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Short communication

Cytochrome c and Chloroplast were Used for an Artificial Approach to Confirming the Irreversible Catalysis by Mitochondrial Cytochrome Oxidase

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Ferricytochrome c was artificially made to receive the aqueous electrons evolved through the influence of illuminated chloroplast. This ferricytochrome c, which was bombarded by electrons, was reduced to ferrocytochrome c by making sure that a certain cytochrome is reduced. This may require an electronic attack that is created by the chloroplast inside the plant cell. The possibility of reversing the oxidation of ferrocytochrome c by cytochrome oxidase was examined using a contrived redox system composed of cytochrome oxidase, ferricytochrome c and chloroplast with illumination. We recognized that the oxidase is unserviceable for the reversibleness in spite of the existence of chloroplast.

Keywords: Chlorolplast, Cytochrome oxidase, Ferricytochrome c.

Cytochrome c not only contributes to electron transports driven by mitochondrial inner and outer membranes, but also serves as an electron shuttle between the two membranes (Matlib and O'Brien, 1976; Bernarde and Azzone, 1981; Kirillora *et al.*, 1985; Lofrumento *et al.*, 1991). The electrons transported through each of the membranes commonly converge into cytochrome c whose electrons are ultimately funneled into molecular oxygen with the assistance of cytochrome oxidase (Stryer, 1995a).

Hill discovered that isolated chloroplasts evolve oxygen when they are illuminated in the presence of ferric ion (Stryer, 1995b). This discovery strongly suggests that ferricytochromes would be reduced to corresponding ferrocytochromes in the presence of water, a reductant, and the chloroplasts illuminated.

In this study, the ferricytochrome c, a commercially available cytochrome, was made to get a possible electron bombardment from water that is activated by the chloroplast, which is illuminated without any other enzyme. This study also examined the possibility of reversing the well-known catalysis of cytochrome oxidase that handles the substrate of ferrocytochrome c. This reversion was attempted using a contrived redox system containing cytochrome oxidase, ferricytochrome c and chloroplast in the presence of photons. We constructed a completely artificial redox system that was considered to be physiologically impossible. However, it appeared to accelerate electron bombardment from water to the cytochrome more intensely than the system involving no enzyme, but only if the enzyme drove reversible catalysis. This artificial system was constructed solely for the purpose of confirming the irreversibility chosen by the redox catalyst. The supposition was that the cytochrome could be reduced even more if it accepted the electrons with the support of the enzyme.

Materials and Methods

Chloroplast separation The methods used by Napier and Barnes (1995), and several other workers (Cockburn *et al.*, 1968; Morgenthaler *et al.*, 1974; Takabe *et al.*, 1979), were combined for the chloroplast separation from spinach and subsequent density-gradient centrifugation.

The midrib of 30 g spinach leaves was deribbed to wash the leaves clearly with cold distilled water. The washed sample was homogenized, blending it with a grinding medium that consisted of 330 mM Sorbitol, 2 mM Na_2-EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 20 mM NaCl, 2 mM isoascorbic acid and 50 mM HEPES-HCl (pH 6.7) for filtration using cheesecloth. The filtrate from the homogenate was centrifuged in the shortest possible time at 2,500 × g in order to pellet crude chloroplasts. The process for the chloroplast separation was completed at a low temperature of 4°C within 7 minutes. We performed a density-gradient centrifugation for the crude chloroplast preparation. For this centrifugation 3% polyethylene glycol 6,000, 1% bovine serum albumin and 1% Ficoll were dissolved in Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) to obtain a PBF-Percoll solution. A buffer solution containing 330 mM Sorbitol, 5 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 50 mM HEPES-HCl (pH 8.0) was

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prepared first. Mixing the PBF-Percoll and the buffer solutions gave us 40% and 85% Percoll solutions. These solutions were provided for making a two-layer density gradient.

Cytochrome oxidase preparation and assay Cytochrome oxidase was purified from the bovine-heart mitochondria, which were separated using differential centrifugation introduced by Ragan *et al.* (1987). The mitochondria separated were then treated first with deoxycholate in order to break them up. Ammonium sulfate was added to the debris obtained to cause a protein precipitation followed by centrifugation. We introduced this detergent treatment, protein precipitation and then the centrifugation to purify cytochrome oxidase (Errede *et al.*, 1978). Purified cytochrome oxidase was dialyzed for 12 hrs against 0.01 M Tris-HCl (pH 8.0) to use in the enzyme assay, which employed ferrocytochrome c substrate that was obtained by reducing ferricytochrome c (from Horse Heart, Sigma, St. Louis, USA) with a small amount of sodium dithionite reductant.

