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The switching of electron flux from the cyanide-insensitive oxidase to the cytochrome pathway in mung-bean (*Phaseolus aureus* L.) mitochondria

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The activities of the mung-bean (*Phaseolus aureus* L.) mitochondrial cyanide-insensitive oxidase and cytochrome pathways have been measured simultaneously. The results show that electrons can be diverted both from the alternative pathway to the cytochrome pathway and from the cytochrome to the alternative pathway. The competition of the two pathways for the available electron flux is discussed.

INTRODUCTION

Plant mitochondria have a branched electron-transport chain. One branch, the conventional cytochrome chain, terminates in cytochrome a_3 and is inhibited by low concentrations of cyanide, antimycin A and related inhibitors. The remaining branch, which originates at the ubiquinone level, terminates in a cyanide-insensitive oxidase whose nature is unknown. Cyanide-insensitive respiration has been extensively studied and frequently reviewed (see, e.g., Solomos, 1977; Day *et al.*, 1980; Palmer, 1981; Laties, 1982). The cyanide-insensitive oxidase system was extensively discussed at the Second International Congress on Plant Mitochondria (Moore & Beechey, 1987). Although it is generally accepted that electrons can be diverted to the cyanide-insensitive alternative oxidase, no evidence is available concerning the diversion of electrons from the alternative oxidase to the cytochrome chain. The present paper describes an experiment that demonstrates such a diversion of electrons from the alternative oxidase to the cytochrome oxidase.

MATERIALS AND METHODS

Mitochondria were isolated and purified from the hypocotyls of 5-day-old etiolated-mung-bean (*Phaseolus aureus* L.) mitochondria grown in the laboratory essentially as described previously (Gauvrit & Wilson, 1983). All reagents were AR or the highest available grade and were obtained from BDH or Sigma, both of Poole, Dorset, U.K.

Spectrophotometry was carried out in a dual-wavelength spectrophotometer specially constructed by the Medical School Workshops, University of Bristol, Bristol, U.K., using a cuvette fitted with a small motor-driven stirrer and a Clark-type oxygen electrode of 3 mm diameter. The cuvette was sealed, apart from a small hole to permit the injection of reagents, and the cuvette housing was flushed with N_2 to exclude O_2. Results were recorded on a 10 mV Watanabe Multi-pen recorder. Ferricyanide was monitored by using the wavelength pair 420–470 nm.

RESULTS

Mung-bean mitochondria prepared as described above have about 30–40% of their succinate-supported O_2-uptake rate insensitive to cyanide (see Wilson, 1980) and have ADP/O ratios indicative of coupled and intact plant mitochondria. In the presence of succinate and KCN, O_2 uptake can be used to measure the alternative-pathway activity, whereas electron flux using the cytochrome *b–c* segment of the conventional respiratory chain is observed by spectrophotometric monitoring of ferricyanide reduction. Confirmation that ferricyanide is monitoring electron flux through the cytochrome segment of the respiratory chain is provided by the sensitivity of the flux to antimycin A (Fig. 1*a*). Ferricyanide is, however, an excellent electron sink and decreases the proportion of the total electron flux passing to the alternative oxidase. Fig. 1(*b*) shows that additions of ADP increase the electron flow to ferricyanide, whereas the corresponding flux to oxygen via the alternative oxidase falls almost to zero. The ferricyanide trace shows a typical ADP control trace after repeated limiting ADP additions, whereas the O_2 trace indicates the reverse effect: O_2 uptake increasing when ADP has been depleted. If the electron flux to oxygen in the presence of cyanide is stopped by allowing the mitochondria to become anaerobic before the addition of KCN and ferricyanide, the traces shown in Figs. 1(*c*) and 1(*d*) are produced. These show that, in the absence of ADP, the addition of either catalase and H_2O_2 or O_2-saturated ethanol to increase the O_2 concentration restarts electron flow via the alternative oxidase and simultaneously diminishes the electron flux through the cytochrome chain monitored by ferricyanide reduction. If only a small O_2 pulse is added (Fig. 1*d*), then the decreased rate of ferricyanide reduction only persists until the O_2 is depleted. In some experiments, under aerobic conditions, the addition of the alternative-oxidase inhibitor salicylhydroxamic acid ('SHAM') increased electron flow to ferricyanide in the presence of cyanide. However, since salicylhydroxamic acid is not completely specific, at low concentrations acts slowly and the time during which the traces were linear was short, its effects were not always repeatable.

