H⁺ production by antimycin-inhibited mitochondria

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1. Oxygen-pulse experiments on antimycin-treated liver mitochondria from young rats in the absence of added substrates indicate the presence of an endogenous hydrogenated reductant on the oxygen side of the antimycin inhibition site. This reductant has been estimated to be present in a 20–50-fold molar excess over cytochrome *aa*₃. Oxidation of the reductant by small bursts of electron flow to oxygen gives a ratio H⁺ translocated:O atoms taken up of 2.1 ± 0.2 . 2. The endogenous hydrogenated reductant competes with added ferrocyanide and with ascorbate/*NNN'**N'*-tetramethyl-*p*-phenylenediamine as a source of reducing equivalents to oxygen. 3. When the contribution of endogenous substrate to H⁺ production is taken into account, there still remains some respiration-dependent H⁺ translocation consistent with a H⁺ pumping activity of cytochrome oxidase. The stoichiometry of this 'extra' H⁺ translocation is variable and depends on the rate of electron flow.

Accurate measurements of H⁺/e stoichiometries for the different coupling sites in the electron transport chain are necessary for a constructive debate on the mechanism of redox-dependent H⁺ movements in mitochondria. One problem complicating the experimental determination of H⁺/e values is the presence of an endogenous hydrogenated reductant situated on the cytochrome *c* side of the antimycin-inhibition site (Papa *et al.*, 1978; Moyle & Mitchell, 1978). This appears to be responsible for some H⁺ production from antimycin-inhibited mitochondria aerobically pulsed with ferrocyanide (Lorusso *et al.*, 1979) or ascorbate plus TMPD (Papa *et al.*, 1980) and interferes with the experimental determination of H⁺/e values for electron flow through cytochrome oxidase (site 3) in mitochondria and submitochondrial particles and *Paracoccus denitrificans* (Moyle & Mitchell, 1978; Wikström & Krab, 1978; Van Verseveld *et al.*, 1981). Thus Moyle & Mitchell (1978) attribute the proton translocation associated with ferrocyanide oxidation in aerobic antimycin-treated mitochondria to a re-reduction of the ferricyanide by the hydrogenated reductant. On the other hand, Wikström (1977) and Wikström & Krab (1978) conclude that such proton translocation is associated

with the proton-pumping function of cytochrome oxidase.

In the present paper we have attempted to assess the contribution of such hydrogenated reductants on the oxygen side of the antimycin-inhibited site to respiration-dependent proton production. We have used the original O₂-pulse method of Mitchell (Mitchell *et al.*, 1979) on antimycin-inhibited mitochondria in the presence and absence of added substrates. As has been pointed out previously (Mitchell & Moyle, 1979) the amount of major endogenous reductants in isolated mitochondria is usually in excess of the usual oxidant pulse and therefore this approach should allow for a direct measurement of proton production from antimycin-insensitive reductants in the absence of any added substrate. The results clearly indicate the presence of a hydrogen carrier situated between the antimycin inhibition site and cytochrome oxidase. However, when account has been taken of proton production from this source, some respiration-dependent proton translocation by cytochrome oxidase is also evident.

Materials and methods

Mitochondria were prepared from the livers of young (<200 g) rats essentially according to Chappell & Hansford (1972) and were suspended in a solution of 75 mM-sucrose/225 mM-mannitol/10 mM-Mops (4-morpholinepropane sulphonic acid) 1 mM-

Abbreviations used: FCCP, *p*-trifluoromethoxy-carbonyl cyanide phenylhydrazone; TMPD, *NNN'**N'*-tetramethyl-*p*-phenylenediamine.

