STUDIES ON THE CYANIDE INSENSITIVE OXIDASE OF PLANT MITOCHONDRIA

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1. Introduction

The proportion of the respiration of isolated plant mitochondria which is inhibited by cyanide varies widely. Bonner [1] has suggested that all plant tissues have the capacity to exhibit this cyanide insensitive respiratory pathway. Recent evidence shows that this alternative respiratory pathway operates via a branch arising from the main respiratory chain between succinate and cytochrome *b* [2]. Wilson [3] has shown that only one energy conservation site occurs on this branch compared with two on the main cytochrome pathways and that this site is coupled to ATP synthesis in the presence of oxygen concentrations greater than about 70 μM.

Recent work has shown that the cyanide insensitive oxidase occurs in tissue culture cells which are about to undergo cell division [4]. Variations occur in the proportion of cyanide insensitive respiration when different substrates are employed even though the apparent specific activities of the dehydrogenases are identical.

The observations presented in this paper are the result of a study of the electron transport system of suspension cultured sycamore (*Acer pseudoplatanus*) cells and show that the synthesis of the cyanide insensitive pathway is initiated by high oxygen concentrations. Evidence is presented to show that some substrate specificity exists between the various respiration chains and that the cyanide insensitive chain is inhibited by inhibitors of non-haem iron electron carriers.

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2. Materials and methods

Suspension cultures of sycamore cells were maintained in 1L bottles on horizontal rotary shakers at 25° as described earlier [5, 6] using synthetic medium at pH 5.2. 21 hr bulk incubations were carried out in 5L Quickfit culture bottles, the oxygen solution rate being varied at a constant gas flow rate by changing the composition of the gas phase or the sparger gas distribution system. Oxygen solution rates were calculated from independent measurements of the rate of sulphite oxidation.

Cells were harvested by filtration through cheese cloth, washed with cold distilled water and finally with 0.3 M mannitol. Mitochondria were isolated using the method of Ikuma and Bonner [7] after disruption of the cells by a 7 sec treatment with a polytron homogeniser.

Oxygen uptake was measured with a Clark type oxygen electrode (Yellow Springs Inc. Ohio) in a magnetically stirred plexiglas curvette of 3 ml capacity. The reaction medium contained 0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$ and 10 mM potassium phosphate buffer (pH 7.2); 8 mM succinate, 16 mM malate or 1.6 mM NADH were used as substrates and supplemented with 1.6 mM ADP or ATP as required. The concentrations of NADH and ADP were determined spectrophotometrically at 340 and 259 nm respectively assuming 6.2 and 15.4 as the respective mM extinction coefficients. Results were calculated on the basis of 240 μM oxygen in air saturated medium. Michaelis constants were calculated from double reciprocal plots and inhibitor constants according to the method of Dixon [8]. The effects of inhibitors were examined by adding the inhibitor dissolved in a small volume of water or ethanol and adjusted to pH 7.2 during the

second state 3. Respiration rates were measured after the inhibited rate became established.

Piericidin A was kindly supplied by Dr. N. Takahashi of the University of Tokyo, Japan.

3. Results and discussion

3.1. *Control of the cyanide insensitive oxidase system*

The results presented earlier [4] indicate the existence of a regulatory mechanism to control the proportion of the two terminal respiratory pathways of the plant mitochondria. Table 1 shows that sycamore cell cultures incubated for 21 hr at a stage of groth when the proportion of the cyanide insensitive oxidase was starting to decline (after 5 days of culture) or was at a low level (after 7 days culture) had different proportions of the two oxidases dependent on the oxygen supply during this period. Increases in the oxygen supply rates above that normally used to maintain cultures in active growth (7 nmoles/ml/min resulting in an oxygen concentration for a 7 day culture of 180 μM) reversed the decline in the activity of the cyanide insensitive oxidase while reduction of the oxygen supply accelerated the decline in activity of this oxidase. Replacement of the culture medium by fresh medium, at the same pH or at the original pH of the culture medium, did not initiate increases in the proportion of the insensitive oxidase. Possible involvement of a volatile regulatory factor [9] is eliminated by the use of gas mixtures containing different oxygen concentrations supplied at constant flow rates through identical sparges.

These oxygen induced changes in the proportion

Table 1

The proportion of cyanide insensitive respiration in sycamore cell mitochondria isolated after 21 hr incubation under varying aeration conditions. Mitochondrial reaction medium as described in the text.

Cell age (days)	Oxygen solution rate (nmoles/ml/min)	% State 3 resistant to 130 μM KCN		
		Succinate	Malate	NADH
5	–*	51	46	12
7	–*	30	32	10
5	25	51	62	–
5	1.5	28	37	–
7	40	46	67	10
7	7	19	38	8

* No preincubation.

of the cyanide insensitive respiratory system take place in a short time compared with the mean generation time of the culture (60–120 hr). The results presented above appear to result from synthesis or activation of the respiratory chain since any immediate influence of oxygen concentration in controlling an active pathway would be apparent during oxygen electrode studies of isolated mitochondria.