Electron transport reaction One mg/ml ferricytochrome c was prepared for use in the electron-transport reaction involving cytochrome c. Purified cytochrome oxidase was dialyzed for 24 hrs against the reaction buffer solution containing 330 mM sorbitol, 50 mM HEPES-HCl (pH 8.0), 2 mM EDTA, 1 mM $MnCl_2$ and 1 mM $MgCl_2$. The concentration of the dialyzed enzyme was 0.97 mg/ml.

Chloroplasts were suspended in the reaction buffer solution so that the chlorophyll level in the suspension was 5.315×10^{-2} mg/ml. These two solutions and one suspension were combined to constitute the following reaction systems.

(i) Hundred μl of ferricytochrome c solution and the identical volume of cytochrome oxidase solution were mixed with the reaction buffer solution in a test tube to make a final 1.1 ml reaction system for a 10 min reaction.

(ii) Hundred μl of ferricytochrome c solution and the identical volume of cytochrome oxidase solution were mixed with the reaction buffer solution in a test tube to make a final 1 ml mixture to which 100 μl of chloroplast suspension was added for a 10 min reaction.

(iii) Hundred μl of ferricytochrome c solution was mixed with the reaction buffer solution in a test tube to make a final 1 ml mixture to which 100 μl of chloroplast suspension was added for a 10 min reaction.

Each effective or nominal redox reaction for the systems described above was repeated ten times. The thirty test tubes containing reaction mixtures were placed at a room temperature of 24-27°C under a fluorescent lamp until the reaction was stopped by adding 100 μl of 0.1 M KCN per tube. The cyanide-treated redox mixtures were settled down for 2 mins at 11,400 rpm with a centrifuge (Eppendorf, Centrifuge 5415C). The 550 nm absorbencies were measured for the pellet-free aqueous phases from the centrifugation by using the Cary spectrophotometer (Varian).

Concentration determinations Protein concentrations were determined by the biuret method using the standard protein of bovine serum albumin (Rendina, 1971). Chlorophyll was estimated by the method of Napier and Barnes (1995).

Results and Discussion

The significant roles linking not only cytochrome reductase to cytochrome oxidase but also NADH-cytochrome b_5 reductase to cytochrome oxidase in mitochondrial electron transport is mediated by a carrier cytochrome c (Mokhova *et al.*, 1977; Stryer, 1995a; Skulachev, 1998). The reducibility of ferricytochrome c by illuminated chloroplast was examined. This examination was followed by confirming whether or not the cytochrome reducibility given by the chloroplast was urged by cytochrome oxidase. Nicholls (1982) conducted this confirmation to verify the following statement: "Unlike the remainder of the respiratory chain, cytochrome oxidase is irreversible."

The cytochrome oxidase provided in this experiment was purified by the method of combining the separation of mitochondria from bovine heart and the treatment of the organelles with a detergent of doxycholate. This detergent treatment was followed by treatment with a neutral salt for precipitation. This purified enzyme was dialyzed against a buffer solution of 0.01 M Tris-HCl (pH 8.0) to remove the

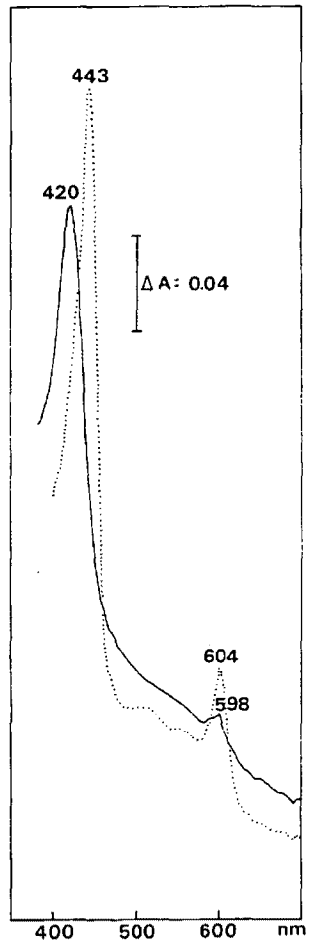

Fig. 1. Absolute absorption spectra of reduced (dot line) and oxidized (solid line) cytochrome oxidase preparations. Purified cytochrome oxidase was reduced by the addition of sodium dithionite.

detergent and salt. The dialyzate that was taken contributed to the enzyme source used. We confirmed that this enzyme source was cytochrome oxidase by spectrophotometric observations (Fig. 1). It was significant that the overall appearance of the absorption spectrum for the enzyme purified was identical to that for cytochrome oxidase (Maeshima and Asahi, 1978; Bill *et al.*, 1980; Ragan *et al.*, 1987; Sinjorgo *et al.*, 1987). The activity of the purified cytochrome oxidase for ferrocytochrome c substrate was determined in order to estimate the important kinetic parameters that are given below. The Michaelis constant Km of the enzyme in question for ferrocytochrome c, an electron donor substrate, was 0.04 mM and the maximum velocity Vmax was estimated to be 0.02 in terms of $\Delta A_{550\,nm}$/min. These kinetic parameters estimated were the same ones that are reported elsewhere (Lee and Lee, 1995).