DISCUSSION

The observation that, under aerobic conditions, cyanide-insensitive O_2 uptake is decreased when ADP increases the flux of electrons through the cytochrome chain, supports the hypothesis that the alternative oxidase can serve as an overflow when the cytochrome system is

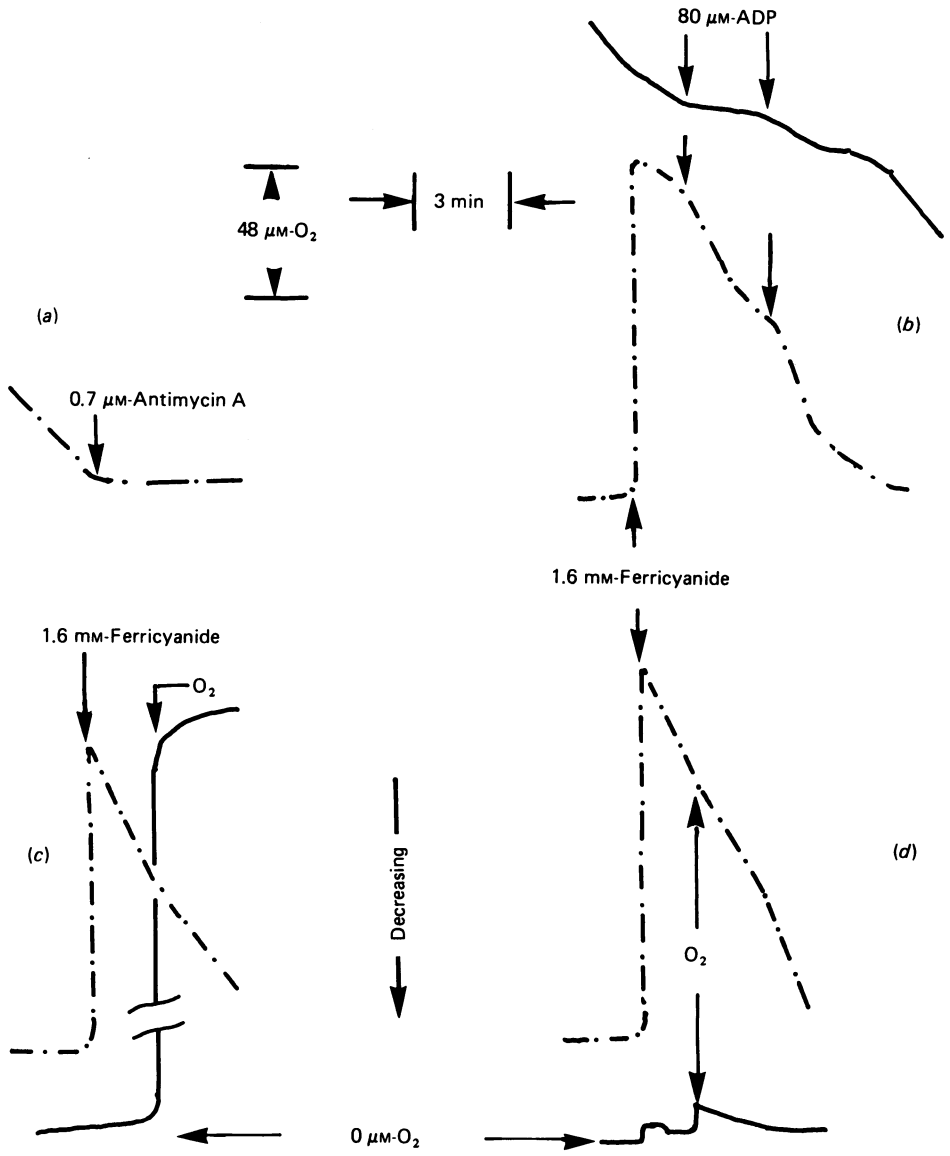

Fig. 1. Simultaneous observation of oxygen (——) consumption and ferricyanide (–·–·) reduction (measured spectrophotometrically at 420–470 nm) in the presence of 130 μM-KCN

The reaction mixture (3.2 ml) contained 0.3 M-mannitol, 10 mM-KCl, 5 mM-$MgCl_2$, 10 mM-potassium phosphate buffer, pH 7.2, 8.3 mM-potassium succinate and approx. 1 mg of mitochondrial protein. All other additions are shown. (*a*) The effect of antimycin A on ferricyanide reduction (O_2 trace not shown); (*b*) aerobic conditions; (*c*) in the presence of bovine catalase (130 units·ml^{-1}); the reaction mixture was allowed to become anaerobic before the addition of KCN and ferricyanide. O_2 was added as 2 μl of 10-volume H_2O_2; (*d*) the reaction mixture was allowed to become anaerobic before the addition of KCN and ferricyanide; O_2 was added as 5 μl of O_2-saturated ethanol.

saturated and unable to carry a sufficiently high electron flux, as originally suggested by Bahr & Bonner (1973*a*, *b*). However, if, when the mitochondria are anaerobic, electrons can be diverted to the cytochrome pathway, then the addition of O_2 should lower the rate of ferricyanide reduction. Such a decrease in flux through the cytochrome pathway was observed in these experiments, and, when the oxygen was again depleted, the reverse occurred. Thus the alternative oxidase is not simply a passive electron sink, but actively competes for electrons, and electrons can be diverted from the alternative oxidase to the cytochrome system if the alternative oxidase is saturated or otherwise restricted. Such a diversion of flow is likely to occur during the thermogenic phase of development of the