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ELECTRON TRANSPORT IN BEEF HEART MUSCLE PREPARATION

THE INHIBITION OF UBIQUINONE-STIMULATED SUCCINATE OXIDASE ACTIVITY BY ANTIMYCIN AND A QUINOLINE-*N*-OXIDE DERIVATIVE

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SUMMARY

1. The succinate oxidase activity of acetone extracted heart muscle preparation could be restored by the addition of ubiquinone homologues.

2. Where short chain ubiquinone homologues were employed to restore succinate oxidase activity, the reconstituted system was found to be no longer fully inhibited by antimycin.

3. When the acetone extracted preparation was preincubated with ubiquinone homologues and then washed free of unbound quinone, the succinate oxidase of the reconstituted particle was found to be completely inhibited by antimycin.

4. Previous reports had proposed that the quinone mediated antimycin insensitive electron was due either to an electronic by-pass around the antimycin sensitive site or to the displacement of inhibitor by the added quinone.

These two alternatives were investigated by measuring the sensitivity of acetone extracted heart muscle preparation at different concentrations of inhibitors in the presence of quinone.

5. The results appear to support an electron by-pass mechanism of electron flow mediated by hydrophilic quinones Q-0 and methyl-Q-0. The pattern of the reversal of antimycin inhibition by the higher ubiquinone homologues Q-2 and Q-3 suggests that displacement of inhibitor is the controlling factor.

6. The Q-0 and methyl-Q-0 mediated antimycin insensitive rate of succinate oxidation of both extracted and unextracted heart muscle was compared and results suggest that this pathway of electron flow does not involve the endogenous ubiquinone of the respiratory chain.

7. These findings are further considered in relation to the hydrophilic nature of short chain ubiquinone homologues.

INTRODUCTION

Ubiquinone (coenzyme Q) has been clearly implicated in mitochondrial electron transport although the precise location and mode of interaction of this carrier is not

Abbreviation: HQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide.

yet completely understood[1-3]. In a recent paper KLINGENBERG AND KRÖGER[4] have presented a comprehensive and convincing study in support of the concept that ubiquinone is on the main pathway of electron transfer and that there are no by-paths of electron transfer associated with the quinone pool. In other approaches, attempts have been made to clarify the precise site of interaction of ubiquinone in the multienzyme electron transport system by the use of inhibitors[5,6], and by examining the effects of removing the endogenous ubiquinone by solvent extraction procedures[7,8].

Inhibitor studies have revealed that ubiquinone appears to mediate electron transport between the NADH and succinate flavoproteins and cytochrome c_1. The relationship of ubiquinone and cytochrome *b* is not yet clear[9,10].

The use of solvent extraction procedures has revealed that removal of the endogenous ubiquinone from respiratory enzyme particles leads to the loss of succinate and NADH oxidase activities. Attempts to restore these activities were made using the naturally occurring ubiquinones, synthetic short chain ubiquinones and various lipid extracts. The results have broadly indicated that the hydrophobic, naturally occurring long chain ubiquinones require to be solubilised before they will effectively reactivate the solvent extracted system[11].

The short chain ubiquinone homologues are more hydrophilic and will readily restore the succinate oxidase activity of extracted respiratory enzyme particles.

The requirement or otherwise for lipid supplement appears to be dependent on the nature of the extraction procedure[12].

The work of AMBE AND CRANE[13] using succinate dehydrogenase complex, electron transport particle and electron transport particle residue indicated that the succinate oxidase and succinate–cytochrome *c* reductase activities of the acetone extracted particles could be stimulated by ubiquinone homologues. However, the antimycin sensitivity of the quinone-stimulated succinate–cytochrome *c* reductase activity of acetone extracted succinate dehydrogenase complex and electron transport particle residue was different from the antimycin sensitivity of the succinate oxidase activity of acetone extracted electron transport particle. AMBE AND CRANE[13] suggest that the antimycin insensitive stimulation of succinate–cytochrome *c* reductase activity of acetone extracted succinate dehydrogenase complex by Q-1 and methyl-Q-0 could be explained by the direct reaction of the reduced form of these quinones with cytochrome *c*, by-passing the antimycin sensitive site.

