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Redox status of cytochrome oxidase in darkened leaf of C_4 crop plants

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Abstract

Light is essential for growth, development and various metabolic processes in plant. One-third of the whole intact leaf blades of pearlmillet and maize were covered (treated leaf) with a black opaque plastic sheet at the middle region for 15 days. The leaf samples were taken from three regions: basal, middle and distal; from treated and parallel untreated leaves (control). Oxygen uptake was measured from all the three regions by taking randomized leaf discs. Oxygen uptake was nearly the same in all the regions of treated and parallel untreated leaf in pearlmillet and maize. Carbon monoxide used at 0.5 mM concentration with pearlmillet inhibited oxygen uptake slightly (22%) in covered leaf blade, whereas the inhibition with maize leaf at 1.12 mM CO was significantly higher (45%). However, CO did not inhibit oxygen uptake in untreated leaf from pearlmillet and maize. In contrast, cyanide brought about 33% inhibition in oxygen uptake at 0.25 mM with pearlmillet and 60% with maize at 0.4 mM, irrespective of the fact whether a portion of the leaf blade was covered or not with an opaque sheet. The results indicate that removing light from a portion of the leaf blade alters the redox state of the whole leaf in terms of an increase in the level of the ferrocytochrome a_3 component of cytochrome *c* oxidase (cytochrome aa_3). © 2000 Published by Elsevier Science Ltd.

Keywords: *Pennisetum americanum*; *Zea mays*; Gramineae; Oxygen uptake; CO; Cyanide; Cytochrome *c* oxidase

1. Introduction

Cytochrome *c* oxidase (EC 1.9.3.1), the multiunit enzyme complex consisting of cytochrome *a* and cytochrome a_3 and associated CuA and CuB components, is the terminal oxidase in the mitochondrial electron transport and contains site 3 of oxidative phosphorylation (Wikström, 1989). CuA accepts the electrons from cytochrome *c*. Electrons are then transferred via heme *a* to the heme a_3-CuB binuclear center where oxygen reduction takes place (Michel, 1998). Carbon monoxide is a specific inhibitor of the enzyme, as it forms a complex with ferrocytochrome a_3 (reduced cytochrome a_3) (Brunori et al., 1985; Denis and Richaud, 1985), whereas cyanide combines with both oxidized and reduced forms of cytochrome a_3 (ferri- and ferrocytochrome a_3) (Stryer, 1988). The open coordination site on the heme iron in subunit I of cytochrome *c* oxidase, where oxygen binds, provides a binding site for CO and cyanide (Calhoun et al., 1994). In our earlier experiments, cytochrome *c* oxidase has been studied by blocking the electron transport chain in mitochondria under aerobic conditions by CO and, thus, making NADH available in the cytosol for the reduction of nitrate to nitrite (Naik et al., 1991, 1992; Sawhney et al., 1978). The estimate of nitrite formed provided an estimate of cytochrome oxidase inhibited by CO in dark, which, in turn, was a reflection of the reduced state of cytochrome a_3 during steady state respiration. During the course of our studies on nitrate assimilation and respiration in C_4 crop plants, significant differences were observed in the rate of oxygen uptake by partially darkened leaf in presence of CO. In this study, the leaf blades of pearlmillet and maize were divided into three segments, and

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oxygen uptake in vivo was studied polarographically to analyze directly the extent of inhibition of cytochrome oxidase respiration in presence of CO.