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EDTA, pH 7.2–7.4, at a concentration equivalent to 50–80 mg of protein/ml. Relative concentrations are expressed in terms of cytochrome aa_3 content, using an absorbance coefficient of $27\text{ mm}^{-1}\cdot\text{cm}^{-1}$ for the latter, reduced minus oxidized, 605–630 nm. pH changes were monitored by using a Radiometer 64 meter with RC-100 recorder adapted to a scale expansion of 0.1 pH units, and a Radiometer semi-micro combination electrode inserted through a perspex jacker into approx. 4.0 ml of reaction mixture in a glass vessel with magnetic stirrer, water-jacketed at 30°C . Oxygen pulses were introduced by injecting small (μl) volumes of reaction medium (100 mM-KCl in 25 mM-sucrose) which had been air-equilibrated at 30°C with intermittent shaking for a minimum of 2 h. H^+/O ratios were calculated from extrapolations of the decay curves to the middle of the respiratory pulse, according to Mitchell *et al.* (1979). All reagents used were of the highest purity available.

Results

O_2 and ferricyanide experiments

H^+ pulses can be induced by small ($<15\text{ ng atom}$) O_2 pulses to an anaerobic suspension of rat liver mitochondria treated with the respiratory inhibitors rotenone and antimycin, in the presence of valinomycin plus K^+ (Fig. 1). The pulse of acid slowly decays back to the base line level and the overall

pulse is sensitive to uncoupler. O_2 pulses in the presence of uncoupler produce no net pH changes. For these experiments, relatively high amounts ($>25\text{ mg}$ of protein) of mitochondria are necessary as the endogenous reductant responsible for H^+ production is easily exhausted by repetitive pulses of O_2 (Fig. 1b). After a few minutes anaerobiosis, however, further O_2 pulses will again elicit proton production. Above an antimycin concentration of $0.1\text{ }\mu\text{g}/\text{mg}$ of protein, the H^+/O ratio is constant at 2.1 ± 0.2 until the endogenous reductant becomes exhausted and H^+ production ceases (Fig. 2a). In terms of XH, the amount of reductant present before exhaustion varied with the size (age?) of the rats. In preparations from young ($<200\text{ g}$) rats, the reductant was usually in 20–50-fold molar excess over cytochrome aa_3 (Fig. 2a). This amount decreased in mitochondria from rats of greater body weight. The amount of reductant was not affected by centrifugation and resuspension of the mitochondria in fresh medium. In the absence of antimycin but with rotenone present, the rate of H^+ production from endogenous reductants is faster (approx. 5-fold) and gives a constant H^+/O ratio of 4.0 ± 0.3 (Fig. 2b), as found for ubiquinol by Lawford & Garland (1973). Exhaustion by repeated O_2 pulsing still occurs after approximately the same net H^+ production as with antimycin-inhibited mitochondria (Fig. 2b). H^+ production from the endogenous reductant in the presence of antimycin plus

Fig. 1. O_2 pulse traces from antimycin-inhibited rat liver mitochondria in the absence of added substrates. Anaerobic suspension (4 ml) contained mitochondria (about 7 mg of protein/ml) in 100 mM-KCl/25 mM-sucrose/2.5 μM -rotenone/antimycin (2.5 $\mu\text{g}/\text{ml}$)/1 mM-EGTA/valinomycin (0.13 $\mu\text{g}/\text{ml}$)/oligomycin (2.5 $\mu\text{g}/\text{ml}$) at 30°C , pH 7.0. (a) and (b) are separate experiments, recorded with different time scales.

Fig. 2. H^+/O ratios (○—○) and total H^+ production (●—●) by O_2 pulsed anaerobic suspensions of rat liver mitochondria

(a) Anaerobic suspensions (8 ml) containing mitochondria (20 mg) treated with 5 μ M-rotenone, antimycin (1.3 μ g/ml), valinomycin (0.6 μ g/ml) and oligomycin (1 μ g/ml). Successive O_2 pulses were used. Other conditions were as in Fig. 1. (b) As in (a) but minus antimycin.

