Biochimica et Biophysica Acta, 368 (1974) 71–85

BBA 46810

ACTIVITY OF REDUCED UBIQUINONE : CYTOCHROME *c* OXIDOREDUCTASE WITH VARIOUS UBIQUINOL-ISOPRENOLOGUES AS SUBSTRATE AND CORRESPONDING INHIBITORY EFFECT OF ANTIMYCIN IN YEAST

BERND LANG, GERTRAUD BURGER and WOLFHARD BANDLOW

Institut für Genetik der Universität München, D-8 München 19, Maria-Ward-Str. 1a (G.F.R.)

(Received April 22nd, 1974)

SUMMARY

1. Reduced ubiquinones-1, -2, -3, -4 and -6 were used as substrates for ubiquinol : cytochrome *c* oxidoreductase.

2. The portion of antimycin-sensitive activity depends on the concentration of ubiquinol and on the pH. Only reduced ubiquinone-2 and reduced ubiquinone-3 show high activities the main part of which is sensitive to antimycin.

3. The antimycin effect curve of ubiquinol : cytochrome *c* oxidoreductase is linear in shape with reduced ubiquinone-2 as substrate but sigmoidal with reduced ubiquinone-3 and succinate. Ubiquinol-3 : cytochrome *c* oxidoreductase activity contains a portion scarcely affected by antimycin. About 300 pmoles of antimycin per mg protein, enough to inhibit succinate, NADH- and reduced ubiquinone-2:cytochrome *c* oxidoreductase almost totally, affect ubiquinol-3 : cytochrome *c* oxidoreductase to only about 80 % and another 300 pmoles of antimycin are needed for the next 10 % of inhibition.

4. The activities of succinate- and NADH : cytochrome *c* oxidoreductase are stimulated by ubiquinones-2 and -3. The shapes of the inhibition curves by antimycin of the stimulated activities are sigmoidal. About twice the amount of antimycin is necessary to inhibit stimulated activities to the same value as the unstimulated.

5. The non-ionic detergent Lubrol WX is not effective in stimulating enzymatic activities. However, in the presence of 0.6 M sorbitol, it converts the linear antimycin effect curve with reduced ubiquinone-2 as substrate, into sigmoidal.

6. NADH- and succinate : cytochrome *c* oxidoreductase activities and reduced ubiquinone-2 and reduced ubiquinone-3 : cytochrome *c* oxidoreductase activities become deactivated with increasing concentrations of the non-ionic detergent Lubrol WX. The activity with reduced ubiquinone-2 as substrate is less resistant to the action of the detergent than with reduced ubiquinone-3. The *b*-cytochromes do not become CO-reactive by this treatment.

Abbreviation: QH_2, reduced ubiquinone.

7. Deoxycholate in low concentrations does not stimulate ubiquinol : cytochrome *c* oxidoreductase activity. It converts the inhibition curve by antimycin from sigmoidal to linear with increasing concentrations of the detergent with all substrates tested. The amount of antimycin needed for 90 % inhibition of reduced ubiquinone activities is about the same under these conditions as with succinate, NADH or reduced ubiquinol in untreated particles.

8. The results are discussed with respect to the theories of the electron transport mechanism and of the inhibition by antimycin of the electron flow through the bc_1-segment of the respiratory chain in beef heart.

INTRODUCTION

Berden and Slater [1] found with Complex III from beef heart a stoichiometry of 2 cytochrome *b* : 1 cytochrome c_1 : 1 antimycin binding site (for review see ref. 2). Recent work revealed that the binding site of antimycin is obviously not cytochrome *b*-566 [2–6] as was formerly proposed [7], but a distinct protein of 11 500 *M* [8] carrying no heme.

The mechanism of inhibition is not yet clear. Klingenberg and Kröger [9–11] interpret sigmoidal inhibition curves of the enzymatic activity of the bc_1-segment of the respiratory chain in terms of the pool-function of the natural ubiquinone. Bryła et al. [12], Slater [13] and Berden and Slater [14] found evidence that antimycin acts as an allosteric effector in terms of the theory of Monod et al. [15]. Wikström and Berden [16] postulate different electron acceptors for reduced ubiquinone (QH_2) and the QH·-radical and a split electron transport chain for the bc_1-segment. Only the pathway which receives electrons from QH_2 and which contains the cytochromes *b* and c_1, is inhibited by the antibiotic. The QH·-radical is oxidized by an antimycin-insensitive pathway with oxygen as the final acceptor, thus shifting the equilibrium to the side of the reduced cytochrome *b* in the presence of antimycin. The donor for this reduction is, in their theory, QH_2 or a flavin. This mechanism formally parallels the earlier statement of Baum et al. [17] and Rieske [18]. These authors postulate the existence of a component X which they localize on the oxygen side of cytochrome *b*. For the exertion of the inhibitory effect of antimycin, X must be in the oxidized state.