2. Results and discussion

There was no change in the rate of oxygen uptake in treated and untreated leaves of pearlmillet and maize (Tables 1 and 2) (Atkin et al., 1997). In pearlmillet, CO (0.25 mM) inhibited the respiration in leaf discs of treated leaf (middle segment, one-third of the whole leaf was darkened) from all the three regions of the leaf tested. The inhibition was 17, 25 and 23% from base, middle and distal segments of pearlmillet leaf, respectively, which was significant at 0.2% level (Table 1). Similarly, in maize, CO used at 1.12 mM significantly inhibited the rate of respiration in treated leaf, which was 50, 44 and 41% in base, middle and distal segments, respectively, the level of significance was 0.05% (Table 2). Thus, CO inhibited oxygen uptake in partially darkened leaf from both pearlmillet and maize, and the pattern of inhibition at two concentrations tested was similar. In contrast, the inhibition of oxygen uptake by cyanide in pearlmillet (cyanide, 0.25 mM) and maize (cyanide, 0.4 mM) was nearly the same in basal and distal segments of the leaf blade, irrespective of the fact whether a segment of leaf blade was covered or not, except the middle segment. The inhibition of oxygen uptake was 39 and 64% in covered middle segment of leaf as against 22 and 49% in the uncovered from pearlmillet and maize at 0.25 and 0.4 mM cyanide, respectively. Nevertheless, the extent of inhibition of oxygen uptake in covered leaf segment against uncovered was significantly far more higher with CO as compared with that of cyanide (4.2- and 3.4-times with CO as against 77 and 31% with cyanide in pearlmillet and maize, respectively) (Tables 1 and 2). Differential effects of CO and cyanide inhibition of oxygen uptake in leaf segments of pearlmillet and maize could be due to the fact that CO selectively combines with ferrocytochrome a_3, while cyanide combines with both ferri- and ferrocytohrome a_3 (Stryer, 1988).

Cytochrome a_3 is reduced by accepting electrons from cytochrome a and is subsequently oxidized by transferring these electrons to oxygen. During steady-state respiration, difference between the rates of these two reactions would determine the extent of reduction of cytochrome a_3 and, hence, its capacity to complex with CO. The results in Tables 1 and 2 clearly demonstrate that the response to CO in covered and uncovered leaf in both pearlmillet and maize underwent a significant change in the darkened leaf (covered), which indicated that a-a_3 electron transfer step was not inhibited, but a_3-O_2 electron transfer step was impaired (Nicholls et al., 1999). Covering a one-third portion of the leaf blade affected the response of the whole leaf, from base to the tip, towards CO; in other words, show that the flux of electron transfer via cytochrome c oxidase to O_2 has been significantly modified in darkened leaf. The dry content per unit weight did not show any change in the treated leaf whether estimated on the basis of whole leaf or different segments.

Table 1
Effect of CO and cyanide on respiration in darkened leaf of pearlmillet[a]

Additions (mM)	Oxygen uptake (mμmol O_2 min^{-1} 200 mg fr. wt^{-1})	
	Leaf: Light* (Control)	Leaf: Dark*
	Base	
Nil	38.52 ± 4.0	40.36 ± 4.3
+ CO 0.5	37.22 ± 3.7	33.42 ± 3.3
+ Cyanide 0.25	26.94 ± 3.0	26.88 ± 2.9
	Middle	
Nil	48.48 ± 5.0	41.76 ± 4.5
+ CO 0.5	45.40 ± 4.5	31.42 ± 3.2
+ Cyanide 0.25	37.68 ± 4.6	25.52 ± 3.0
	Distal	
Nil	51.12 ± 6.1	45.44 ± 5.4
+ CO 0.5	47.82 ± 6.0	35.12 ± 3.5
+ Cyanide 0.25	30.72 ± 3.5	30.52 ± 3.5

[a] Middle portion (about one-third) of the experimental leaf blade was covered (dark) with opaque black sheet and each leaf blade was divided into three segments; namely, base, middle (covered in experimental) and distal (tip). The randomized leaf discs were taken from each segment for oxygen uptake study. Other details are as in the text. Values are mean of four individual experiments ± SD. For Student's t test, the values of three segments; basal, middle and distal; from CO treated and untreated leaves were taken (paired-test). *Light, parallel uncovered leaf blade (control). *Dark, experimental covered leaf blade (one-third).

