Cyanide-Insensitive Oxidation of Ascorbate + *NNN'*N'-Tetramethyl-*p*-phenylenediamine Mixture by Mung-Bean (*Phaseolus aureus*) Mitochondria

AN ENERGY-LINKED FUNCTION

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Freshly prepared washed or purified mung-bean (*Phaseolus aureus*) mitochondria utilize oxygen with ascorbate/tetramethyl-*p*-phenylenediamine mixture as electron donor in the presence of KCN. ATP control of the oxygen uptake can be observed with very fresh mitochondria. The electron flow, which is inhibited by antimycin A, salicylhydroxamic acid or octylguanidine, takes place by reversed electron transport through phosphorylation site II and thence to oxygen through the cyanide-insensitive pathway. Oligomycin and low concentrations of uncoupler partially inhibit the oxygen uptake in a manner similar to that observed for other energy-linked functions of plant mitochondria. An antimycin A-insensitive oxygen uptake occurs if high concentrations of uncoupler are used, indicating that the pathway of electron flow has been altered. The process of cyanide-insensitive ascorbate oxidation is self-starting, and, since it occurs in the presence of oligomycin, it is concluded that the reaction can be energized by a single energy-conservation site associated with the cyanide-insensitive oxidase pathway.

Respiration by plant mitochondria uses a branched respiratory chain. In addition to a cytochrome pathway there is a cyanide-insensitive branch with an independent non-cytochrome terminal oxidase (Bendall & Bonner, 1971). In some plants this latter pathway accounts for a substantial proportion of the total respiratory capacity of the mitochondria. Mitochondria that are substantially cyanide-insensitive appear to lack the tight coupling and respiratory control exhibited by other mitochondria. Until recently, evidence that the cyanide-insensitive branch could conserve energy was lacking. Consequently the concept arose that this pathway served mainly to generate heat, as observed in aroid spadices. Recent evidence shows that the cyanide-insensitive oxidase system of some plant mitochondria that contain cytochrome *b₇* is capable of conserving energy either for the production of ATP or for various energy-linked functions (Wilson, 1970; Bonner & Bendall, 1968; Dawson & Gains, 1969). Storey (1971) has suggested that those mitochondria that lack cytochrome *b₇* may also lack the ability to conserve energy with the cyanide-insensitive system.

In most mitochondria, oxygen uptake by using

electrons donated to the respiratory chain at the cytochrome *c* level by artificial donors is totally cyanide-sensitive. The present paper describes a cyanide-insensitive oxygen uptake by the partially cyanide-insensitive mung-bean (*Phaseolus aureus*) mitochondria with such an electron-donor system. The mechanism suggested for this process involves reversed electron transport energized by the subsequent transport of electrons to oxygen via the cyanide-insensitive oxidase. A preliminary report of these findings has been presented (Wilson & Moore, 1973).

Materials and Methods

Mitochondria were prepared from the hypocotyls of 5-day-old etiolated mung-bean (*Phaseolus aureus* L.) seedlings grown in the laboratory. After preliminary chopping and washing with ice-cold water, the tissue was homogenized with a Polytron homogenizer. The mitochondria were isolated as described by Ikuma & Bonner (1967a), except that the preliminary low-speed centrifugation was replaced by sieving the homogenate through a nylon cloth of approx. 50 μ m pore size. For some experiments mitochondria were isolated and purified by the method of Douce *et al.* (1972). Mitochondria were only used if they were tightly coupled, with respiratory-control ratios and ADP:O values close to those normally observed with

Abbreviations used: Tes, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulphonic acid; compound 1799, 2,6-dihydroxy-1,1,1,7,7,7-hexafluoro-2,6-bis(trifluoromethyl)heptan-4-one; Kemithal, sodium 5-allyl(cyclohex-2-en-1-yl)-2-thiobarbiturate.

intact washed plant mitochondria, i.e. ADP:O = 1.6 with succinate and 2.3–2.4 with malate as substrates. Purified mitochondria had higher ADP:O values, a higher specific activity and a high proportion of intact membranes.