The mechanism of inhibition by antimycin of the electron flow through the bc_1-segment, is difficult to investigate mainly for two reasons: first, because of the highly complex structure and the great number of hemoproteins and non-heme proteins assembled in this segment (for review see refs 19, 20); and, secondly, because of the difficulty of directly measuring the enzymatic activity of the bc_1-segment due to the water-insolubility of the natural ubiquinol (QH_2-6 in yeast) and due to the fact that the addition of any quinols alters the natural ubiquinone pool. Despite these obvious limitations in the prediction of the in vivo situation, it appears that the use of artificial substrates for ubiquinol : cytochrome *c* oxidoreductase is highly efficient in testing its activity. In addition, the use of artificial ubiquinones provides two advantages: (i) the extension of the ubiquinone pool by exogenously added ubiquinol may eliminate a possible rate limitation by the diffusion of ubiquinol from the site(s) of acceptance to the site(s) of delivery of its electrons; and (ii) ubiquinol-isoprenologues differing in the length of the lipophilic side chain will affect the potential of total

ubiquinone. Consequently, the site of donor function of artificial ubiquinones might not be identical with that of the natural QH_2-6, as was observed earlier for the acceptor functions of other synthetic quinones in NADH : quinone oxidoreductase by Ruzicka and Crane [21]. Alteration of the properties of the ubiquinone pool thus provides a means of investigating the influence of these differences on the electron flux through the bc_1-segment and its inhibition by antimycin, as will be described in this paper.

The experiments described in the following have been performed with yeast mitochondria. Mitochondria from this organism provide two major advantages over those from mammals: (i) They contain a second type of NADH dehydrogenase localized on the outer surface of the inner mitochondrial membrane and linked to the extrinsic NADH pool [22] in addition to the one on the inner surface which regenerates the NAD^+ needed for the maintenance of the intrinsic citric acid cycle, the latter being not accessible for exogenous NADH. Since both of them are linked to the cytochromes of the respiratory chain via the ubiquinone pool, respiratory activity and its inhibition by antimycin can be determined with exogenously added NADH. (ii) Yeast provides the possibility of attacking the problem of antimycin binding and antimycin inhibition by way of drug-resistant mutants as have been described by Butow and Zeydel [23] and by Grimmelikhuijzen and Slater [24]. The fission yeast *Schizosaccharomyces pombe* was used throughout the experiments described since this yeast does not give rise to petite mutation, which may complicate the interpretation of results obtained with *Saccharomyces* strains.

MATERIALS AND METHODS

Chemicals

Ubiquinones-1, -2, -3 and -4 were graciously donated by Dr Solms, ubiquinone-6 was obtained from Serva, cytochrome *c* from Boehringer, antimycin from the Nutritional Biochemical Corporation, Lubrol WX from Imperial Chemical Industries, and deoxycholate from Merck.

Cell growth and preparation of mitochondrial particles

Schizosaccharomyces pombe was grown in a medium containing 3 % glycerol, 1 % yeast extract, 0.5 % peptone, as described by Heslot et al. [25], except that the medium was adjusted to pH 5.5–6.0. Cells were harvested in late logarithmic phase of growth yielding about 10 g wet weight cells per 1 medium. The harvesting procedures, the disintegration of the cells by shaking with glass beads and the purification of mitochondrial particles by differential centrifugation, were the same as described earlier [5]. The time of shaking with glass beads was decreased so that only 20–30 % of the cells were disrupted in order to obtain better qualities of mitochondrial particles.

Determination of enzymatic activities

All enzymatic activities were assayed in cuvettes of 1 cm light path at 25 °C. Succinate- and NADH : cytochrome *c* oxidoreductase were determined in a buffer containing 0.1 M potassium phosphate, 25 mM Tris–HCl, 1 mM EDTA and 0.1 mM cytochrome *c*, pH 7.4. The mitochondrial particles were preincubated for 15 min in the test buffer. KCN was added at a concentration of 0.9 mM 1 min before starting the reaction with succinate or NADH. For stimulation by ubiquinones and for inhibi-

tion by antimycin, mitochondria were preincubated for 15 min with the respective substance(s).

Ubiquinol : cytochrome *c* oxidoreductase activities were determined as above, except that the buffer contained additional 0.6 M sorbitol and the pH was adjusted to 6.5, except otherwise stated. The activities measured were corrected for the rate of direct reaction of ubiquinols with cytochrome *c*. Succinate- and NADH activites which were stimulated by exogenous ubiquinones, were corrected for antimycin insensitive activity (0.3 μmoles antimycin/mg mitochondrial protein). The final concentrations of substrates were: succinate, 7.5 mM; NADH, 0.5 mM; ubiquinol-2, 0.065 mM; ubiquinol-3, 0.13 mM. Concentrations used for stimulation of NADH- and succinate : cytochrome *c* oxidoreductase were 12 μM for both ubiquinone-2 and ubiquinone-3. Antimycin was applied as an ethanolic solution. Cytochrome *c* reduction was determined by using an absorption coefficient $\varepsilon_{550} = 18.5\ mM^{-1} \cdot cm^{-1}$.

Reduction and determination of the concentration of ubiquinones

Ubiquinones were reduced by the method of Rieske [26] except that $NaBH_4$ was used after acidification with HCl instead of $Na_2S_2O_4$. Concentrations of ubiquinones were determined as described by Mayer and Isler [27] using an absorption coefficient of $\varepsilon_{272} = 14.7\ mM^{-1} \cdot cm^{-1}$.

Low-temperature spectroscopy

Difference absorption spectra were recorded with a Cary Model 14 spectrophotometer at 77 °K, as described previously [5].

Protein determination

Protein was determined according to the method of Lowry et al. [28].

