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ENERGY CONSERVATION ASSOCIATED WITH CYANIDE-INSENSITIVE RESPIRATION IN PLANT MITOCHONDRIA

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SUMMARY

1. Using succinate as substrate, conservation of energy as ATP has been observed in the presence of KCN by mitochondria from suspension-cultured sycamore cells and from the *Arum* spadix. ATP production in the presence of KCN is inhibited and uncoupled by oligomycin and 1799 (bis-hexafluoroacetonyl acetone), respectively.

2. No phosphorylation is observed at O_2 concentrations of less than 70 μM in the presence of KCN.

3. Phosphorylation is observed using CN^--insensitive sub-mitochondrial particles which could not utilise malate.

4. It is suggested that only one phosphorylation site is connected with the CN^--insensitive respiratory chain. The control of phosphorylation by O_2 concentration is suggested as a mechanism for controlling heat production in the *Aroid spadix*.

INTRODUCTION

CN^--insensitive respiration in plants is a widely distributed phenomenon[1]. The proportion of a plant's mitochondrial oxidative capacity which is CN^--insensitive varies from a few percent in sources such as cauliflower buds to 100% in specialised tissues like the spadices of aroid plants. Evidence is available to support the conclusion that this insensitive respiration represents an alternative oxidase pathway perhaps branching from the main respiratory pathway between succinate and cytochrome *b* (ref. 2) and terminating in an oxidase with a high affinity for O_2 (apparent $K_m < 5\ \mu M$).

Previous experiments have led to the belief that respiration in the presence of CN^- is not coupled to the production of ATP[3], the purpose being merely the production of heat. While this may be correct for the fly pollinated skunk cabbage and aroid flowers, recent data such as the guanidine inhibition of the CN^--resistant respiration of mung bean mitochondria[4], the reversed electron transport and energy-dependent swelling observed in the presence of CN^- in aroid mitochondria[5,6] and reversed electron transport in mung bean mitochondria (S. B. WILSON, unpublished

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results) suggest that CN^--resistant respiration may involve energy conservation processes.

Recent observations[7] which show that mitochondria prepared from suspension-cultured cells of sycamore can be largely CN^--insensitive, have enabled examination of the question of energy conservation by the alternative oxidase without the problem of working during the very short season associated with obtaining spadices from flowers.

The results presented in this paper show that phosphorylation of ADP to ATP is associated with CN^--resistant respiration in mitochondria from cultured cells of sycamore (*Acer pseudoplatanus*) and from the spadices of *Arum maculatum* flowers.

MATERIALS AND METHODS

Mitochondria were prepared as described by IKUMA AND BONNER[8] from suspension-cultured cells of sycamore and from the spadices of *Arum maculatum* collected locally. Sycamore cells were cultured in 500 ml of synthetic medium[9] in 1-l bottles as described earlier[10,11] and harvested 5 days after inoculation. Mitochondria prepared from cultures of this age were 50–60% resistant to 130 μM CN^- when succinate was the substrate and, in the absence of CN^-, exhibited respiratory control. Only mitochondria which met these requirements were used for this study. Tissues were disrupted by a 7-sec treatment with a 'Polytron' high frequency homogeniser. (Northern Media Supply Co., Hull).

Submitochondrial particles were prepared from mitochondria as described by WILSON AND BONNER[12] using Arum mitochondria which for a brief period of the year could be obtained in the high yields necessary to prepare such particles.

O_2 uptake by approx. 0.8 mg mitochondrial protein or 0.3 mg submitochondrial particle protein was measured with a Clark type O_2 electrode in a magnetically stirred plexiglass cuvette of 3-ml capacity and the O_2 uptake calculated on the basis of 240 μM O_2 in air-saturated medium. ADP and ATP for use in incubations were estimated spectrophotometrically at 259 nm the concentration being calculated using $\varepsilon_{mM} = 15.4$. ATP produced by mitochondrial activity was measured in small samples from the O_2 electrode cuvette using the firefly luciferase assay[13], the light emission being measured by counting in a Beckman LS 100 liquid scintillation counter. Activity of the sample from the O_2 electrode cuvette was stopped by diluting the sample with 10 vol. of water at 100° and maintaining the diluted material at this temperature for 0.5–1 min. Samples were then rapidly cooled by freezing with a solid CO_2–ethanol mixture and stored for a maximum of overnight at −15°. Samples were only thawed immediately prior to assay.