Fig. 3. Acidification by antimycin-induced rat liver mitochondria on the addition of (a) large O_2 pulses or (b) ferricyanide

Mitochondria (about 1 mg of protein/ml) were incubated under the conditions described in Fig. 1 above. In (b), after repetitive pulses of air-saturated medium (see Fig. 1b) to exhaust H^+ production, KCN (1 mM) was added. Ferricyanide was added 5 min after cyanide addition. Other additions were as indicated.

rotenone was insensitive to *N*-ethylmaleimide (125 μ M). This is in agreement with the results of Moyle & Mitchell (1978) where exogenous reductants were used.

If larger amounts of O_2 are introduced into the anaerobic suspension of antimycin-treated mitochondria, rather than successive additions of small quantities of O_2 , exhaustion of the endogenous reductant still occurs after approximately the same total amount of H^+ production but the shape of the H^+ pulses are different (Fig. 3a). The initial rapid change in acidity (approx. 30 μ mol of electron equivalents/min per g of mitochondrial protein) is followed by a slower phase (approx. 7 μ mol of electron equivalents/min per g of mitochondrial protein), and when the suspension becomes anaerobic, the pH changes no longer decay back to the original level, indicating that some net H^+ production has occurred. The presence of succinate did not alter the rates of respiration-dependent H^+ production in antimycin-inhibited mitochondria but did prevent the exhaustion of H^+ production by repetitive O_2 pulses.

It has been shown previously (Mitchell & Moyle, 1978; Lorusso *et al.*, 1979) that ferricyanide can be reduced by a cyanide-insensitive system in antimycin-treated mitochondria, at a rate proportional

to the leakage of electrons through the antimycin-inhibited site. This reduction is accompanied by a H^+ release which has been attributed to the oxidation of endogenous hydrogenated reductants.

Fig. 3(b) shows that the addition of ferricyanide to mitochondria in the presence of rotenone, antimycin and cyanide induces a rapid acidity change followed by a slower H^+ production similar to the changes seen on the addition of O_2 (Fig. 3a). The initial rapid acidity change induced by ferricyanide depends, in magnitude, on the duration of anaerobic incubation after cyanide addition. After a minimum of 10 min anaerobic incubation before ferricyanide addition, the magnitude of the initial acidity change corresponds approximately to one-third of the amount of hydrogenated reductant present, as estimated by the O_2 pulse exhaustion method described above. The H^+ release following ferricyanide addition is uncoupler-insensitive and unaffected by further additions of rotenone or antimycin A.

Experiments with exogenous substrates

With a knowledge of the endogenous H^+/O stoichiometry for antimycin-treated mitochondria, it is possible to quantify the possible effects of any hydrogenated reductants on the H^+/O stoichiometries of exogenous substrates, assuming a direct

competition for reducing equivalents by cytochrome oxidase. Fig. 4 shows typical O_2 pulse experiments using antimycin-inhibited mitochondria in the presence of added ferrocyanide (Fig. 4a) or ascorbate plus TMPD (Fig. 4b). The transient and net changes in acidity can be compared for systems in the presence and absence of uncoupler (Table 1). For both substrates, there is an uncoupler-sensitive initial H^+ production followed by a net alkalization. However, the alkalinity changes are consistently less than those expected from the net chemical reactions for oxidation of the two substrates (eqns. 1 and 2).

This shortfall in the expected net alkalinity changes suggests a competition for reducing equivalents between the endogenous and the exogenous reductants. If the H^+/O ratio is 2 for the antimycin-insensitive hydrogenated reductant then the exogenous reductants are providing approx. 70% of the electron flow under the conditions used. In the

presence of an uncoupler, the contribution to electron flow provided by exogenous reductants rises to 90%. However, even when the 30% contribution of endogenous substrate to H^+ production under energized conditions is taken into account, there still remains some uncoupler-sensitive transient H^+ production. This shows an H^+/O stoichiometry of 0.7 when ferrocyanide is present and 1.2 with ascorbate/TMPD (Table 1).

Table 1. H^+/O ratios for antimycin-treated mitochondria in the absence and presence of exogenous substrates. Conditions of measurement are as in Fig. 1. H^+/O indicates the stoichiometry of the initial H^+ pulse and H^+/O (net) the residual acidity after the collapse of the initial pulse.