Oxygen uptake was measured with a Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.) in a magnetically stirred Plexiglass cuvette of 3 ml capacity. Up to 2 mg of mitochondrial protein/ml was used in experiments with cyanide present.

The reaction mixture contained 0.3 M-mannitol, 10 mM-KCl, 5 mM-MgCl₂, 10 mM-potassium phosphate buffer, pH 7.2; 10 mM-ascorbate + 1 mM-tetramethyl-*p*-phenylenediamine mixture was added as substrate donating electrons at the cytochrome *c* level (Jacobs, 1960). To initiate State 3 (Chance & Williams, 1955) 0.6 mM-ADP was added, and for State-4 and reversed-electron-transport experiments 0.16–0.8 mM-ATP was added. Where phosphate was omitted, 10 mM-Tes was added as a buffer. Inhibitors and substrates were dissolved in water or ethanol and added to the cuvette in a small volume. Compound 1799, a potent uncoupler of oxidative phosphorylation, was the gift of Dr. P. Heytler, Du Pont de Nemours, Wilmington, DE, U.S.A., and octylguanidine was the gift of Dr. B. Pressman, Johnson Foundation, University of Pennsylvania, Philadelphia, PA, U.S.A. Kemithal (I.C.I. Pharmaceuticals, Manchester, U.K.), a potent inhibitor acting at the site-I level, was the gift of Professor J. W. Dundee, Department of Anaesthetics, Queen's University, Belfast, Northern Ireland, U.K.

Steady-state reduction states of the redox carriers

flavoprotein and cytochrome *b* were measured by using a specially constructed spectrophotometer (Medical School workshops, University of Bristol). Flavoprotein was monitored by fluorescence at 510 nm excited by 436 nm light and cytochrome *b* by absorption at 560 nm–540 nm (Chance, 1957). Wavelengths for fluorescence measurements were selected by using appropriate combinations of interference (Balzers Liechtenstein, O.E.C. Ltd., Reigate, Surrey, U.K.) and Wratten gelatine absorption filters (Kodak, London W.C.2, U.K.). Some measurements of flavoprotein were also carried out by absorption by using the 470 nm–520 nm wavelength pair. The carriers were considered to be totally oxidized after depletion of the mitochondria for 5 min with 0.16 mM-ADP and fully reduced after the addition of a small amount of dithionite.

Results

The addition of ascorbate + tetramethyl-*p*-phenylenediamine mixture to mitochondria pretreated with sufficient KCN to cause maximum inhibition results in an oxygen uptake that can be stimulated by ATP (Fig. 1). In the presence of KCN the oxygen-uptake rate is about 15% of that observed in the absence of KCN. When very fresh washed or purified mitochondria and limiting amounts of ATP are used, this stimulation is transient and resembles the normal State-3–4 cycle induced by ADP in the absence of KCN, the oxygen uptake declining apparently on exhaustion of ATP. As in the ADP control of respiration, the addition of a further amount of ATP restimulates the oxygen uptake until the approach of

Fig. 1. Cyanide-insensitive oxygen uptake by mung-bean mitochondria

Freshly washed mung-bean mitochondria (7 mg of mitochondrial protein) were supplied with ascorbate + tetramethyl-*p*-phenylenediamine (TMPD) as substrate. Reaction mixture contained 0.3 M-mannitol, 10 mM-KCl, 5 mM-MgCl₂, 10 mM-potassium phosphate, pH 7.2. Numbers alongside trace give oxygen-uptake rates (nmol of O₂/min per mg of protein). Respiratory-control (RC) ratios and O:ATP ratios (calculated by analogy to Estabrook, 1967) were: cycle I, RC = 1.2, O:ATP = 0.67; cycle II, RC = 1.4, O:ATP = 0.67.