RESULTS

In quite a variety of mitochondrial preparations tested, the specific activity of NADH-oxidation was much higher than that of succinate oxidation. The amount of antimycin needed for 90 % inhibition is the same in both cases; however, the inhibition curves show two cross-over points as demonstrated by Fig. 1. Moreover, with freshly prepared mitochondrial particles the inhibition curve by antimycin of NADH : cytochrome *c* reductase activity was only slightly sigmoidal in contrast to the effect curve with succinate as substrate. With yeast mitochondrial particles no further activation of succinate : cytochrome *c* oxidoreductase was observed, neither after mild detergent treatment nor after anaerobic preincubation with succinate. The latter procedure leads, on the contrary, to a loss of activity due to aggregation and lowers the antimycin binding constant [14]. The activity measured thus reflects the maximum velocity of succinate : cytochrome *c* oxidoreductase. The sigmoidicity of the antimycin inhibition curves revealed by Fig. 1 is consistent with the assumption of different rate limiting steps in the dehydrogenase segments of NADH- and succinate : ubiquinol reductase in connection with a Q-pool.

Sigmoidicity of the antimycin effect on succinate : cytochrome c oxidoreductase activity

The amount of antimycin necessary for inhibition of succinate : cytochrome *c*

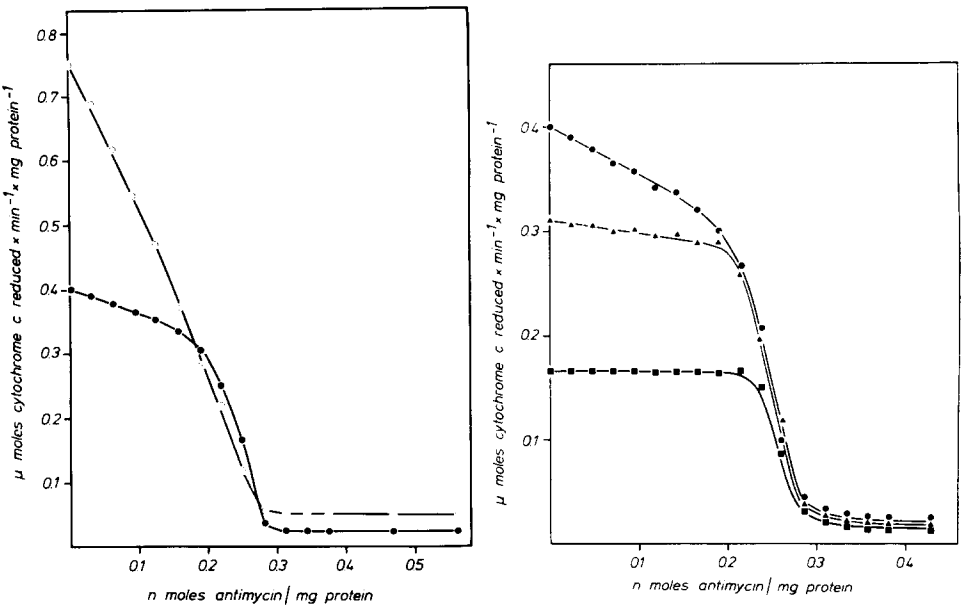


Fig. 1. Inhibition of succinate- and NADH: cytochrome *c* oxidoreductase by antimycin. Mitochondrial particles (6 μg/ml) were incubated as described in Methods. The reaction was started by quickly adding the respective substrate under stirring. The amounts of antimycin indicated in the figure, were added 15 min, and 0.9 mM KCN 1 min before the reaction was started. The concentration of alcohol was kept constant in all samples. (○, NADH-; ●, succinate : cytochrome *c* oxidoreductase activity).

Fig. 2. Effect of aging on succinate : cytochrome *c* oxidoreductase activity and on the shape of the antimycin effect curve. Experiments were carried out as described in legend for Fig. 1. Mitochondrial particles were aged at 4 °C in a closed Eppendorf reaction vessel. (●, freshly prepared; ▲, 16 h after preparation; ■, 49 h after preparation).

oxidoreductase is 250–320 pmoles/mg protein. It is not diminished proportional to the loss in activity after mechanical treatment, e.g. prolonged shaking with glass beads, or aging by storage at 4 °C, which has a similar effect. As revealed by Fig. 2, the sigmoidicity increases concomitantly with the loss of enzyme activity. This reflects the increasing rate-limitation in combination with the Q-pool by succinate dehydrogenase with the propagation of aging. Furthermore, the number of antimycin inhibition sites is not dependent on the state and on the conditions of growth. On the other hand, the specific activity of NADH : cytochrome *c* oxidoreductase of cells from stationary phase exceeds that of logarithmically growing cells by a factor of up to 4 (cf. Table I).

Extension of the ubiquinone-pool by oxidized exogenous ubiquinones

In order to prove a possible rate limitation by the diffusion of intrinsic ubiquinones, we tested the influence of the alterations of the natural pool of ubiquinone by exogenously added ubiquinones on the activities of NADH- and succinate : cytochrome *c* oxidoreductases and on the cross-over points in the antimycin effect curves as shown in Fig. 3.

TABLE I

SPECIFIC ACTIVITIES OF SUCCINATE- AND NADH : CYTOCHROME *c* OXIDOREDUCTASE WITH AND WITHOUT STIMULATION BY Q-2 AND Q-3, RESPECTIVELY, DEPENDING ON GROWTH CONDITIONS AND MODE OF DISINTEGRATION

Logarithmically growing cells of Type I experiment (first line) were harvested at a titer of $2–4 \cdot 10^7$ and disintegrated by shaking with glass beads for 10 s at maximum speed in a Merkenschlager homogenizer [29]. Types II and III were harvested from stationary phase ($3–6 \cdot 10^8$) and homogenized for 10 and 45 s, respectively. Activities are expressed as μmoles cytochrome *c* reduced/min per mg protein.