The electron-transport reactions caused by the combinative systems composed of cytochrome c, cytochrome oxidase and chloroplast were assayed as described. It was confirmed that the control system (i.e., ferricytochrome c in a reaction buffer solution) could not be changed in an absorbency of 550 nm. If the absorbency at this wavelength had increased, the increase would have been in accordance with the reduction of ferric cytochrome. The measured absorbency for this control system containing ferricytochrome c only was 0.054 at the same wavelength. Other control systems without the electron acceptor of ferricytochrome c (i.e., cytochrome oxidase and chloroplast in reaction buffer solution, chloroplast in reaction buffer solution and cytochrome oxidase in reaction buffer solution) exhibited 0.013, 0.016 and 0.003 in absorbencies at 550 nm, respectively. These absorbency values were smaller than the absorbency increases obtained from the reaction systems, which are described under "Electron transport reaction". Examining the absorbency increases for these reaction systems might be troublesome, even though the control systems gave strikingly smaller absorbencies. We were convinced, nevertheless, that examining the absorbency increases rendered by the reaction systems could derive highly probable characteristics which were inherent in the catalysis managed by the cytochrome oxidase. This derivation, which was intended to pursue a large trend, ignored the markedly small absorbencies for the control systems. A nominal electron-transport system, composed of ferricytochrome c and cytochrome oxidase, was organized to measure its 10 minute reaction progress, if any, in terms of 550 nm absorbency (Table 1). We performed this reaction 10 times under identical conditions and with identical compositions. The average for the 550 nm absorbencies that measured 10 times gave a mean value of 0.045. This was smaller than the 0.054 for the control system containing ferricytochrome c only. Since this result did not correspond with the fact that cytochrome oxidase did nothing toward ferricytochrome c, we deduced that the commercial ferricytochrome c (Sigma) that we used might contain some reduced form. It was recognized that cytochrome oxidase had no effect on ferricytochrome c. When we, however, made contact between the oxidized cytochrome c and chloroplast, the average 550 nm-absorbance derived from the electron-transport system was 0.064 (Table 1). We realized from this value that ferricytochrome c was reduced to ferrocytochrome c by chloroplast action, when we compared this value with the 550 nm-absorbance given by the system of ferricytochrome c and cytochrome oxidase, which was recognized to do nothing toward ferricytochrome c, as described above. An electron-transport system, constructed with three components of ferricytochrome c, chloroplast and cytochrome oxidase, was also subjected to the 10-minute redox reaction under conditions identical with the ones already described (Table 1). This assay gave an average 550 nm-absorbance of 0.056. This absorbency is larger than that obtained from the ferricytochrome c-cytochrome oxidase system and smaller than that measured with the ferricytochrome c-chloroplast system. The .550 nm-absorbances, representing the degrees of cytochrome c reductions by the effects of the redox systems, are hitherto described. We have given clear-cut facts that chloroplast does artificially possess the potential to reduce ferricytochrome c. We have also shown that the reducing potential displayed by chloroplast upon cytochrome c is diminished by the oxidizing effect given by cytochrome oxidase. We can assert that the reducing potential given by chloroplast is not urged by cytochrome oxidase. This assertion is in accord with the report describing the irreversibility property (Nicholls, 1982) that is held by the oxidoreductase. We could undoubtedly predict that a certain cytochrome that is accessible within a plant cell would be reduced due to its contact with an illuminated chloroplast.

We used the chloroplasts that had an intact value greater than 95% (Napier and Barnes, 1995). Describing the

Table 1. The electron transport reactivities caused by the artificial and combinative redox systems composed of ferricytochorme c (ferricyt.c), cytochrome oxidase (CcO) and chloroplast (Chl)

Reaction system	Absorbance (550 nm)	
	mean	s. d.
ferricyt. c	0.054	0.0015
CcO+ferricyt. c	0.043	0.0033
Chl+ferricyto. c	0.064	0.0034
CcO+Chl+ferricyt. c	0.056	0.0020

The redox reaction systems composed of 100 μl ferricyt. c (1 mg. protein/ml) and equal volume of CcO (0.97 mg. protein/ml) and Chl (5.315×10^{-2} mg. chlorophyll/ml) were incubated in 330 mM sorbitol, 50 mM HEPES-HCl (pH 8.0), 2 mM EDTA, 1 mM $MnCl_2$, and 1 mM $MgCl_2$ for 10 minutes. To terminate the reaction, each system was made to contain 7 mM KCN. The reaction was repeatedly measured ten times in terms of optical density at 550 nm. Other conditions for the measurement were the same as described in Materials and Methods.

cytochrome c-chloroplast interaction would have been more competent if we had used the 100% intact chloroplast.

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