EFFECT OF ANTIMYCIN ON THE RAPID REDUCTION OF CYTOCHROME c_1 IN THE bc_1 REGION OF THE MITOCHONDRIAL RESPIRATORY CHAIN

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1. Introduction

Antimycin A is a potent inhibitor of mitochondrial and microbial electron-transfer chains [1]; it stoichiometrically binds to the bc_1 complex of such systems [1] inducing a typical crossover in the redox state of cytochromes *b* and *c* [2]. The mechanism of action of antimycin is not yet clearly elucidated, and constitutes a key problem in the interpretation of the redox events in the bc_1 region.

Evidence against a simple linear pathway of electron transfer in this span of the mitochondrial respiratory chain has been collected by a number of experimental observations on the effects of the antibiotic, including the oxidant-induced extra-reduction of cytochromes *b* [3] and the slower reduction of cytochromes *b* when cytochrome c_1 is previously reduced [4]. The protonmotive Q-cycle of Mitchell [5] and variations thereof [6,7] or different cyclic schemes [8] have been proposed to explain the above and other experimental observations. Such schemes postulate that the first oxidant of ubiquinol is a redox center located at the cytoplasmic side of the mitochondrial membrane (generally identified as the iron–sulfur protein of the bc_1 complex [6,7,9]) which delivers electrons to cytochrome c_1 in an antimycin-insensitive way, forming on the other hand a ubisemiquinol species acting as the reductant for cytochromes *b* [5]. According to these models, antimycin inhibits steady-state electron transport to cytochrome *c* by the bc_1 complex because it prevents reoxidation of cytochromes *b* by ubiquinone [5]; however, it should not inhibit the rapid reduction of cytochrome c_1 in a single turnover when a reductant is added to the fully oxidized complex.

We have investigated the effect of antimycin on the rapid reduction of cytochromes *b* and c_1 by either succinate or ubiquinol-1 in a mitochondrial fraction enriched in the bc_1 complex. These results show that cytochrome c_1 reduction is antimycin-sensitive in the first enzymatic turnover, in contrast with the predictions of most current cyclic schemes in their present form.

Abbreviations: AA, antimycin A; DCIP, dichlorophenolindophenol; EDTA, ethylenediaminotetraacetate; Q_1, ubiquinone-1; Q_1H_2, ubiquinol-1; S_1, mitochondrial fraction enriched in bc_1 complex ('crude') succinate-cytochrome *c* reductase); TTFA, thenoyltrifluoroacetone

2. Methods

'Crude' succinate–cytochrome *c* reductase was prepared from bovine heart mitochondria [10] by the method in [11] (fraction S_1 during the isolation of the bc_1 complex [12]). Until used it was collected as a pellet and stored −70°C. Absorption spectra of cytochromes were measured in a Perkin-Elmer 559 spectrophotometer at room temperature in 0.1 M Na-phosphate buffer (pH 7.4) containing 1% deoxycholate. Ubiquinol-1 was obtained from ubiquinone-1 as in [12]. Protein was determined by a biuret method [12].

Succinate–ubiquinone-1-dichlorophenolindophenol (DCIP) activity and succinate–cytochrome *c* reductase activity was assayed as in [13]; ubiquinol-1-cytochrome *c* reductase activity was assayed as in [12]. The initial rates of DCIP and cytochrome *c* reduction were measured at 600 and 550 nm, respectively, in a Cary 15 spectrophotometer equipped with a rapid mixing apparatus with small amounts of S_1 (4–10 nM cytochrome c_1). Rapid reduction of cyto-

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chrome b and c_1 was monitored at room temperature with a Biochem Sigma ZWS11 dual wavelength spectrophotometer equipped with a stopped-flow apparatus completing mixing at a 1:1 ratio in ~3 ms. The traces were stored in the digital memory of the instrument and recorded in an $X-Y$ recorder at the desired amplification; the bandpass of the two wavelength channels was 1–1.5 nm, and the time response of the photomultiplier 5 ms. The reduction of cytochrome b was followed at 562–575 nm and that of cytochrome c_1 at 552.5–540 nm, using the extinction coefficient of 25.6 and 17.5 mM^{-1}, respectively [4]. When exogenous cytochrome c (horse heart, type III Sigma) was added to the reaction mixture, the wavelength pair was shifted to 551–540 nm to equally follow the redox changes of cytochrome c + cytochrome c_1 [14]. One syringe contained the 'crude' reductase, oxidized with 1–2 μM K-ferricyanide [14], suspended in 0.25 M sucrose, 20 mM Tris–HCl, 1 mM EDTA, 1 mM KCN (pH 7.4), containing 0.5 μg/mg protein of rotenone, at 1–3 μM cytochrome c_1; the other syringe contained 10 mM Na-succinate or different concentrations of ubiquinol-1 dissolved in the same buffer.