	Enzyme tested:	Succinate : cytochrome *c* reductase			NADH : cytochrome *c* reductase		
	Additions:	None	12 μM Q-2	12 μM Q-3	None	12 μM Q-2	12 μM Q-3
I	Log phase 10 s disintegration	0.3–0.4	0.6 –0.75	0.55–0.75	0.6–0.9	1.2–1.5	1.3–1.8
II	Stationary phase 10 s disintegration	0.5–0.9	0.95–1.3	0.9 –1.4	0.4–3.2	0.7–3.5	0.8–3.9
III	Stationary phase 45 s disintegration	0.2–0.5	0.4 –1.0	0.4 –0.95	—	—	—

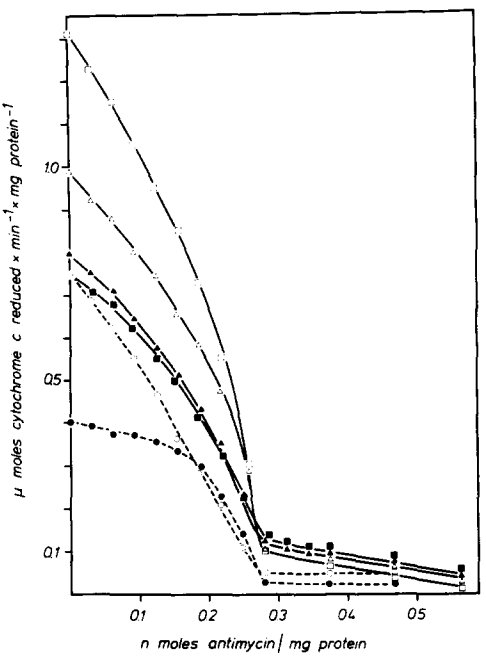


Fig. 3. Inhibition by antimycin of stimulated NADH- and succinate : cytochrome *c* oxidoreductase activity. The concentration of mitochondrial protein was 3 μg/ml. The respective ubiquinones were incubated together with the protein for 15 min before the reaction was started. Open symbols: NADH : cytochrome *c* oxidoreductase; closed symbols: succinate : cytochrome *c* oxidoreductase (○, NADH, unstimulated; △, stimulated by 12 μM ubiquinone-2; □, stimulated by 12 μM ubiquinone-3; ●, succinate, unstimulated; ▲, stimulated by 12 μM ubiquinone-2, ■; stimulated by 12 μM ubiquinone-3). Stimulated activities have been corrected for antimycin insensitive activity as described in Methods.

It can be seen from this figure that the activities are, in fact, stimulated by ubiquinone-2 and -3 by a factor of up to 2, demonstrating a pool-function of the natural ubiquinone [10]. The data suggest that after stimulation, the velocity of the dehydrogenases may be rate limiting rather than the diffusion rate of ubiquinol. On the other hand, the cross-over points in the antimycin effect curves of succinate- and NADH : cytochrome *c* oxidoreductase are abolished with the addition of ubiquinone-2 or -3, the shapes of all inhibition curves becoming superimposible. Exogenously added ubiquinone did, in no case, change the inhibition curve by antimycin to linear.

It is evident from Fig. 3 that ubiquinone-2 was much less effective in stimulating NADH : cytochrome *c* reductase activity than ubiquinone-3. The opposite effect is observed with the stimulation of succinate activity.

The stimulated activity contains a portion less sensitive to antimycin in contrast to the unstimulated one. From the difference in the effect of antimycin on the enzymatic activities of the bc_1-segment with different substrates and under various pool conditions of ubiquinone, we were prompted to examine these effects directly in more detail by measuring ubiquinol : cytochrome *c* oxidoreductase activity.

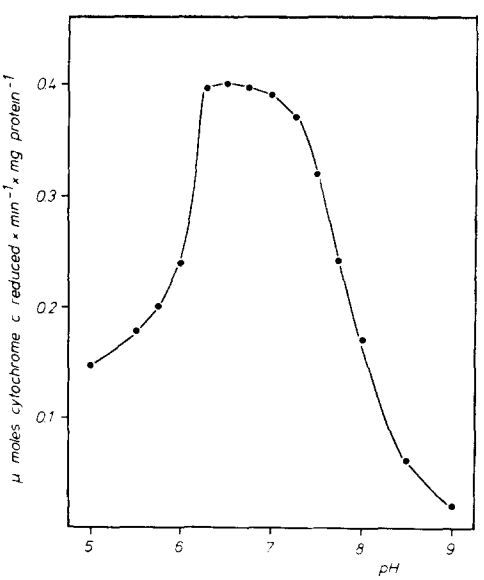
Ubiquinol : cytochrome c oxidoreductase and inhibition by antimycin

A possibility to investigate electron flux through the bc_1-segment directly and free of kinetic influences of the other segments of the respiratory chain, is to measure ubiquinol : cytochrome *c* oxidoreductase activities with various synthetic ubiquinol isoprenologues as substrates, the activities of some of which are compiled in Table II.