More recently, work by TAKEMORI AND KING[14] on the reactivation effects of coenzyme Q-2 on antimycin inhibited succinate–cytochrome *c* reductase particle has been interpreted in terms of the displacement of the inhibitor by the added quinone and not as the result of an electron by-pass mechanism. The preparation used by these authors was not solvent extracted to remove the endogenous ubiquinone.

From earlier work[15] it is clear that the respiratory enzyme particle will bind considerable amounts of added ubiquinone homologues. A study of the antimycin sensitivity of heart muscle preparation was undertaken in an attempt to clarify the nature of quinone mediated activation.

MATERIALS AND METHODS

Solvents. All solvents were distilled before use. Acetone was first dried over Na_2SO_4; light petroleum (b.p., 40–60°) over Na wire. Ethanol was refluxed over Zn

dust and NaOH before distillation. Water was double glass distilled and used where appropriate in solutions, preparations and assays.

Chemicals. All reagents were of analytical grade. Antimycin A from *Streptomyces kitazawaensis*, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) and horse heart cytochrome *c* (type III) were obtained from Sigma Chemical Co. Ubiquinone homologues were obtained from Dr. O. Isler, Hoffman La Roche. Aurantiogliocladin (2,3-dimethoxy-5,6-dimethylbenzoquinone) was obtained from Professor Thompson, University of Aberdeen.

Heart muscle preparation. This was prepared from ox heart according to the procedure previously described[16].

Acetone extraction procedure. This procedure was modified from the method previously described[15]. Dry acetone was cooled to −15° and 100 ml measured into a 250-ml flask. A suspension of heart muscle preparation (10 ml having a protein concentration of about 40 mg/ml) in 0.25 M sucrose–0.01 M KH_2PO_4–Na_2HPO_4 (pH 7.4) buffer was added dropwise to the solvent and the whole stirred for 40 min. The temperature of the acetone was allowed to rise to 0° in the 40-min period. The flocculent mass of protein was then allowed to settle, the milky acetone supernatant decanted into a suitable flask and the residue transferred with 0.1 M phosphate buffer (pH 7.4) to a Potter–Elvehjem homogeniser. The extracted heart muscle preparation was homogenised with 50 ml of 0.1 M sodium–potassium phosphate buffer (pH 7.4) and recentrifuged. The washing with buffer was repeated four times to ensure complete removal of acetone from the preparation. The washed preparation was finally resuspended in phosphate buffer to give a protein concentration of about 10 mg/ml.

The final product was paler in colour than the unextracted heart muscle preparations and on standing quickly sedimented. When the preparation was stored at −15°, reproducible assay results were obtained over a period of 1–2 weeks. When stored at 0–4° the preparation was stable for about 2 days.

Incubation of quinones with heart muscle preparation and estimation of bound quinone. These processes were carried out as previously described[15].

Succinate oxidase assay. Succinate oxidase activity was measured polarographically at 23° using a Clark type electrode. The reaction mixture contained 67 mM KH_2PO_4–Na_2HPO_4 buffer (pH 7.4); 37 mM sodium succinate; 30 μM cytochrome *c*; heart muscle preparation 1–2 mg protein. The final reaction volume was 3 ml.

RESULTS

Fig. 1 shows that heart muscle preparation, extracted with acetone to remove the endogenous ubiquinone and then incubated with quinone, binds varying and in some cases considerable amounts of the added quinone, *e.g.*, Q-2, Q-3.

The bound quinone is able to stimulate the succinate oxidase activity of the extracted preparation. The stimulation of activity observed with higher ubiquinone homologues (Q-6 to Q-10) is less than maximal since supplementary lipid materials are required for full restoration of electron transport in acetone extracted preparations[17]. It was also found that the addition of antimycin at a concentration of 100 μg/g enzyme protein completely inhibited electron transport in the reconstituted preparations.