Fig. 2. Effects of electron- and energy-transfer inhibitors on the cyanide-insensitive ascorbate oxidation

The basic reaction mixture contained 0.3 M-mannitol, 10 mM-KCl, 5 mM-MgCl₂ and 10 mM-potassium phosphate, pH 7.2; all other additions are as shown. (a) Effect of 5 μ g of antimycin A, with 7 mg of mitochondrial protein (freshly washed mung-bean mitochondria). (b) Effects of 4 μ g of oligomycin and 0.75 mM-octylguanidine, with 3 mg of mitochondrial protein. (c) Effect of 13 μ M-compound 1799, with 3 mg of mitochondrial protein. (d) Ascorbate + tetramethyl-*p*-phenylenediamine (TMPD) oxidation after 20 min preincubation with 13 μ M-compound 1799 and 0.2 mM-ATP, with 3 mg of mitochondrial protein.

anaerobiosis causes a decline in oxygen uptake. The 'respiratory' control ratio and O:ATP ratios calculated (by analogy to Estabrook, 1967) from these data (1.2 and 0.7 respectively for washed mitochondria, and slightly higher values for purified mitochondria) are similar to those observed for the normal operation of a single phosphorylation site with ADP in plant mitochondria (S. B. Wilson, unpublished work). ADP, unlike ATP, causes a slight inhibition (10–15%) of oxygen uptake with ascorbate+tetramethyl-*p*-phenylenediamine in the presence of cyanide. Similarly P_i also partially inhibits the reaction (28% inhibition at 1.6 mM-P_i). If boiled mitochondria are used, a very low rate of oxidation is observed. This rate is approximately equal to that in the presence of antimycin A, indicating that the inhibitor-insensitive rate is non-enzymic. It has not been possible to demonstrate an equivalent oxygen uptake in either turnip (totally cyanide-sensitive) or *Arum maculatum* spadix (totally cyanide-insensitive) mitochondria.

The ATP-supported cyanide-insensitive ascorbate+tetramethyl-*p*-phenylenediamine oxidation can be inhibited by oligomycin (Fig. 2b) and by the uncoupler compound 1799 (Fig. 2c). Prolonged pretreatment (20 min) of the mitochondria with compound 1799 before the addition of substrate almost completely prevents any oxygen uptake (Fig. 2d). The initial rate in the absence of added ATP was also markedly

inhibited by a combination of oligomycin and Kemithal, whereas oligomycin alone had no effect and the effect of Kemithal was small. The effect of compound 1799 varies with the concentration used (Fig. 3); low concentrations markedly inhibit the process in the presence of ATP, but only slightly inhibit it if oligomycin is present to prevent the use of ATP as an energy donor. The concentration for maximum inhibition (0.2–0.5 μ M) by compound 1799 is comparable with that (0.2 μ M) causing half-maximum stimulation of 'State-4' succinate oxidation in the absence of KCN. However, compound 1799 at concentrations above that required to release 'State-4' succinate oxidation partially restores the inhibition caused by low concentrations and stimulates the oxidation in the presence of antimycin A. Thus high uncoupler concentrations or a detergent (Lubrol WX) only slightly inhibit the ATP-supported rate (Fig. 2c) and stimulate the oxygen uptake in the presence of antimycin A or salicylhydroxamic acid. Similar results were observed if carbonyl cyanide *m*-chlorophenylhydrazone was used as uncoupler. Octylguanidine, which appears to behave as an energy-transfer inhibitor in plant mitochondria (Wilson & Bonner, 1970a), decreases the oxygen uptake of the reaction to the non-enzymic value if added in the absence or presence of oligomycin (Fig. 2b). The concentration for half-maximum

Fig. 3. Titration with compound 1799 of ATP-supported cyanide-insensitive ascorbate + tetramethyl-*p*-phenylenediamine (TMPD) oxidation