Added substrate	H^+/O	H^+/O (net)	
		-FCCP	+FCCP
None	$+2.1 \pm 0.2$	0	0
Ascorbate			
+ TMPD	$+2.4 \pm 0.1$	-0.8 ± 0.1	-0.9 ± 0.1
Ferrocyanide	$+1.3 \pm 0.2$	-1.4 ± 0.1	-1.8 ± 0.1

Fig. 4. O_2 pulse traces from antimycin-inhibited rat liver mitochondria in the presence of added substrates, (A) ferrocyanide (2.5 mM) and (B) ascorbate (2.5 mM) plus TMPD (18 μ M)

Conditions were as in Fig. 1 except that the mitochondrial concentration was 2.5 mg of protein/ml. (a) Control pulses in the absence of added substrate and with the endogenous substrate exhausted; (b) in the presence of added substrate; (c) as (b) plus *N*-ethylmaleimide (115 μ M); (d) as (b) plus FCCP (1 μ M).

Discussion

The present results confirm that in antimycin-inhibited mitochondria there is a 'pool' of hydrogenated reductant (XH) which can supply reducing equivalents to O_2 or ferricyanide. The reductant lies on the cytochrome *c* side of the antimycin-inhibition site and can be fully oxidized in anaerobic mitochondria by repeated O_2 pulses or by the addition of ferricyanide. Re-reduction of the oxidized component is slow and occurs via antimycin-insensitive electron flow from the endogenous substrates (SH) normally present in isolated rat liver mitochondria. Re-reduction is not accompanied by any net H^+ production, suggesting the process of eqn. (3):

However, in the presence of ferricyanide, an uncoupler-insensitive H^+ production occurs on the cytoplasmic side of the mitochondrial inner membrane concomitantly with the reduction to ferrocyanide (eqn. 4).

A similar reduction (of the oxidized form of added reductants) has been suggested (Moyle & Mitchell, 1978; Lorusso *et al.*, 1979) as responsible for variations in the measurement of H^+/e stoichiometries of site 3 when substrate pulse and/or steady state methods were used (Brand *et al.*, 1976; Sigel & Carafoli, 1978; Wikström & Krab, 1979; Azzone *et al.*, 1979).

Quantification of H^+/e values by substrate-pulse methods depends on accurate rate measurements of the initial phases of electron flow and H^+ translocation, and fast response techniques should be used (Papa *et al.*, 1980). With the O_2 -pulse approach however an accurate measure of added O_2 is possible and, under suitable conditions, a reasonable estimate of the amount of H^+ translocation can be made (see Mitchell *et al.*, 1979). In the present experiments several H^+ pulses of constant H^+/O stoichiometry were obtained before full oxidation of the endogenous hydrogenated reductant occurred. After a few minutes, sufficient re-reduction had taken place to support further respiration-dependent H^+ movements. In the presence of exogenous substrates the pattern of H^+ production suggests a competition between the pool of endogenous reductant and the added substrates for the supply of electrons to cytochrome oxidase. Thus the net alkalinity changes for the oxidation of ferrocyanide and of ascorbate plus TMPD (eqns. 1 and 2) are less than expected for oxidation of these two substrates in the absence of any endogenous reductants. The 30% contribution of endogenous hydrogenated reductant to ferrocyanide oxidation calculated from the present results agrees with the value of 30% excess O consumption obtained by rate measure-

ments in the presence of ferrocyanide (Moyle & Mitchell, 1978). But re-reduction of the ferricyanide (or dehydroascorbate) formed in the pulse experiments does not appear to be responsible for the initial H^+ production, as suggested by Moyle & Mitchell (1978). The rate and stoichiometry of this H^+ production on the cytoplasmic side of the mitochondrial inner membrane can be simply explained by electron flow from the hydrogenated reductant to oxygen. The increased net alkalinity changes obtained in O_2 pulse experiments with added substrates in the presence of uncoupler indicate that the relative contribution of endogenous and exogenous reductants to electron flow to O_2 can change. The degree of competition for reducing equivalents between exogenous and endogenous reductants appears to depend on the rate of electron flow. Under relatively fast cytochrome oxidase turnover conditions, the antimycin-insensitive endogenous reductant does not contribute significantly as a source of reducing equivalents.