In another experiment (Table I) the acetone extracted preparation was incu-

bated with ubiquinone homologues at $1 \cdot 10^{-4}$ M final concentration. Under these conditions the reconstituted succinate oxidase activity of the preparation was only partly inhibited by antimycin at 100 μg/g protein concentration. Methyl-Q-0 and Q-0 showed the lowest percentage inhibition by antimycin while Q-1 and Q-2 showed a proportionally greater degree of inhibition with increasing side chain length.

Under the conditions of assay the amount of enzyme protein added to the system containing quinone at 10^{-4} M concentration would bind only about 4% of the Q-1 or about 24% of the added Q-2. Methyl-Q-0 and Q-0 would not be bound to any significant extent. That complete inhibition of succinate oxidase by antimycin

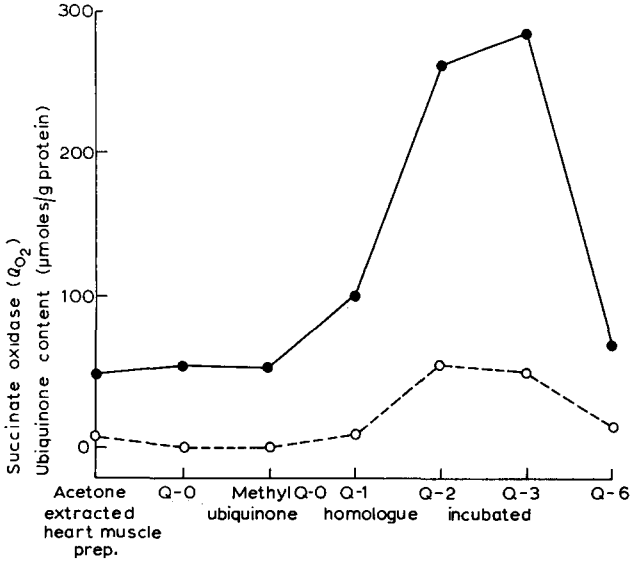

Fig. 1. The binding of ubiquinone homologues and the succinate oxidase activity of acetone extracted heart muscle preparation. ●—●, succinate oxidase activity; O---O, ubiquinone content. The succinate oxidase activity was 100% inhibited in all cases by the addition of 100 μg antimycin/g heart muscle preparation protein. The incubation with quinone and the acetone extracted heart muscle preparation was incubated with excess quinone and subsequently washed with phosphate buffer to remove any unbound quinone. The succinate oxidase and bound quinone determinations are as described in the text.

TABLE I

ANTIMYCIN INHIBITION OF THE SUCCINATE OXIDASE ACTIVITY OF ACETONE EXTRACTED HEART MUSCLE PREPARATION IN THE PRESENCE OF UBIQUINONE HOMOLOGUES

	Acetone extracted heart muscle preparation						
Ubiquinone homologue added (final concn., 10^{-4} M)	None	Q-0	Methyl-Q-0	Q-1	Q-2	Q-3	Q-6
Succinate oxidase activity (Q_{O_2})	52	148	160	212	218	220	51
Inhibition (%) of succinate oxidase activity by antimycin (concn., 100 μg/g heart muscle preparation protein)	100	22	40	87	97	100	100

is no longer obtained may therefore be related to the presence of unbound short chain ubiquinones in the assay medium.

Table II shows the effects of increasing concentration of ethanolic solutions of ubiquinone homologues when added to the washed, acetone extracted heart muscle preparation preincubated with the same ubiquinone. The addition of the lower homologues methyl-Q-0, Q-1 and Q-2 caused further stimulation of the succinate oxidase activity of the heart muscle preparation already preincubated with these

TABLE II

ANTIMYCIN INHIBITION OF THE SUCCINATE OXIDASE ACTIVITY OF ACETONE EXTRACTED–UBIQUINONE INCUBATED HEART MUSCLE PREPARATION IN THE PRESENCE OF VARYING CONCENTRATIONS OF UBIQUINONE

Acetone extracted heart muscle preparation incubated with ubiquinone (Q-*n*) and washed free of unbound quinone. Figures in parentheses indicates % inhibition of succinate oxidase activity by antimycin (concn., 100 μg/g heart-muscle preparation protein).

