text{Na}_2\text{S}_2\text{O}_4$  after mixing), the spectrum recorded within 20 ms after mixing showed absorption peaks at 534 and 561 nm with a broad absorption around 633 nm. This spectrum was almost unchanged for at least 5 s except for a shift of the 534-nm peak to 531 nm (data not shown). After 5 min standing, however, the reaction mixture gave the spectrum of ferric CcP. When the dithionite concentration was three times higher (0.7 mM  $\text{O}_2$ /2.4 mM  $\text{Na}_2\text{S}_2\text{O}_4$  after mixing), the initial product with absorption maxima at 534 and 561 nm was no longer stable, and its spectrum changed mostly to that of ferric CcP in 0.6 s, showing isobestic points at 517 and 649 nm (Fig. 1B). Then, the ferric form was taken over by the ferrous one. As the dithionite concentration was increased further, the amount of the initial product became small, and its decay was accelerated. Fig. 2B illustrates the absorbance changes at 531, 561, and 590 nm. At 561 nm the initial decrease in absorbance apparently corresponds to the decay of the initial compound to the ferric species, although a lag phase suggests that the process involves more than one species. As the dissolved oxygen is exhausted, the reduction of the ferric enzyme starts, as indicated by absorbance increases at 561 and 590 nm. It should be noted that the reduction of CcP also contributes to an absorbance decrease at 531 nm.

The spectral profiles of the initial compound with twin peaks at 534–531 and 561 nm suggest that it would be compound ES. Also, there is another possibility that it is already an oxy-ferryl compound

(one oxidizing equivalent less than compound ES) since both are indistinguishable spectrally from each other (Anni and Orii, unpublished results, but see [16]). Compound ES and the oxy-ferryl species are formed by reaction of  $\text{H}_2\text{O}_2$  with ferric and ferrous CcP, respectively. However, kinetic considerations eliminate the possibility that the initial compound is the oxy-ferryl species; the rate of reduction of ferric CcP by dithionite is too low ( $k_{\text{app}} = 2.4 \text{ s}^{-1}$  under anaerobic conditions) to allow accumulation of an enough amount of ferrous CcP prior to its reaction with  $\text{H}_2\text{O}_2$ . Therefore, it is concluded that the initial spectrum represents compound ES formed between ferric CcP and  $\text{H}_2\text{O}_2$ , which is produced by reduction of  $\text{O}_2$  by dithionite. Afterwards dithionite must have acted as an electron donor to compound ES, yielding ferric CcP possibly via the oxy-ferryl species. To confirm this possibility compound ES was prepared directly from ferric CcP and 1.2 equivalents of  $\text{H}_2\text{O}_2$ , and allowed to react with dithionite under anaerobic conditions. The spectral changes observed were similar in general profile to those shown in Fig. 1B (data not shown). The absorbance at 561 nm, however, decreased exponentially without a lag phase. This contrasting result suggests that in the previous reaction (Fig. 1B) both formation and decay of compound ES proceed at the same time as long as oxygen remains in the system. The rate constant for the formation of compound ES from ferric CcP and  $\text{H}_2\text{O}_2$  is reported to be  $4.5 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$  at pH 6 and  $25^\circ\text{C}$  [17]. By using this value and with an assumption that within 10 ms 99% of CcP is transformed into compound ES, a time-average concentration of  $\text{H}_2\text{O}_2$  during this period was calculated to be  $10.2 \times 10^{-6} \text{ M}$ , well comparable to the concentra-

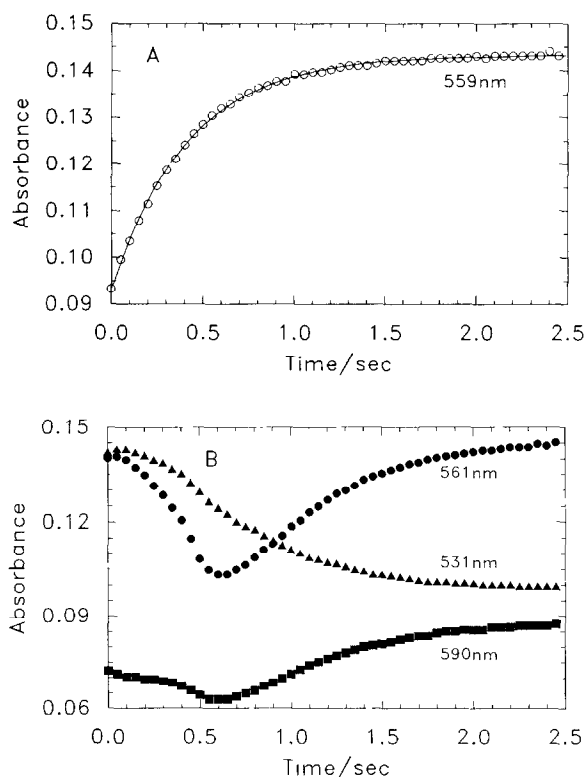


Fig. 2. Absorbance changes of CcP during reaction with dithionite in the (A) absence and (B) presence of oxygen. (A) The absorbance at 559 nm was taken from the data in Fig. 1A. A solid line was drawn based on  $A = 0.143 - 0.05\exp(-2.4t)$ , where  $A$  and  $t$  are absorbance and time, respectively. (B) The absorbances at different wavelengths were taken from the data in Fig. 1B.

tion of CcP. The transient formation of compound ES upon mixing of an aerobic solution of CcP with dithionite was thus established.