Since it proved to be impossible to solubilize enough of the highly lipophilic ubiquinols-4 and -6 in an aqueous buffer system, and since ubiquinol-1 reacts very fast directly with cytochrome *c*, only ubiquinols-2 and -3 are useful substrates for measuring the enzymatic activity of the bc_1-segment. The application of ubiquinol-2 has already been described by Rieske [30]. In order to get optimal activities with yeast mitochondrial particles, we changed the buffer as described in Methods and took into account that the pH optimum of both ubiquinol-2 and -3 activity is in the region of 6.5 in yeast, as demonstrated by Fig. 4. The determination of the enzymatic activity of the bc_1-segment with both substrates was hampered by the fact that ubiquinol-3 is not soluble in excess over the acceptor in each buffer system (Fig. 5) and that ubiquinol-2, on the other hand, reacts directly with cytochrome *c*.

Most surprising with the oxidation of ubiquinol-2 and -3 was the fact that the specific activities were about the same with both substrates in freshly prepared and highly active preparations (cf. Table II). The inhibition curve by antimycin of ubiquinol-2 activity proved to be linear, as published earlier by Rieske [30] for beef heart mitochondria, but it was sigmoidal with ubiquinol-3 as substrate, as can be seen from Fig. 6.

The linear curve obtained with ubiquinol-2 could be converted into sigmoidal when Lubrol WX, a non-ionic detergent, was present in the buffer (Fig. 7). This



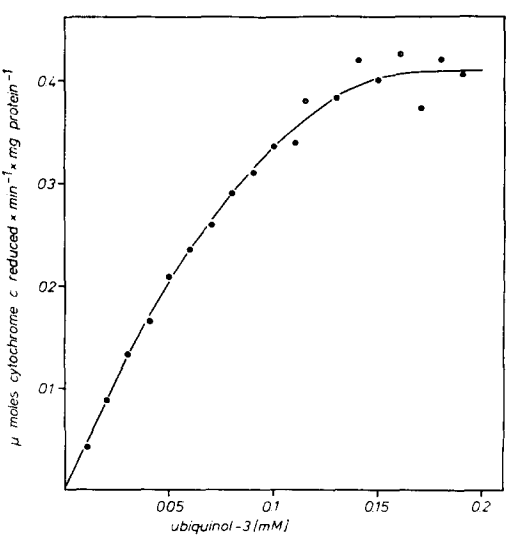


Fig. 4. Effect of pH on ubiquinol-3 : cytochrome *c* oxidoreductase. The experiment was carried out as described in legend for Fig. 1. The activities were corrected for antimycin insensitive reaction in the presence of 0.3 mmoles antimycin/mg particles (about 8 % of the total).

Fig. 5. Influence of substrate concentration on ubiquinol-3: cytochrome *c* oxidoreductase. The conditions were the same as described under Fig. 1. The activity was corrected for the rate measured in the absence of mitochondrial particles due to direct reaction of ubiquinol-3 with cytochrome *c* and the increase in turbidity after the addition of ubiquinol-3.

detergent prevented part of the direct reaction of ubiquinol-2 with cytochrome *c*, but did not stimulate the specific reaction.

TABLE II

SPECIFIC ACTIVITY OF UBIQUINOL : CYTOCHROME *c* OXIDOREDUCTASE WITH VARIOUS UBIQUINOL-ISOPRENOLOGUES AND DIFFERENT PREPARATIONS OF MITOCHONDRIAL PARTICLES

Experimental conditions were described in Methods and under Fig. 5. Activities are given as μmoles cytochrome *c* reduced/min per mg protein. Concentrations used were 65 μM for ubiquinols-1, -2, -4 and -6, and 130 μM for ubiquinol-3.

Substrate	Specific activity	
	Antimycin-sensitive	Antimycin-insensitive
QH_2-1*	0.3–0.5	—
QH_2-2	0.35–0.54	0.05–0.07
QH_2-3	0.32–0.56	0.03–0.05
QH_2-4**	0.12	0
QH_2-6***	0.14	0

* Sample and reference cuvettes contained the same concentration of ubiquinol-1 in order to correct for the very high rate of antimycin insensitive reaction. The reference contained no mitochondrial protein.

** No substrate saturation could be achieved.

*** Particles were sonicated in the presence of substrate *plus* KCN and the reaction started by the addition of cytochrome *c*. Without sonication no activity was observed.