Ubiquinone concn. (*M*)	*Succinate oxidase* (Q_{O_2})		
	Methyl-Q-0	*Q-1*	*Q-2*
$5 \cdot 10^{-5}$	121 (49)	172 (91)	321 (100)
$1 \cdot 10^{-4}$	161 (40)	345 (87)	333 (97)
$5 \cdot 10^{-4}$	199 (24)	378 (78)	343 (96)

same lower homologues. The addition of increasing concentrations of Q-1 to the extracted Q-1 incubated preparation caused a stimulation of succinate oxidase activity up to a maximum value reached at $5 \cdot 10^{-4}$ M concentration of Q-1. Higher concentrations of added Q-1 began to inhibit the succinate oxidase of the preparation. The percentage inhibition by a fixed concentration of antimycin (100 μg/g protein) began to decline as the concentration of the added quinone was increased. This effect was noted for methyl-Q-0, Q-1 and Q-2 treated samples although in the latter case the percentage inhibition by antimycin showed only a slight decrease. The higher homologues with side chains longer than 15 C failed to significantly increase the succinate oxidase activity and the antimycin sensitivity remained at 100 %. Q-3 behaved in an intermediate manner producing significant stimulation of the succinate oxidase activity but at the added concentration, antimycin remained completely inhibitory in the system.

These results suggested that where the succinate oxidase activity of an acetone extracted preparation was restored by added ubiquinone, all of which is bound to the particle, then the pathway of electron flow is *via* the bound ubiquinone and the antimycin sensitive site of the electron transport system. However, where the ubiquinone homologue was added in amounts in excess of the binding capacity of the enzyme then it appears that the hydrophilic molecules in the external medium are able to mediate electron transport even in the presence of antimycin.

The reversal of antimycin inhibition by the addition of short chain ubiquinone homologues could be explained in terms of either (a) displacement of antimycin by the quinone molecules or (b) a by-pass mechanism.

These possibilities were investigated by measuring the sensitivity of acetone extracted heart muscle preparation at different concentrations of inhibitor in the

presence of a fixed concentration of quinone. Expressed in Fig. 2 are the results obtained.

This experiment was repeated using HQNO, an inhibitor that is reported to act at the same site as antimycin. The results in Fig. 3 indicate a close similarity of behaviour between antimycin and HQNO in the systems investigated.

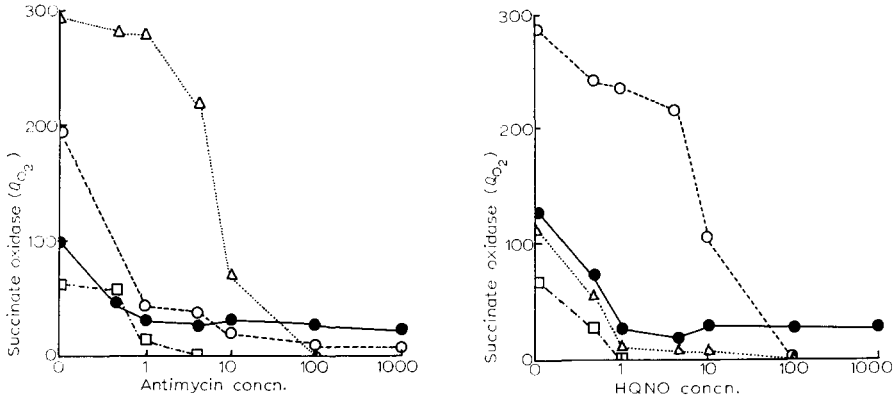

Fig. 2. Antimycin inhibition of ubiquinone stimulated succinate oxidase activity of acetone extracted heart muscle preparation. ●———●, Q-0; O— — —O, Q-1; △·····△, Q-2; □–·—·–□, Q-6. Methyl-Q-0 and Q-3 results omitted for clarity; see Fig. 3 and text. Assay as described in text. The extracted heart muscle preparation was pretreated with the appropriate quinone at $5 \cdot 10^{-4}$ M final concn. Unit concentration of inhibitor was the minimum required to abolish succinate oxidase activity in the untreated control.