Antimycin A (AA) from Sigma was dissolved in ethanol at 0.1–1 mM, and its concentration was controlled spectrophotometrically using the extinction coefficient of 4.8 mM^{-1} at 320 nm [15].

3. Results

Fraction S_1 is a crude concentrate of the mitochondrial inner membrane essentially devoid of ATPase, cytochrome c and cytochrome oxidase, displaying a 3–4-fold enrichment in the bc_1 complex with respect to mitochondrial particles [12]. Our preparations of S_1, in fact, contain 0.6–0.8 nmol cytochrome c_1/mg protein and 1.2–1.5 nmol cytochrome b/mg protein, with almost undetectable traces of cytochrome oxidase. In the presence of KCN to block these contaminations, and of rotenone to inhibit complex I, which is also present in S_1 [11], this fraction can be well considered as a 'crude' succinate-cytochrome c reductase preparation. In parallel to the enrichment in the bc_1 concentration, in fact, all the specific electron-transfer activities of the succinate-cytochrome c region are increased >3-fold (table 1), reaching values not much different from those usually reported with isolated succinate-cytochrome c reductase prepared by other methods [4]. The activities are TTFA and/or antimycin sensitive; furthermore the turnover numbers of succinate-cytochrome c reductase and of Q_1H_2–cytochrome c reductase, calculated from initial rates approaching zero-order kinetics at high substrate concentrations (18–22 s^{-1} and 70–150 s^{-1}, respectively), are even higher than those found in intact mitochondria. Such 'crude' reductase preparation is particularly suitable for stopped-flow techniques, which require large amounts of materials, since it can be obtained with a high yield [12] and presents much lower turbidity than intact mitochondrial particles.

Table 1
Electron transfer activities of the 'crude' succinate-cytochrome c reductase[a] (S_1)

Activity	Rates (μmol . min^{-1}, mg $protein^{-1}$)	Sensitivity to TTFA	AA
Succinate-Q_1-DCIP[b]	0.54	≥95%	–
Succinate-cytochrome c[c]	0.62	≥95%	90–95%
Q_1H_2-cytochrome c[d]	2.52	–	~100%

[a] Final [cytochrome c_1] concentration of a frozen and thawed S_1 preparation was 5.7 nM
[b] With 110 μM Q_1, 40 μM DCIP and 5 mM succinate
[c] With 16 μM cytochrome c and 5 mM succinate
[d] With 16 μM cytochrome c and 57 μM Q_1H_2

In the stopped-flow experiments the enzyme is working under substrate-like conditions, and its reduction is expected to follow kinetics of order higher than zero and to be slower than the turnover of the enzyme when the cytochrome c acceptor is present. In fact addition of succinate to the fully oxidized reductase (fig.1A) causes reduction of cytochrome c_1, which is completed with half-times of 200–250 ms and appears to be first order in cytochrome c_1. The reduction of cytochromes b is slower and only partial, and apparently occurs when cytochrome c_1 is already reduced (fig.1B). Antimycin A preincubated at 3:1 ratio with cytochrome c_1 induces a 78% inhibition of the initial rate of cytochrome c_1 reduction, as shown in fig.1A, but does not alter the maximal extent of reduction, which is reached at much longer times than in the absence of the inhibitor. On the other hand, the same antimycin concentration enhances the extent of cytochrome b reduction speeding up its rate by a factor >10 (fig.1B). In