When ATP production was to be measured in the presence of CN^- the medium was oxygenated to raise the O_2 concentration to 480 μM and the mitochondria allowed to respire in the presence of substrate and phosphate acceptor until a steady rate was reached before KCN was added. Samples for ATP assay were removed after the steady inhibited rate was achieved until anaerobiosis was reached. P/O ratios were calculated from plots of ATP against O_2 utilised.

The incubation medium contained 0.3 M mannitol, 0.01 M KCl, 0.01 M potassium phosphate buffer (pH 7.2) and 5 mM $MgCl_2$; 8 mM succinate or 16 mM malate

was used as substrate with 0.3 mM ADP or AMP as phosphate acceptor. 0.16 mM ATP was added to submitochondrial particles to improve succinate oxidation[12]. All experiments were carried out at room temperature (22–24°). 130 μM KCN was used as inhibitor except as mentioned in the text. Care was taken to ensure that the level of KCN employed was always in excess of that required to cause maximum inhibition of cytochrome mediated respiration.

1799 (bis-hexafluoroacetonyl acetone) a potent uncoupler of oxidative phosphorylation was kindly supplied by Dr. P. Heytler, Du Pont de Nemours, Wilmington, Del.

RESULTS

Phosphorylation occurred when sycamore mitochondria were oxidising succinate in the presence of 130 μM KCN the ATP/O ratio being in the region of 0.2–0.5 (Fig. 1). Subsequently it was shown (Fig. 2) that the ATP/O ratio was dependent on the KCN concentration at levels of KCN greater than those required to maximally inhibit O_2 uptake, suggesting an uncoupling action by high concentrations of the inhibitor. The plateau region of the curve in Fig. 2 occurred at an ATP/O ratio of 0.6 a value which has been approached using *Arum* mitochondria.

The phosphorylation observed in the presence of KCN was never maintained at O_2 concentrations of less than 70–100 μM, whereas in the absence of KCN respiratory control and phosphorylation occur at much lower concentrations. Re-oxygenation of the reaction medium restored phosphorylation after some loss of ATP had occurred. Similarly if the O_2 concentration was reduced to 70 μM before starting the experiment no phosphorylation was observed until the reaction medium was oxygenated. Phosphorylation has been maintained for long periods by initially oxygenating the reaction medium to 480 μM or by adding small amounts of O_2 to maintain the concentration in the region 200–240 μM. The O_2 uptake was always linear over the range of O_2 concentrations employed in these experiments, the rate only declining below 50 μM O_2.

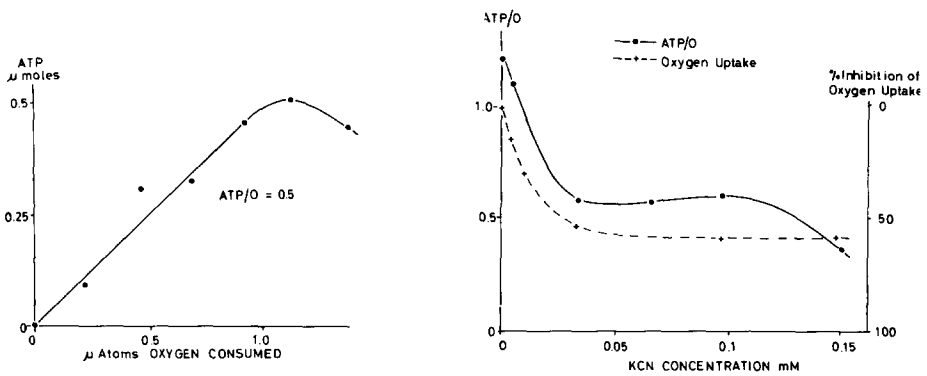

Fig. 1. Amount of ATP formed as a function of O_2 consumed in the presence of 130 μM KCN. Sycamore mitochondria, 0.3 mM ADP as phosphate acceptor. Reaction medium as described in the text.

Fig. 2. The effect of KCN concentration on ATP/O ratios. Sycamore mitochondria supplied with succinate as substrate and ADP as phosphate acceptor. Reaction mixture as described in the text.