The basic reaction mixture contained 0.3 M-mannitol, 10 mM-KCl, 5 mM-MgCl₂ and 10 mM-potassium phosphate, pH 7.2; 5 mM-ascorbate, 0.5 mM-tetramethyl-*p*-phenylenediamine, 130 μM-KCN and 0.8 mM-ATP were present in all experiments, with no further addition (●) or followed by oligomycin (4 μg, ▲) or antimycin A (2.5 μg, ■), and then compound 1799 in increasing amounts. For comparison the uncoupling effect of compound 1799 on State-4 succinate oxidation is shown (○). The increased rate of oxidation is expressed as percentage of the initial State-4 rate. Measurement was made in the basic reaction medium containing 0.8 mM-ATP and 8 mM-succinate, followed by compound 1799 in increasing amounts.

inhibition by octylguanidine is 0.37 mM. This value is close to that previously reported for the octylguanidine inhibition of phosphorylation site II when the inhibitor is added to mitochondria in the energized State 4.

Antimycin A causes a complete inhibition of the enzymic cyanide-insensitive oxygen uptake (Fig. 2a). Salicylhydroxamic acid, a specific inhibitor of electron transport through the cyanide-insensitive oxidase branch (Schonbaum *et al.*, 1971), likewise abolishes the enzymic cyanide-insensitive oxygen uptake with ascorbate + tetramethyl-*p*-phenylenediamine as electron donor. The half-maximum inhibitor concentration for salicylhydroxamic acid is 0.2 mM, similar to values previously reported for the inhibition of cyanide-insensitive oxidations. However, at high uncoupler concentration or in the presence of detergent (Lubrol WX), the oxygen uptake is largely insensitive to both antimycin A and salicylhydroxamic acid. Although plant mitochondria are partially

inhibited by inhibitors acting at the site-I level, rotenone (8 μM) was without any effect, and 0.3 mM-Kemithal, at a concentration that inhibits 60% of malate but not succinate oxidation, gave only 9% inhibition of the cyanide-insensitive ascorbate oxidation.

The electron carriers cytochrome *b* and flavoproteins occur on the low-potential side of the antimycin A and the salicylhydroxamic acid sites respectively. The branch point for the cyanide-insensitive chain lies between these two components (Storey & Bahr, 1969). Since these components are both complex mixtures, they were monitored by using as examples the wavelength pair 560 nm–540 nm (cytochrome *b*₅₅₇) and fluorescence excitation at 436 nm for the cytochrome *b* and flavoproteins respectively. Preliminary experiments with other wavelength pairs showed that the other cytochromes *b* gave results similar to those for cytochrome *b*₅₅₇. Difference spectra (Fig. 4a) obtained after the addition of antimycin A showed a peak in the region of the cytochrome *b* absorption band, confirming that the wavelength pair chosen was suitable for monitoring the changes associated with the cyanide-insensitive oxidation of ascorbate + tetramethyl-*p*-phenylenediamine. Fig. 4(b) shows that after the addition of substrate mixture to previously depleted mitochondria in the presence of KCN both carriers became only partially reduced. The presence of salicylhydroxamic acid further enhances the reduction, particularly of the flavoprotein, by restricting the flow of electrons to oxygen via the cyanide-insensitive oxidase. Antimycin A, however, causes an oxidation of the cytochrome, but not the flavoprotein, which can still receive electrons from endogenous substrates. Oligomycin, by inhibiting the ATP-supported electron flow at the coupling sites, oxidizes the cytochrome components, but reduces the flavoprotein component. Conversely the uncoupler compound 1799 at a high concentration (7 μM) causes an oxidation of both components. Antimycin A added after either of the two previous reagents produces some reduction of the flavoprotein and a further slight oxidation of the cytochrome (Fig. 5). The effect of oligomycin in causing a reduction of the fluorescent flavoprotein is in accord with the location of this carrier on the low-potential side of phosphorylation site I. The oxidation of the cytochrome *b* with oligomycin and of both components by the uncoupler indicates that the redox state of both carriers is dependent on the energy state of the mitochondria. Cytochrome *c* did not appear in difference spectra obtained when the cyanide-insensitive ascorbate + tetramethyl-*p*-phenylenediamine oxidation was inhibited by antimycin A, and measurements at the wavelength pair 550 nm–540 nm showed that cytochrome *c* was fully reduced under the conditions of these experiments.