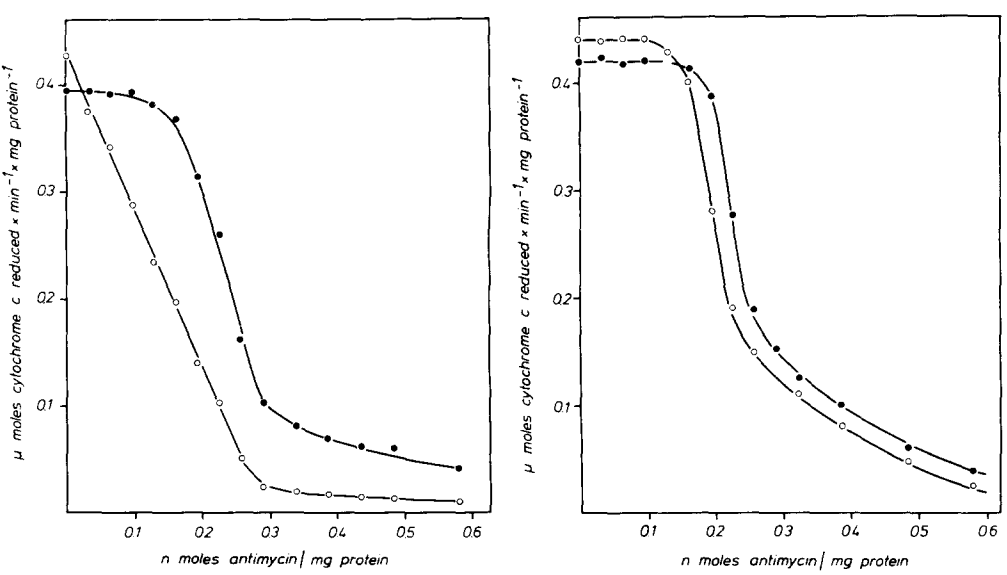


Fig. 6. Inhibition of ubiquinol-2- and ubiquinol-3 : cytochrome *c* oxidoreductase activity by antimycin. Experiments were performed as described in Methods, and the activities corrected for antimycin insensitive reaction as described under Fig. 4 (11 % of the total for ubiquinol-2; 8 % of the total for ubiquinol-3) (○, ubiquinol-2; ●, ubiquinol-3).

Fig. 7. Inhibition of ubiquinol-2- and ubiquinol-3 : cytochrome *c* oxidoreductase activity by antimycin in the presence of Lubrol WX. Experimental conditions were the same as described under Fig. 5, except that Lubrol WX (1 mg/mg mitochondrial protein) was present and preincubated together with the protein for 15 min. (Correction for antimycin insensitive activity with ubiquinol-2, 4 % of the total; and ubiquinol-3, 0.5 % of the total). (○, ubiquinol-2; ●,ubiquinol-3).

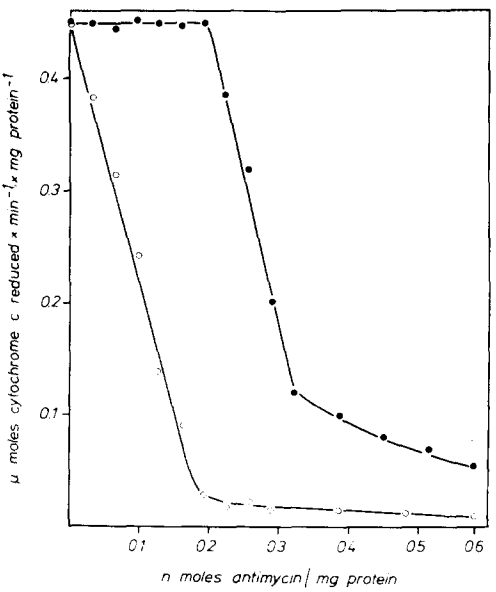


Fig. 8. Inhibition of ubiquinol-2 and ubiquinol-3 activity by antimycin with a particular preparation of mitochondrial particles. Experimental conditions described in legend for Fig. 5 were followed, except that mitochondrial particles were prepared after prolonged disintegration of the cells (45 s) and further purified by sucrose-density centrifugation as described previously [31]. The band with highest ubiquinol activity was diluted and sedimented by recentrifugation. (○, ubiquinol-2; ●, ubiquinol-3).

In mitochondrial preparations obtained from cells after prolonged disintegration, ubiquinol-2 activity was usually 2–4 times higher than ubiquinol-3 activity. Furthermore, the inhibition curve changed dramatically. An example obtained with mitochondrial particles after sucrose gradient centrifugation (performed as described in ref. 31) is given in Fig. 8. It reveals that the reduction of cytochrome *c* by ubiquinol-2 is fully inhibited by amounts of antimycin that scarcely affect ubiquinol-3 activity.

The curve with QH_2-3 as substrate is not principally affected by the prolonged disintegration except that the sigmoidicity increases with this treatment. However, QH_2-2 : cytochrome *c* oxidoreductase activity, though exhibiting the same activity as QH_2-3 : cytochrome *c* reductase (like in untreated particles) is inhibited by about half the amount of antimycin as QH_2-3 : cytochrome *c* oxidoreductase. Although this effect might be due to an artifact of preparation and may not reflect in vivo conditions, and although the 2 : 1 ratio may be accidental, it is of great interest to realize that under certain conditions, much more antimycin is needed for full inhibition of ubiquinol-3 oxidation activities than is needed for ubiquinol-2 oxidation.

Also with freshly prepared mitochondrial particles, a less antimycin sensitive portion has been observed. About 300 pmoles of antimycin inhibit the reduction of cytochrome *c* byNADH, succinate and QH_2-2 almost totally while only about 70–80% of the activity are inhibited with QH_2-3 as substrate. Another 300 pmoles of antimycin are (reproducibly) needed for full inhibition in the presence of QH_2-3.