Table 2
Effect of CO and cyanide on respiration in darkened leaf of maize[a]

Additions (mM)	Oxygen uptake (mμmol O_2 min^{-1} 200 mg fr. wt^{-1})	
	Leaf: Light* (Control)	Leaf: Dark*
	Base	
Nil	35.64 ± 4.2	35.52 ± 4.1
+ CO 1.12	31.87 ± 3.2	17.76 ± 1.6
+ Cyanide 0.4	12.03 ± 1.4	13.95 ± 1.4
	Middle	
Nil	33.48 ± 3.6	30.00 ± 2.8
+ CO 1.12	29.13 ± 3.2	16.80 ± 1.5
+ Cyanide 0.4	17.24 ± 1.8	10.92 ± 1.0
	Distal	
Nil	35.94 ± 3.8	30.96 ± 3.8
+ CO 1.12	31.16 ± 3.4	18.24 ± 1.6
+ Cyanide 0.4	12.70 ± 1.0	14.88 ± 1.2

[a] Values are mean of four individual experiments ± SD. Other details are as in Table 1.

It is known that mitochondrial electron transport chain is inhibited at site 3, by the inhibition of cytochrome *c* oxidase by CO, since a cytochrome a_3-CO adduct is formed (Wikström, 1989; Brunori et al., 1985; Denis and Richaud, 1985). CO forms a complex with cytochrome a_3 only when it is in the reduced form (ferrocytochrome a_3) (Brunori et al., 1985; Denis and Richaud, 1985). It, therefore, appears that significantly higher degree of response of a partially darkened leaf to CO, was probably a direct consequence of the altered redox state of cytochrome a_3 (reduced form) in mitochondria (Naik and Nicholas, 1986a). Elimination of light for prolonged period of time might have loosened the tight coupling between electron transport and oxidative phosphorylation resulting into more of cytochrome oxidase remaining in the reduced state as compared to that during steady-state respiration by coupled mitochondria (Naik and Nicholas, 1986b). Pre-treatment with uncouplers of oxidative phosphorylation such as 2,4-dinitrophenol or carbonyl cyanide *m*-chlorophenylhydrazone had been shown to make otherwise non-responsive semi-dwarf wheat cultivars respond to CO (Naik and Nicholas, 1986c). The plausible explanation is that darkness has a direct impact on oxidation–reduction state of the mitochondrial electron transfer chain, as well as in situ tightness of the coupling of oxidation to phosphorylation, which results into more of cytochrome a_3 remaining in the reduced form (loosened coupling) rather than getting oxidized rapidly during steady-state respiration. This reduced cytochrome a_3 readily forms cytochrome a_3-CO adduct in the presence of CO, thus, resulting into the inhibition of oxygen uptake by darkened leaf. To test this hypothesis, experiments are in progress to analyze quantitative amounts of cytochrome a_3 (reduced and oxidized) by the use of absorption spectra techniques in purified mitochondria isolated from covered and uncovered leaves.

3. Experimental

Pearlmillet (*Pennisetum americanum* L.) and maize (*Zea mays* L. cv. Ratna) were grown in the Institute plots under normal sunlight and irrigation conditions. The seedlings were supplied with nitrogen fertilizer (urea, equivalent to 150 kg nitrogen ha^{-1}) in the beginning and then at the interval of 1-month period. After 40 days of age of plants, apex leaf blades were selected carefully and their mid-region (12–16 cm, ca. one-third) was covered with opaque (black) plastic sheet without injuring the leaf. After 15 days, treated whole leaf was removed from the plant; and for control, leaf from nearby placed plant of the same age and size group was used. Each leaf was divided into three nearly equal segments; namely, basal (emerging from the stalk), middle (covered with black sheet in case of experimental) and distal (tip) (Fig. 1).