Fig. 4. (a) Difference spectrum [oxidized - (oxidized + 5 μ g of antimycin A)] for cyanide-sensitive ascorbate + tetramethylene-*p*-phenylenediamine (TMPD) oxidation, and (b) simultaneous spectrophotometric observations of the redox states of cytochrome *b* and flavoprotein measured by transmission (*T*) and fluorescence (*F*) respectively during the cyanide-insensitive ascorbate + tetramethyl-*p*-phenylenediamine oxidation

(a) Both sides contained the basic reaction mixture 0.3M-mannitol, 10mM-KCl, 5mM-MgCl₂ and 10mM-potassium phosphate buffer, pH7.2, with 0.8mM-ATP, 5mM-ascorbate, 0.5mM-TMPD, and 140 μ M-KCN. 550nm is the wavelength for cytochrome *c*. (b) Basic reaction mixture was as for (a). The starting value is for the ADP-depleted mitochondria. All other additions are marked on the Figure and show the effects of inhibitors of electron transport. Abbreviation used: SHAM, salicylhydroxamic acid.

Discussion

The results presented above show that electrons must be donated at the cytochrome *c* level by the substrate mixture, as observed in heart mitochondria (Tyler *et al.*, 1966). If electrons were supplied at the cytochrome *b* level or at some lower potential then these components would not become oxidized on the addition of antimycin A, and cytochrome *c* would not remain fully reduced. The effects of salicylhydroxamic acid provide clear evidence of the involvement of the cyanide-insensitive oxidase. Sufficient cyanide was present maximally to inhibit the cytochrome oxidase (Ikuma & Bonner, 1967b). It is not possible to exclude totally the involvement of site I in this reaction, since there are no inhibitors that totally and specifically inhibit site-I oxidations in mung-bean mitochondria. Although the mechanism of action of octylguanidine is not fully understood, it inhibits oxidation through sites I and II at concentrations characteristic of the site involved (Wilson & Bonner, 1970a). Since the cyanide-insensitive oxidation of ascorbate + tetramethyl-*p*-phenylenediamine mixture is insensitive to concentrations of rotenone and Kemithal that cause substantial inhibition of site-I oxidations and, since octylguanidine inhibits at concentrations that are characteristic of site II rather than the more sensitive site I, it is concluded that site I is not directly involved in this reaction. The small inhibition observed with Kemithal probably results from the uncoupling that usually results from the use of barbiturates. The reaction studied, however, was sensitive both to antimycin A and to salicylhydroxamic acid; thus electrons must flow from the cytochrome *c* level through the antimycin-sensitive phosphorylation site II to the cytochromes *b*, and to oxygen via the cyanide-insensitive oxidase branch. Fig. 6 shows the proposed path of electron transport. Such a pathway would be energy-requiring.

The ATP stimulation and the measured ATP stoichiometry agree with the reversal of electron flow through a single phosphorylation site, thus supporting the proposed pathway. The results obtained with ATP, ADP, phosphate and the inhibitors of energy transfer show that the cyanide-insensitive ascorbate + tetramethyl-*p*-phenylenediamine oxidation has properties identical with those of the other energy-linked functions of plant mitochondria (Wilson & Bonner, 1970b). Other energy-linked functions in plant mitochondria when energized by aerobically generated high-energy intermediates are sensitive to inhibition by octylguanidine, but not to uncouplers in the absence of preincubation. Only octylguanidine or preincubation with uncoupler completely inhibited the cyanide-insensitive ascorbate + tetramethyl-*p*-phenylenediamine oxidation in the absence of ATP, showing that this system is also supported by aerobically generated