Fig. 3. Inhibition of ubiquinone stimulated succinate oxidase of acetone extracted heart muscle preparation by HQNO. ●———●, methyl-Q-0; O— — —O, Q-2; △·····△, Q-3; □–·—·–□, Q-6; Q-0 and Q-1 results omitted for clarity; see Fig. 2 and text. Conditions as described under Fig. 2.

The effects of adding Q-0 and methyl-Q-0 to extracted and unextracted heart muscle preparation treated with a high concentration of antimycin are shown in Table III. The absolute rates of succinate oxidation expressed as Q_{O_2} in the presence of 500 μg antimycin/g heart muscle preparation protein are similar in both control and acetone extracted preparations treated with Q-0 and methyl-Q-0. The addition of Q-1 or Q-2 was without effect at this concentration of inhibitor.

TABLE III

ANTIMYCIN INSENSITIVE SUCCINATE OXIDASE ACTIVITY OF ACETONE EXTRACTED AND UNEXTRACTED HEART MUSCLE PREPARATION IN THE PRESENCE OF Q-0 AND METHYL-Q-0

Antimycin, final concn., 500 μg/g heart muscle preparation protein. Quinone, final concn., $2 \cdot 10^{-4}$ M.

	Additions		
	None	*Q-0, antimycin*	*Methyl-Q-0, antimycin*
Succinate oxidase of heart muscle preparation			
Q_{O_2} (control)	350	38	49
Q_{O_2} (acetone extracted)	49	35	31

The by-pass hypothesis is linked to the concept of the quinone molecules involved being essentially water soluble and hence able to participate in electron transport in the external medium. The solubility of the quinones used in the investigation was measured under the conditions of the experiments. The results are shown in Table IV.

TABLE IV

THE SOLUBILITY OF UBIQUINONE HOMOLOGUES IN PHOSPHATE BUFFER

The solubility was determined by injecting a 10^{-2} M ethanolic solution of quinone (100 μl) into 3 ml of 67 mM KH_2PO_4–Na_2HPO_4 buffer (pH 7.4). The solution was mixed, centrifuged and the clear aqueous layer removed. The fall in the absorbance of the solution at 275 mμ was measured after the addition of reducing agent. From this total amount of quinone in true solution was calculated and expressed as a percentage of the total quinone injected.

Ubiquinone homologues	*Solubility* (%)
Q-0	100
Methyl-Q-0	100
Q-1	100
Q-2	30
Q-3	18
Q-6	3
Q-10	0

DISCUSSION

When the acetone extracted heart muscle preparation was incubated with short chain ubiquinone homologues Q-1, Q-2, Q-3 and all unbound quinone removed from the system then there was a stimulation of succinate oxidase activity. The reconstituted preparations behaved similarly to heart muscle preparation containing the full complement of Q-10 in that the succinate oxidase activity was completely inhibited by relatively low concentrations of antimycin. This suggests that, as with the intact preparation, the electron flow is *via* the bound quinone and the antimycin sensitive site.

In the experiments reported by AMBE AND CRANE[13] it can be calculated (by assuming a binding capacity similar to that of heart muscle preparation) that the amount of quinone added to the succinate dehydrogenase complex preparation was in excess of the binding capacity of the enzyme protein present, *i.e.*, the quinone molecules would have been present in the external medium and able to participate in the proposed by-pass mechanism of antimycin insensitive electron transport.

However, in the investigation of TAKEMORI AND KING[14] the high concentrations of Q-2 employed would also appear to be in excess of the binding capacity of their unextracted enzyme preparation but the results obtained by these workers have been interpreted in terms of a displacement mechanism.

The mode of action of antimycin is not yet fully understood[18] but it appears that it inhibits electron transport by binding at a site between cytochrome *b* and cytochrome *c*.

Displacement of antimycin might therefore be expected to result from the com-

petition of those ubiquinone molecules that have a high affinity for and are extensively bound to the enzyme particles.