The effect of varying concentrations of sodium dithionite on the decay of compound ES was examined under anaerobic conditions by following the absorbance decrease at 424 nm. Fig. 3 shows that the apparent rate constant increases linearly with the square root of the dithionite concentration up to 26 mM, suggesting that the electron donor to compound ES is  $\text{SO}_2^-$  rather than  $\text{S}_2\text{O}_4^{2-}$  [7]. If we use the dissociation constant of  $1.4 \times 10^{-9}$  M for the dissociation of  $\text{S}_2\text{O}_4^{2-}$  into monomeric  $\text{SO}_2^-$  at pH 8 [7] to estimate the effective concentration of  $\text{SO}_2^-$  in our case, the second order rate constant of its reaction with compound ES was calculated to be  $5.6 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ . It is to be noted that this value is apparently higher than the bimolecular rate constant of  $3.8 \times 10^{-5} \text{ M}^{-1} \cdot \text{s}^{-1}$  for the decay of compound ES with ferrocyanide [18]. However, this analysis should be taken as operational because, contrary to the ferrocyanide effect which showed biphasicity, we could not differentiate kinetically the step A from B in the reaction scheme below. If we consider that step B is more likely to be rate-limiting as usual,

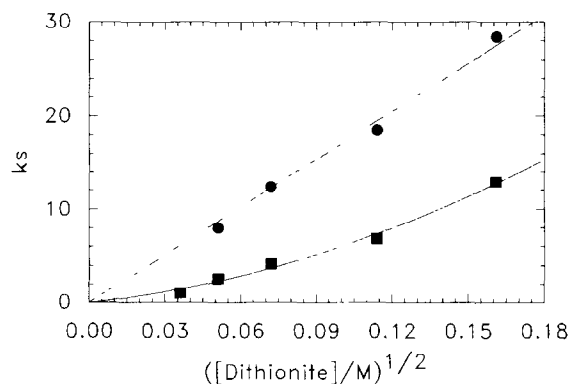
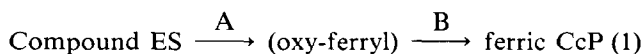


Fig. 3. The decay of compound ES and reduction of ferric CcP by dithionite. (●) Compound ES was prepared by adding  $8 \mu\text{M}$   $\text{H}_2\text{O}_2$  to  $4 \mu\text{M}$  CcP in a reservoir of the stopped-flow apparatus and allowed to react with dithionite under anaerobic conditions. The absorbance change was followed at 424 nm and at least three traces were accumulated prior to determination of apparent first order rate constants,  $k$ . A solid line was calculated according to  $k = 170.87 \text{ M}^{-1/2} \cdot \text{s}^{-1} [\text{dithionite}]^{1/2}$ . (■) Ferric CcP was reduced with dithionite under anaerobic conditions. The absorbance change was followed at 436 nm. A solid line was drawn based on  $k = 29.53 \text{ M}^{-1/2} \cdot \text{s}^{-1} [\text{dithionite}]^{1/2} + 308 \text{ M}^{-1} \cdot \text{s}^{-1} [\text{dithionite}]$ .

the above rate constant would be for the decay of the oxy-ferryl species to ferric CcP. Compound ES and the oxy-ferryl form are reduced by a number of one electron donors [18,19], but the present result demonstrates for the first time that  $\text{SO}_2^-$  is also a very efficient electron donor.



On the contrary, the apparent rate constants for the anaerobic reduction of CcP by sodium dithionite were generally lower than those for the decay of ES, and the data were rather fitted by an equation of the form of  $k_{\text{app}} = a[\text{dithionite}]^{1/2} + b[\text{dithionite}]$  (Fig. 3). Therefore, it is possible that  $\text{S}_2\text{O}_4^{2-}$  as well as  $\text{SO}_2^-$  participates in the reduction of ferric CcP [7].

In conclusion, a combined use of CcP and rapid scanning spectroscopy has proved its usefulness in probing even a transient formation of  $\text{H}_2\text{O}_2$ , at least up to  $10 \mu\text{M}$ , in a time range from a few ms to tens of ms. This would find its application in related studies.

*Acknowledgements:* This work was supported in part by a research grant of the Fujiwara Foundation of Kyoto University, Grants-in-Aid for Scientific Research on Priority Area of Bioenergetics, and Grant 63870102 from the Ministry of Education, Science and Culture of Japan (Y.O.). H.A. has been supported by a European Community fellowship during her stay at Kyoto University (1989–1990).

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