Influence of detergents on the enzymatic activities of the bc_1-segment and on the special properties of the b-cytochromes

From the data presented we cannot exclude that electrons from ubiquinol-2 and -3 may use different pathways for electron transport. This view is confirmed by experiments with the non-ionic detergent Lubrol WX. At a concentration of 1–1.5 mg Lubrol WX per mg of mitochondrial particles, most of the succinate and NADH activity is abolished in contrast to the antimycin sensitive activity of the ubiquinols. With higher concentrations of the detergent, the activity with QH_2-2 decreases more rapidly than with QH_2-3. This result is not easily explained by the dissociation of Complex III from the Complexes I and II, due to the action of the detergent. The cytochromes *b* and c_1 are left native and reducible by substrate under these conditions, and the *b*-cytochromes do not become CO-reactive concomitantly with the loss of succinate, NADH, and QH_2-2 activity (cf. Fig. 9). The reducibility of *b*-cytochromes by substrates is, however, lower in the presence of Lubrol WX than in its absence. Concomitantly, a blue shift of about 1 nm of the absorption maximum found at 560.7 nm in the low temperature spectrum (77 °K) is observed when Lubrol WX is added (cf. Fig. 9). This indicates that this peak is composite (cf. ref. 5) and the

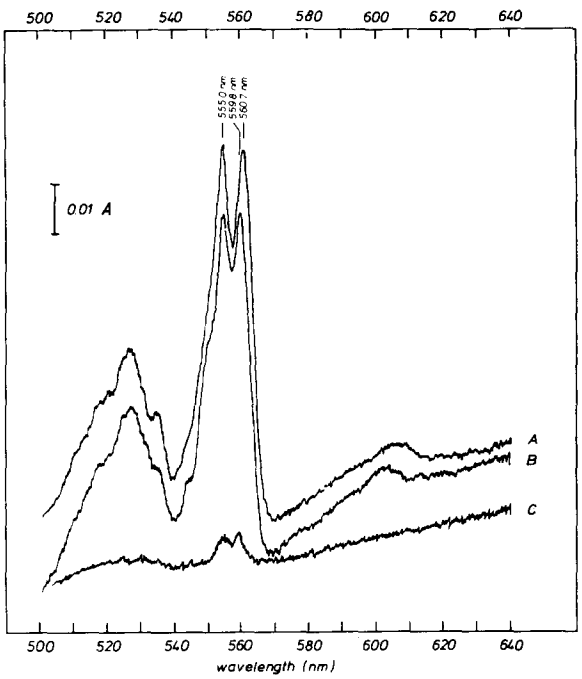


Fig. 9. Difference spectra of *b*-cytochromes at 77 °K (reduced minus oxidized) in the absence and presence of Lubrol WX and the influence of CO. Mitochondrial particles (4 mg/ml) were suspended in a buffer containing 0.6 M mannitol, 0.1 M potassium phosphate, 0.025 M Tris-HCl, 1 mM EDTA, pH 7.4. The light path was 3 mm. A: The sample was reduced by a few crystals of S_2O_4 = and the reference by 10 mM ascorbate plus 0.3 mM TMPD. In B, the sample was reduced by dithionite in the presence of 1 mg/ml Lubrol WX and measured against the same reference as A. In C, both sample and reference were reduced by dithionite in the presence of Lubrol WX, the reference being bubbled with CO for 1 min in addition.

component absorbing at higher wavelength (i.e., *b*-566) is less reducible (even by dithionite) in the presence of the detergent. Despite these obvious effects on the spectral properties of cytochrome *b*, the inhibition curve by antimycin of the ubiquinol-3 : cytochrome *c* oxidoreductase activity is not principally affected under these conditions.

Deoxycholate on the other hand, converts sigmoidal inhibition curves with NADH, succinate and QH_2-3 to linear, as was shown earlier for succinate : cytochrome *c* oxidoreductase from beef heart by Bryła et al. [32]. Simultaneously, part of cytochrome *b* is rendered CO-reactive. This effect was found by Berden [33] with preparations of Complex III from beef heart after treatment with deoxycholate, and was interpreted as a result of conformational changes occurring after the solubilization of cytochrome *b* from the membrane. An activation of cytochrome *c* reduction with NADH, succinate, QH_2-2 and QH_2-3 as substrates by low concentrations of either Lubrol WX or deoxycholate was not observed.

DISCUSSION

Ubiquinol : cytochrome *c* oxidoreductase activity is sensitive to antimycin with each of the isoprenologues tested; however, the effect of the drug concentration on the inhibition of electron transport activity differs with the length of the isoprenic side chain. The results obtained concerning the mechanism of electron flow through the bc_1-segment and its inhibition by antimycin are: (i) titration with antimycin linearily affects ubiquinol-2 : cytochrome *c* oxidoreductase activity, whereas the effect on ubiquinol-3 : cytochrome *c* oxidoreductase activity is sigmoidal. The specific activity is about the same with QH_2-2 and QH_2-3. (ii) the linear curve with QH_2-2 as substrate is converted into a sigmoidal one in the presence of Lubrol WX. (iii) NADH : cytochrome *c* oxidoreductase activity is stimulated by externally added Q-2 and Q-3. The stimulation yields an increase in sigmoidicity of the antimycin effect curve. (iv) NADH and succinate : cytochrome *c* oxidoreductase activities are differently stimulated by either Q-2 or Q-3, the latter being more effective with NADH : cytochrome *c* oxidoreductase, while succinate : cytochrome *c* oxidoreductase is stimulated to a higher extent by Q-2. The latter result is consistent with the findings of Castelli et al. [34] that the activity of NADH : ubiquinone oxidoreductase depends on the length of the isoprenic side chain of Q, while that of succinate : ubiquinone oxidoreductase does not.