Fig. 2 indicates that Q-0 and methyl-Q-0, which are not bound, stimulate the succinate oxidase activity of antimycin inhibited acetone extracted heart muscle preparation. At and above minimum concentration of inhibitor the succinate oxidase activity is reduced to 40–50 % of the initial rate without inhibitor, and this figure is relatively independent of increases in the inhibitor concentration. That Q-0 and methyl-Q-0 inhibitor insensitive rates of oxidation are not markedly influenced by a 100-fold increase in inhibitor concentration suggest that this is not a displacement effect but rather the operation of a by-pass mechanism of electron transport around the inhibitor sensitive site.

Heart muscle preparation, like the succinate dehydrogenase complex and electron transport particle residue of AMBE AND CRANE[13], is an 'open' type of preparation which can catalyse electron transport *via* exogenous cytochrome *c*. The results with heart muscle preparation therefore substantiate the earlier suggestion of an antimycin insensitive by-pass mechanism catalysed by Q-0 and methyl-Q-0 in open electron transport particles. The closed type of preparation *e.g.* electron transport particle is readily inhibited by antimycin. On the other hand Q-1, which is lightly bound by heart muscle preparation, stimulates the activity of the preparation in a manner which could be interpreted as displacement of inhibitor.

This is supported by the findings for the increasingly lypophilic series Q-1 to Q-2. Increasing the inhibitor concentration readily produces further inhibition of the succinate oxidase rate of the preparation stimulated by the lightly bound Q-1. Higher concentrations of inhibitor have to be employed to produce the same degree of inhibition of the succinate oxidase rate of the preparation stimulated with strongly bound Q-2. Both Q-1 and Q-2 stimulated electron transport is eventually abolished by sufficiently high concentrations of inhibitor. SZARKOWSKA[19] has observed that the Q-1 stimulated NADH oxidase activity of heptane extracted, lyophilised mitochondria was also completely abolished by high concentrations of antimycin (2 mg/g protein).

It is possible to explain these findings in terms of the displacement hypothesis but is evident that even very high concentrations of inhibitor cannot abolish completely the succinate oxidase activity of the preparations stimulated by the hydrophilic quinones Q-0 and methyl-Q-0. This suggests that the water soluble quinones with little or no binding affinity are unable to compete with inhibitor but are capable of mediating in an electron by-pass mechanism.

Identical experiments were carried out using the inhibitor HQNO. It appears that this compound acts by binding at the same site as antimycin[20] but about 40 times as much HQNO is required to produce the same inhibition of succinate oxidase activity of heart muscle preparation as compared with antimycin. The close similarity between the degrees of inhibition caused by proportional changes in inhibitor concentration could imply a competition between inhibitors and quinone for a reactive site on the electron transport chain. On the other hand it seems more probable that this similarity is fortuitous and that the inhibition is due to the binding of inhibitor into quinone–phospholipid micelles[21] attached to the particles. For example CRANE[22] has shown that the 2-hydroxy-3-alkylnaphthoquinone (SN 5949) inhibition of electron transport particle would be reversed by the addition of relatively large amounts of Q-10, α-tocopherol or vitamin K_1.

Recently RIESKE[18] has reported that the antimycin inhibition of QH–cytochrome *c* reductase activity of a Complex III preparation was not reversed by the addition of Q-2. RIESKE has, however, sought to explain these findings in terms of the low requirements of the active Complex III required to support the maximum rate of succinate oxidation. In this case a relatively small amount of Complex III would be sufficient to support full succinate oxidase activity.

The relationship demonstrated between water solubility and the by-pass function of Q-0 and methyl-Q-0 supports the suggestion that a pathway of electron transport could be explained as an electron shuttle around the antimycin sensitive site which involves hydrophilic quinone molecules in the external medium. It is clear, however, that succinate oxidase of acetone extracted heart muscle preparation reconstituted with these water soluble quinones also catalyse a substantial proportion of the electron flow *via* the antimycin sensitive site.

The findings that both acetone extracted and normal preparations tend to have a similar rate of interaction with the external quinone molecules (see Table III) in the presence of completely inhibitory concentrations of HQNO and antimycin suggests that the site of interaction of the quinone molecules involved in the by-pass does not involve the endogenous ubiquinone site.

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