Fig. 5. Simultaneous spectrophotometric observation of the redox states of cytochrome b and flavoprotein measured by transmission (T) and fluorescence (F) respectively during the cyanide-insensitive ascorbate + tetramethyl-p-phenylenediamine (TMPD) oxidation

The basic reaction mixture contained 0.3 M-mannitol, 10 mM-KCl, 5 mM-MgCl₂ and 10 mM-potassium phosphate, pH 7.2. The starting value is for the ADP-depleted mitochondria; dithionite addition gives the fully reduced state. Additions as marked on the Figure show the effects of adding (a) 4 μ g of oligomycin or (b) 7 μ M-compound 1799 to inhibit energy transfer from ATP. Abbreviation used: SHAM, salicylhydroxamic acid.

high-energy intermediates. Preincubation with uncoupler is apparently required for complete destruction of the endogenous energy reserves and the coupling mechanism. Low concentrations of uncoupler

added during the incubation only partially inhibit the process, but the residual oxidation remains antimycin A-sensitive, showing that the energy-requiring cytochrome c-to-b step is still operative.

Fig. 6. *Electron transport and energy conservation in mung-bean mitochondria (after Bonner, 1973)*

The proposed pathway for the cyanide-insensitive ascorbate + tetramethyl-*p*-phenylenediamine (TMPD) oxidation is indicated by a broken line. $\downarrow$, Energy conservation sites. $\downarrow$, Site of action of inhibitors used in the present study. Abbreviations used: Cyt, cytochrome; Fp, flavoprotein; NHI, non-haem iron; UQ, ubiquinone; SHAM, salicylhydroxamic acid.

High concentrations of uncoupler or detergents, however, modify the electron-transport sequence and remove the antimycin-sensitivity, possibly by disrupting the membrane structure. It can be concluded that the cyanide-insensitive oxidation of the ascorbate + tetramethyl-*p*-phenylenediamine mixture is an energy-linked function capable of utilizing both ATP and aerobically generated high-energy intermediates.

The energy source for a continuous reversal of site II in the absence of added ATP must be provided by an electron flux to oxygen, either from endogenous substrates utilizing site I or by the reversed flow from site II utilizing an energy-conservation site associated with the cyanide-insensitive oxidase. For endogenous substrates utilizing site I to be able to support the reversed electron flow through site II, the endogenous flow must occur at a rate equal to that through site II. However, mung-bean mitochondria, particularly after purification, have very low rates of endogenous respiration compared with the antimycin-sensitive oligomycin-insensitive flux through site II observed in these experiments. This observation and the arguments presented above exclude site I as the energy source for the reaction observed. In the absence of any other energy source, the reversal of site II must be energized by the cyanide-insensitive oxidase. Endogenous reserves using site I do, however, prime the reaction in the absence of added ATP, since self-starting was inhibited by a combination of Kemithal and oligomycin. Individually these reagents did not inhibit self-starting or, when in combination, the reaction itself. The cyanide-insensitive ascorbate + tetramethyl-*p*-phenylenediamine oxidation therefore behaves as an energy siphon, energy required at the site-II level being supplied by the passage of the electrons to oxygen via the cyanide-insensitive oxidase system. It can be concluded that energy conservation can be associated with the operation of the alternative oxidase in the mung bean. Only a

single energy-conservation site must be involved, since either additional sites or perfect coupling at a single site would prevent the observed ATP control of oxygen uptake by rephosphorylating the ADP formed. Phosphorylation of ADP in plant mitochondria has never been observed to give P:O values of 1.0 for a single phosphorylation site; rephosphorylation therefore occurs at a lower rate than ATP utilization, and ATP control of oxygen uptake is observed. Evidence has previously been presented to show that only a single phosphorylation site appears to be involved in energy conservation in other cyanide-insensitive plant systems (Wilson, 1970). Fig. 6 indicates the association of this new energy-conservation site with the salicylhydroxamic acid-sensitive pathway. A preliminary report (Wilson, 1977) has been presented on experiments to study the ability of this site to energize energy-linked functions.

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