The pool-function of ubiquinone

Quinones of sufficiently low potential should equilibrate with the natural Q-6 pool. In this case, a sigmoidal inhibition curve by antimycin is to be expected if the rate limiting step is before the cytochrome *b* reduction [10]. (i) If the diffusion rate of ubiquinol is rate limiting, it is expected that enlargement of the ubiquinone pool stimulates succinate- and NADH : cytochrome *c* oxidoreductase activity concomitant with a decrease in sigmoidicity of the antimycin effect curve. For NADH as substrate this is evidently not the case. (ii) If the activity of the dehydrogenases is rate limiting, stimulation by quinones might occur only in case they act as positive effectors on the dehydrogenases. However, a loss in sigmoidicity of the antimycin effect curve would be expected under these conditions, too.

When the potential of the ubiquinol externally added is considerably higher than that of the natural Q-6 so that the natural pool is by-passed as shown for duroquinol by Ruzicka and Crane [35] and by Boveris et al. [36, 37], linear antimycin effect curves will be expected as have been described by Rieske and Das Gupta [30] for ubiquinol-2 and by Kröger and Klingenberg [11] for duroquinol and dimethoxy-dimethyl-benzohydroquinone. However, no simple explanation is available for the conversion of the linear inhibition curve into a sigmoidal one by Lubrol WX, unless it is assumed that the non-ionic detergent lowers the potential of QH_2-2 considerably; but this is very unlikely. We suggest that an additional reason for the sigmoidicity is inherent to the bc_1-segment, as may be concluded from the slightly sigmoidal shapes of the inhibition curves by antimycin with NADH, or after stimulation by exogenous ubiquinone (cf. Fig. 3). The strong sigmoidicity with unstimulated succinate oxidation may then reflect the additional effect of a rate limiting step in the succinate dehydrogenase segment. Though our results confirm the pool function of the natural ubiquinone (cf. Figs 1, 2) it appears unlikely that the rate limitation by the dehydrogenase segments in combination with a mobile Q-pool, is the only reason for the sigmoidicity of the antimycin effect curve.

The allosteric model

Slater et al. [2, 12–14] have found evidence to assume that the sigmoidicity of the inhibition curve by antimycin is a consequence of a shift of an equilibrium from the non-binding T-state to the R-state in the sense of the theory of Monod et al. [15] caused by an antimycin-induced conformational change in the bc_1-segment. This model easily explains the loss of sigmoidicity in the presence of deoxycholate by the solubilizing effect of detergents destroying the units of cooperativity. If this model is correct, R-state formation in the bc_1-segment should be independent of the substrate used, and the sigmoidicity should be independent of the substrate (NADH or succinate) oxidized by the respiratory chain. This is evidently not the case, since with freshly prepared particles the antimycin effect curve is close to linear with NADH, while it is sigmoidal with succinate. The sigmoidicity of the inhibition curve increases after stimulation by both Q-2 and Q-3. It is, moreover, difficult to understand that cooperativity is established with QH_2-3 as substrate, while it is not with QH_2-2, and that the cooperativity is also induced by QH_2-2 in the presence of Lubrol WX. From our data we cannot find a support for the allosteric model.

Split electron transport chain in the bc_1-segment

Berden [33] and Wikström and Berden [16] propose a model of the bc_1-segment with a split electron transport chain. One pathway contains all the cytochromes and the binding site of antimycin, and receives its electrons from QH_2. The other branch contains the site of inhibition by BAL (2, 3-dimercaptopropanol) [38] and is fed with electrons from the QH · -radical. In terms of this model, sigmoidicity is explained by the assumption that the pathway containing the inhibition site for antimycin is much faster than the other one, dependent on the stability constant for the QH · -radical in a lipophilic environment.

Provided the model of Wikström and Berden is correct, then it seems reasonable to assume that the stability constant of the QH · -radical in a lipophilic environment decreases with the length of the isoprenic side chain. When the stability constant of

QH · -2 is much lower than that of QH · -3 and QH · -6, QH_2-2 will only use that branch of the ubiquinol : cytochrome *c* oxidoreductase which contains the cytochromes *b* and c_1 and the antimycin binding site, while QH · -3 will use the antimycin insensitive pathway, in addition. This model thus explains the linear curve with QH_2-2 and the sigmoidal ones with QH_2-3, succinate and NADH.

Not consistant with this theory is the result that the sigmoidicity of the antimycin effect curve with succinate and QH_2-3 as substrate is relatively strong while it approaches linearity with NADH. It does also not explain that under certain conditions, more antimycin is necessary for the inhibition of QH_2-3 : cytochrome *c* oxidoreductase activity than with NADH-, succinate-, or QH_2-2 : cytochrome *c* oxidoreductase activity, the specific activities with QH_2-2 and QH_2-3, however, being about the same.

Especially for the latter data we cannot exclude the possibility that different pathways for electrons might exist for QH_2-2 and QH_2-3, containing different antimycin inhibition sites, as proposed by Norling et al. [39] and modified by Wikström [19]. In addition, we suggest that the sigmoidicity of the antimycin effect is composite. The rate limitation of the segment of the dehydrogenases seems to contribute part of it, as may be concluded from the increase in sigmoidicity after the application of inhibitors of the dehydrogenases [10], or after aging. The second part of the sigmoidal effect seems, however, to be inherent to the bc_1-segment and must originate from the part of the electron transport chain after the cytochrome *b* reduction.

ACKNOWLEDGEMENTS

The authors are indebted to J. A. Berden for discussion of the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft.

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