While the apparent specific activity of mitochondria from sycamore cells towards different substrates is often constant, the proportion of respiration inhibited by cyanide varies, the NADH oxidation always being more sensitive than either succinate or malate oxidation. Using this disparity in cyanide sensitivity the utilisation of oxygen can be restarted (fig. 1) by adding 16 mM malate or 8 mM succinate to preparations previously supplied with NADH and

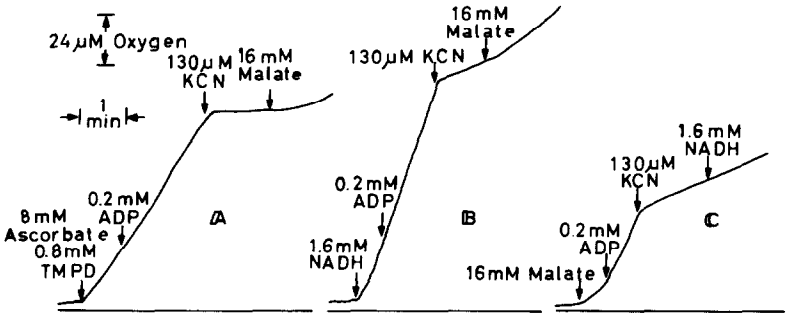

Fig. 1. Pattern of oxygen uptake by sycamore mitochondria reaction medium as described in the text. (A) Action of malate on KCN inhibited Ascorbate TMPD respiration; (B) action of malate on KCN inhibited NADH oxidation; (C) action of NADH on KCN inhibited malate oxidation.

inhibited by cyanide. Similarly malate will increase the oxygen consumption if succinate or ascorbate TMPD (*N,N,N,N'*-tetramethyl-*p*-phenylene-diamine) is the first substrate. NADH added to cyanide inhibited malate supplemented mitochondria causes no increase in respiration. In the absence of cyanide, malate added to mitochondria already supplied with NADH causes only a small increase of respiration. It can be concluded therefore that the pressure of electron flux through the respiratory chain does not result in the observed variations in the proportions of the two oxidases, rather there is a specificity in the proportions of the two respiratory chains available to different substrate dehydrogenases.

3.2. *Variations in the Michaelis constants of the two oxidases of sycamore mitochondria*

An additional quantitative variation in the activity of the two respiratory oxidase systems is observed in the apparent Michaelis constants for the three chosen substrates. Fig. 2 shows that the Michaelis constants vary according to the proportion of cyanide insensitive respiration. The varying affinity for succinate is confirmed by measuring the inhibitor constant K_i for the competitive inhibitor malonate. Thus for preparations with 27% and 80% cyanide insensitive respiration with succinate as substrate K_i values of 1.4 and 5.9 mM were obtained with corresponding K_m values of 155 and 820 μM respectively.

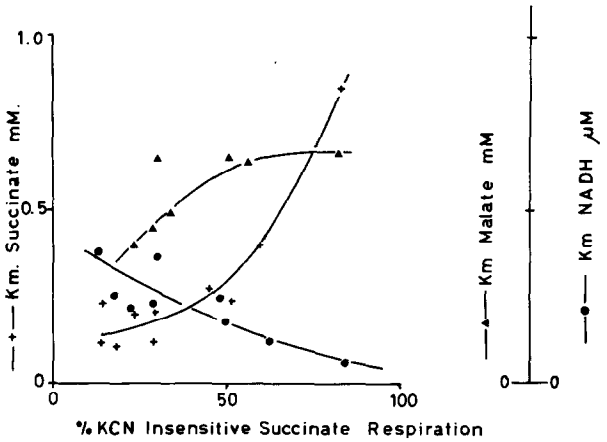

Fig. 2. Variation of K_m values of succinate, malate and NADH with the proportions of KCN insensitive succinate respiration. Reaction medium as described in the text.

3.3. *Inhibition of the cyanide insensitive oxidase*

Fig. 3 shows that the inhibition of oxygen uptake by the ubiquinone analogue piericidin A and the non-haem iron chelating agent TTFA (2-thenoyl-trifluoro-acetone) is greatly enhanced by excess cyanide. This increase in sensitivity is not observed for those substrates selectively inhibited by the inhibitors, malate by piericidin A and succinate by TTFA. It is noteworthy that piericidin A does not inhibit malate respiration of sycamore mitochondria at the very low concentrations reported for mammalian mitochondria [10] supplied with the NAD-linked substrates.

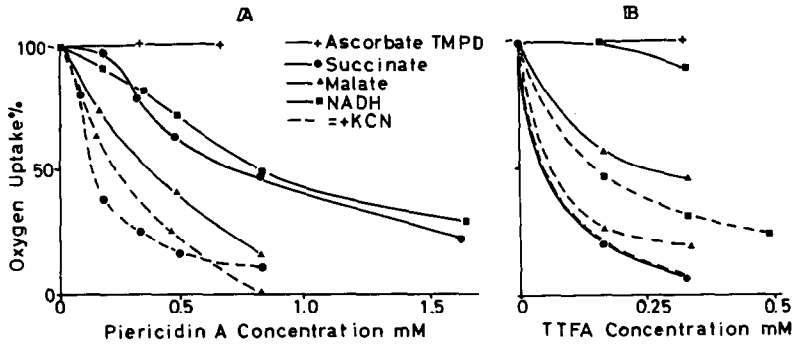

Fig. 3. Inhibition of sycamore mitochondria uptake by piericidin A and TTFA. Reaction medium as described in the text.

Since both piericidin A and TTFA have been reported to interact with non-haem iron electron carriers [11, 12] it may be concluded that such a carrier may exist on the cyanide insensitive respiratory chain of sycamore mitochondria. Because a cyanide induced sensitivity shift is not observed in the presence of malate or succinate as substrates with both inhibitors, inhibition cannot result from cyanide induced changes in the non-haem iron electron carrier. This latter mechanism would result in a change of sensitivity with either substrate towards the preferred inhibitor in addition to the observed shift when the alternative inhibitor is utilised.

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