Phosphorylation in the presence of KCN was inhibited by 1799 and by oligomycin. Fig. 3 illustrates the effect of various concentrations of oligomycin on phosphorylation in the presence and absence of CN^-.

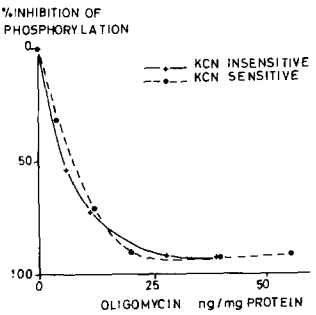

Fig. 3. The inhibition of phosphorylation by oligomycin in the presence and absence of 130 μM KCN succinate as substrate and ADP as phosphate acceptor. Reaction medium as described in the text.

AMP could be utilised as phosphate acceptor in the presence of KCN the ATP/O value being half that observed with ADP (typically 0.14 and 0.31 for AMP and ADP, respectively, in the presence of 130 μM KCN).

Submitochondrial particles which were unable to utilise malate and lacked endogenous NAD^+ were also found to phosphorylate ADP, the ATP/O value in the presence of CN^- being the same as that of the original mitochondria. Using intact sycamore mitochondria which could utilise malate the difference between the ATP/O values with succinate and malate as substrates (0.2–0.3) and attributable to the action of phosphorylation site 1 was identical in the presence and absence of CN^-.

DISCUSSION

The results presented in this paper show that phosphorylation is associated with succinate oxidation in the presence of CN^- thus extending the earlier observations of reversed electron transport and ion transport energised by intermediates from the CN^--insensitive oxidase. Inhibitors and uncouplers of respiratory chain linked oxidative phosphorylation inhibit the energy conservation observed.

It has been shown that CN^--resistant respiration occurs *via* a branch from the cytochrome electron transport chain and that this branch originates on the substrate side of phosphorylation site 2. An adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3) enzyme system cannot result in the phosphorylation observed in these experiments since AMP can be used as the phosphate acceptor without a change in the P/O value.

It is possible that the energy conservation observed results from activity at phosphorylation site 1, electrons being derived by utilisation of the products of succinate oxidation, however, this is unlikely as the K_m for malate in plant mitochondria is relatively high[8]. The lack of phosphorylation at low O_2 concentrations and the demonstration of phosphorylation using submitochondrial particles which could not utilise malate show that site-1 activity is not involved in the energy con-

servation pathway of the CN^--insensitive oxidative system with succinate as substrate.

The energy conservation observed in this study results from electron flow through the CN^--insensitive sytem apparently mediated by one energy conservation site. The ATP/O ratios obtained (0.6, Fig. 2) with sycamore mitochondria are similar to those obtained by calculating the affect of a single active phosphorylation site in the absence of KCN when the succinate was supplied as substrate or by utilising Ascorbate TMPD (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine) as substrate. When malate was supplied as substrate the site 1 phosphorylation merely added to the observed phosphorylation. The results obtained for the oligomycin titre suggests a common phosphorylation system for both the CN^--sensitive and CN^--insensitive systems.

Previous hypothesis for the alternative CN^--insensitive oxidase of plant mitochondria have been based on the belief that energy conservation is absent and that its function is to generate heat[3]. Energy conservation does not, however, preclude this activity, the lower efficiency of a single energy conservation site and the reversible coupling controlled by O_2 concentration would still allow heat production to occur in a manner analogous to that observed in brown fat mitochondria[14]. The reversible coupling operated by O_2 concentration suggests a mechanism for the observed control of heat production by the CN^--insensitive aroid spadix. It is suggested that the normal diurnal temperature rise would increase oxidative activity and thus reduce the O_2 concentration within the spadix, thereby uncoupling the respiration and increasing heat production giving the heating observed during the afternoon. Since the CN^--insensitive oxidase which can support all the respiration in the totally CN^--insensitive *Arum* mitochondria has a relatively high affinity for O_2 (apparent $K_m < 5$ μM), it may be assumed that it can compete effectively with the CN^--sensitive oxidase (apparent $K_m < 1$ μM) over the O_2 concentration range necessary for uncloupling to occur (< 100 μM).

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