The net alkalinity changes for oxidation of exogenous substrates can be used to calculate the contribution of the endogenous hydrogenated reductant to such electron flow. The height of the uncoupler-sensitive pulse of H^+ production gives a measure of the amount of H^+ translocation taking place during the oxygen pulse. The present results show that even after taking into account the contribution of the endogenous hydrogenated reductant to H^+ production (to a maximum H^+/O of 2.1 when only endogenous substrate is being oxidized), extra H^+ translocation is taking place. The stoichiometry of this H^+ translocation is variable and appears to depend on the rate of electron flow to oxygen, giving an extra H^+ movement with a H^+/O value of 0.7 for ferrocyanide oxidation and 1.2 for the relatively faster ascorbate/TMPD oxidation. A close examination of the stoichiometry of charge separation is necessary to determine whether this H^+ translocation is electrogenic. $H^+/2e$ quotients measured with endogenous substrates and with added methanol and ascorbate/TMPD to whole cells of *Paracoccus denitrificans* suggest that electrogenic translocation of protons by cytochrome oxidase does take place (Van Verseveld *et al.*, 1981).

Fig. 5 summarizes the present findings. Two sources of respiration-dependent H^+ production can be detected in antimycin-inhibited mitochondria. The first comes from oxidation of an endogenous hydrogenated reductant which is kept in a reduced state in anaerobic mitochondria by antimycin-insensitive electron flow from a pool of reductant. This reductant cannot be removed by centrifugation and resuspension of the mitochondria in fresh medium. From the relative molar quantities of total reductant and from the experiments of Lorusso *et al.* (1979) using the inhibitors 2-*n*-heptyl-4-hydroxyquinoline

Fig. 5. Diagrammatic representation of the possible sources of respiration-dependent H^+ production in antimycin-treated mitochondria

A hydrogenated reductant (XH) releases protons ($30 \mu\text{mol equiv./min per g}$) to the cytoplasmic side of the mitochondrial inner membrane, upon oxidation, with an H^+/e ratio of 1. A slow ($7 \mu\text{mol equiv./min per g}$) re-reduction takes place via an antimycin-insensitive electron flow from the reduced ubiquinone pool. Re-reduction of ubiquinone depends on endogenous ($0.3 \mu\text{mol equiv./min per g}$) or added substrates. H^+ consumption during the reduction of O_2 to water occurs on the matrix side of the mitochondrial inner membrane, also with an H^+/e ratio of 1. H^+ translocation by cytochrome oxidase appears to have a stoichiometry dependent upon the rate of electron flow through the complex.

N-oxide and dibromothymoquinone it may be concluded that this component is probably reduced ubiquinone. However, the rate of H^+ production from the oxidation of the endogenous reductant, even in the initial phase where H/O values of 2 are observed, is still only 20% of the rate when antimycin is absent. Some slow bypass of the usual functioning of the QH_2/Q couple may be occurring in the presence of antimycin. The second source of H^+ production appears to rise from the H^+ pumping activity of cytochrome oxidase across the mitochondrial inner membrane. The present experiments suggest that this activity is dependent on the rate of turnover of the oxidase, as did some results from reconstitution experiments using purified cytochrome oxidase incorporated into phospholipid vesicles (Casey *et al.*, 1979). H^+ translocation by cytochrome oxidase may depend on the relative amount of high- to low-spin forms of the enzyme (Nicholls & Wigglesworth, 1982) which is known to depend on turnover (Nicholls & Hildebrandt, 1978).

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