aroid spadix, in undamaged potato (*Solanum tuberosum*) tubers and in other plant tissues when the alternative oxidase is the major pathway for oxidation. Since the K_m for O_2 of the alternative oxidase is higher than that for cytochrome oxidase (Solomos, 1977), the ability to switch electron flow from the alternative to the cytochrome system permits oxidation to continue when the O_2 supply is depleted. A decrease in the O_2 supply could, for example, occur during periods of high metabolic activity after

tissue damage, or during the afternoon, when thermogenesis in the aroids is most active, when diffusion limits O_2 entry into bulky tissues and in waterlogged soils. The ability of plant mitochondria to switch electrons between the two oxidase pathways casts further doubt on the inhibitor titrations extensively used to estimate the relative contributions *in vivo* of the two pathways to overall tissue respiration (see, e.g., Day *et al.* 1980; Lambers, 1980).

The observation that electrons can be switched from alternative to cytochrome oxidase and vice versa is perhaps not surprising; both pathways probably derive their electrons from a common ubiquinone pool, and modern control theory (Kacser & Burns, 1973; Kacser, 1983) predicts that, in a branched pathway, restriction of the function of any enzyme will modify the flux through all parts of the system. Reversible switching of the electron flux, the single phosphorylation site that can be observed on the alternative-oxidase pathway between the branch point and O_2 (ADP/O close to 1 for quinone to oxygen in the presence of cyanide) (Wilson, 1970, 1978, 1980) compared with two sites on the corresponding cytochrome pathway, and the branched-dehydrogenase segment of the plant mitochondrial electron-transport system, all indicate a complex and metabolically flexible system which must possess an equally complex and as yet ill-understood regulatory system.

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