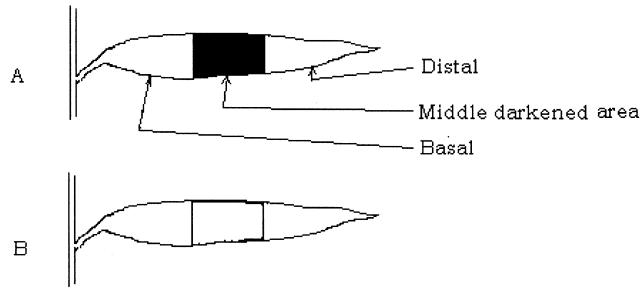

Fig. 1. Covered and uncovered leaf blades of C_4 crop plant. A. Treated leaf. Middle portion (ca. one-third) covered with black opaque sheet. B. Parallel control. Not covered at the middle.

3.1. Leaf respiration

Respiration in vivo of maize leaf discs was measured polarographically at 33°C in the dark (Prakash et al., 1986). Then 0.2 g randomized leaf discs of each portion were placed in an O_2 electrode cuvette containing 5 ml of reaction medium (pH 6.8), made up of: Hepes 50 mM, Mes 10 mM and $CaCl_2$ 0.2 mM. The reaction medium was equilibrated for 3 min before the start of the assay, and then weighed amount of leaf discs in a nylon-net bag was placed in the cuvette. O_2 uptake was calculated (Estabrook, 1967) by taking O_2 concentration in air-saturated medium as 240 μM in a glass cuvette to which a Clark O_2 electrode (YSI Yellow Spirng Instrument, Model 53, OH) mounted on a lucite plunger fitted closely was attached. After addition of leaf discs, the air in the cuvette was expelled through access slot and O_2 uptake was monitored on a 100 mV potentiometer recorder during the steady-state respiration for 5 min.

Cyanide was injected into the cuvette through a narrow access slot with the help of a long narrow needle attached to a calibrated syringe. Similarly, CO was slowly bubbled through the reaction mixture in the chamber with stirring. Solubility of CO in water was taken as 188 μM (Umbreit, 1964).

All experiments were repeated at least four times in duplicate. Respiration data wherever required from partially darkened and control leaf were analyzed by Student's *t* test.

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References

Atkin, O.K., Westbeek, M.H.M., Cambridge, M.L., Lambers, H., Pons, T.L., 1997. Plant Physiol. 113, 961.
Brunori, M., Bickar, D., Bonaventura, I., Bonaventura, C., 1985. J. Biol. Chem. 260, 7165.
Calhoun, M.W., Thomas, J.W., Gennis, R.B., 1994. Tibs 19, 325.
Denis, M., Richaud, P., 1985. Eur. J. Biochem. 147, 533.
Estabrook, R.W., 1967. Method Enzymol. 10, 41.
Michel, H., 1998. Proc. Natl. Acad. Sci. USA 95, 12819.
Naik, R.M., Dhage, A.R., Munjal, S.V., Singh, P., Desai, B.B., Mehta, S.L., Naik, M.S., 1992. Plant Physiol. 98, 984.
Naik, R.M., Munjal, S.V., Singh, P., Desai, B.B., Mehta, S.L., Naik, M.S., 1991. Phytochemistry 30, 1061.
Naik, M.S., Nicholas, D.J.D., 1986a. Theor. Appl. Genet. 72, 735.
Naik, M.S., Nicholas, D.J.D., 1986b. Plant Cell. Report 5, 259.
Naik, M.S., Nicholas, D.J.D., 1986c. Plant Sci. 45, 51.
Nicholls, P., Sharpe, M., Torres, J., Wilson, M.T., Cooper, C.E., 1999. Biochem. Soc. Trans. 26, S323.
Prakash, S., Singh, P., Naik, M.S., 1986. J. Plant Physiol. 122, 303.
Sawhney, S.K., Naik, M.S., Nicholas, D.J.D., 1978. Nature 272, 647.
Stryer, L., 1988. Biochemistry. Freeman, New York, p. 1089.
Umbreit, W.W., 1964. In: Umbreit, W.W., Burris, R.H., Stauffer, J.F. (Eds.), Manometric Techniques: A Manual Describing Methods Applicable to the Study of Tissue Metabolism. Burgess Publishing Co, MN, p. 305.
Wikström, M., 1989. Nature 338, 775.