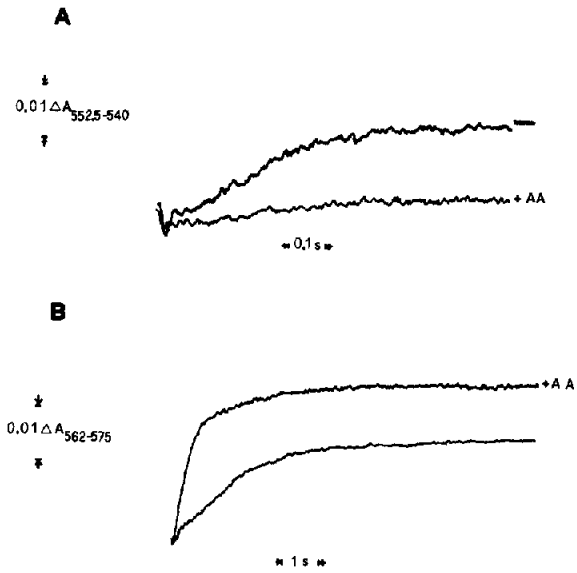

Fig.1. (A) Rapid reduction of cytochrome c_1 by succinate (final [cytochrome c_1] 1.14 μM). The lower trace refers to the reduction pattern obtained in the presence of 3 mol AA/mol cytochrome c_1 (+AA). The upper separated line indicates the maximal extent of reduction, coincident to that obtained with ascorbate; such extent was identical for the 2 traces. (B) Reduction of cytochromes *b* in the absence (lower trace) and in the presence (upper trace, +AA) of 3 mol AA/mol cytochrome c_1 under the same conditions as in A. The maximal extents of reduction correspond to the final level of the traces.

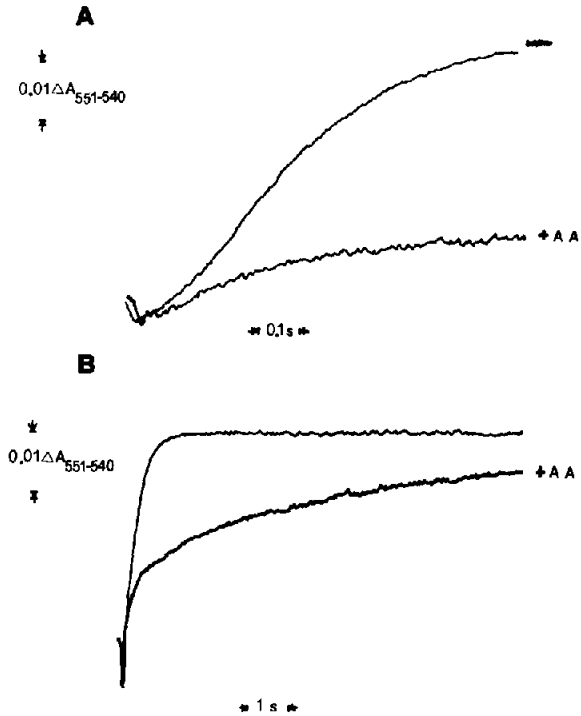

Fig.2. (A) Reduction of cytochrome c_1 (1.14 μM) plus 1.23 μM cytochrome *c* in the absence (upper trace) and in the presence (+AA) of 6 mol AA/mol cytochrome c_1. The recorded time-scale is 1 s. (B) Same conditions as in (A) but with a recorded time-scale of 10 s. The maximal extent of reduction correspond for both traces to the final level of the upper trace.

the presence of the inhibitor the biphasic behaviour of cytochrome *b* reduction becomes more clear, in accordance with [7,16].

Addition of stoichiometric amounts of cytochrome *c* to S_1 results in a different reduction pattern of cytochromes $c + c_1$, which is no more monotonic as with cytochrome c_1 alone (cf. fig.1A), remaining antimycin-sensitive as before (fig.2); even with high antimycin/cytochrome c_1 ratios, however, the slower reaction proceeds until reaching the same extent of reduction, as it is better shown using a larger time-scale (fig.2B).

We have titrated the antimycin inhibition of the steady-state activity of succinate-cytochrome *c* reductase of S_1 under the same assay conditions employed in the stopped-flow experiments. The inhibition curve appears to be sigmoidal (fig.3), in accordance with the typical sigmoidal inhibition of succinate cytochrome *c* reductase and succinate oxidase activities in mitochondrial particles [17]. The extent of inhibition

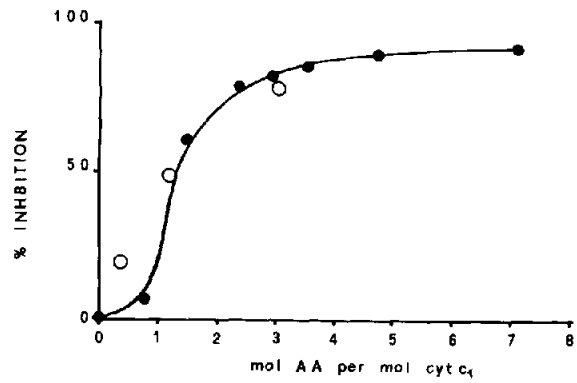

Fig.3. Inhibition curve of succinate-cytochrome *c* reductase activity in S_1 with 5 mM succinate and 16 μM cytochrome *c*. Antimycin was preincubated with an S_1 suspension containing 1.76 μM of cytochrome c_1 at different concentrations, and small aliquots of the reductase were then added to the assay mixture to a final cytochrome c_1 level of 4.4 nM. (●——●) Inhibition curve of the initial rates of succinate-cytochrome *c* reductase activity; (○) extents of inhibition of the initial rates of cytochrome c_1 reduction with the same S_1 preparation as monitored by stopped-flow experiments.

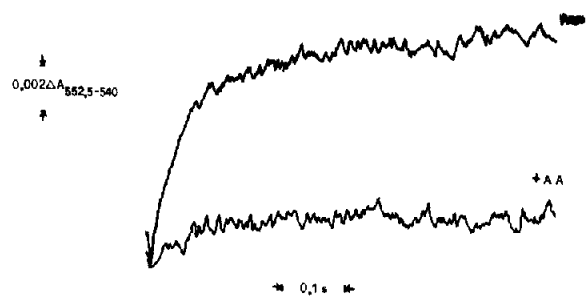

Fig.4. Rapid reduction of cytochrome c_1 by ubiquinol-1. The final concentrations are 0.57 μM and 13 μM for cytochrome c_1 and Q_1H_2, respectively. The lower trace (+AA) represents the reduction pattern obtained in the presence of 5 mol AA/mol cytochrome c_1 (~86% inhibition of the initial rate). The upper separated line indicates the maximal extent of reduction, which was reached in a few seconds in the absence of the inhibitor, but at much longer times in the presence of AA.

of the initial rates of cytochrome c_1 reduction appear close to those observed in the steady-state activity of the reductase (fig.3).

The antimycin titer of ubiquinol-1 cytochrome *c* reductase activity in S_1 is somewhat lower than for succinate-cytochrome *c* reductase activity, with a linear curve of inhibition [18]. The rapid reduction of cytochrome c_1 with ubiquinol-1 as the electron donor to the 'crude' reductase is again almost completely antimycin sensitive (fig.4).

4. Discussion

We have shown by fast kinetics that antimycin inhibits reduction of cytochrome c_1 by both succinate and ubiquinol-1 in the first turnover of a mitochondrial fraction enriched in bc_1 complex. This effect agrees with [19] which shows that antimycin at concentrations equimolar with cytochrome *b* inhibits cytochrome c_1 reduction by ~90% in a purified succinate-cytochrome *c* reductase preparation. Also in agreement with [19], we have found that the reduction of cytochrome *b* by saturating succinate levels occurs after cytochrome c_1 is reduced (cf. fig.1). The antimycin inhibition of cytochrome c_1 reduction is strongly in contrast with the data [14], which were, however, obtained without stopped-flow techniques, thus missing the initial stages of the redox changes.

The extents of inhibition of the initial rates of cytochrome c_1 reduction are roughly coincident with those of subsequent turnovers, and of the steady-state succinate-cytochrome *c* reductase activity (cf. fig.3). This indicates that the inhibitory effect on the electron transfer to cytochrome c_1 is responsible for the inhibition of the steady-state redox cycles of bc_1 complex. The reduction pattern of cytochrome $c + c_1$ is no more of first-order than of cytochrome c_1 alone, becoming of sigmoidal type (cf. fig.2A); such a phenomenon, possibly related to the pre-steady-state kinetics of the enzyme, is presently under investigation in our laboratory.

Under the same assay conditions cytochrome *b* reduction is highly stimulated by antimycin (cf. fig.1B), thus excluding that the antibiotic effect on cytochrome c_1 reduction is secondary to inhibition of cytochromes *b*. It is well known that antimycin enhances the reducibility of cytochromes *b* when the iron–sulfur center of the bc_1 complex and cytochrome c_1 are fully oxidized, whereas the cytochrome *b* reduction is slowed, in the presence of the inhibitor, when such redox centers are previously reduced [16], or when the iron–sulfur protein is lacking or damaged [7,9]. The results of this investigation are not readily explained by any cyclic model in which antimycin inhibits reoxidation of cytochrome(s) *b* in a pathway not directly linked or subsequent to cytochrome c_1 reduction. Although our data do not allow us to draw an alternative scheme, they suggest that the binding of antimycin to the bc_1 complex induces a block of the electron transfer leading to cytochrome c_1 either in a linear sequence, or in a cyclic system where the reduction of cytochrome c_1 does not precede that of cytochromes *b*. The slower reduction of cytochromes *b* with respect to that of cytochrome c_1 by succinate in the absence of antimycin (cf. fig.1) could be due to the fact that succinate dehydrogenase is rate-limiting in the electron flow to cytochrome c_1 [1,4]. If cytochromes *b* are located in an intermediate position of the electron transfer occurring in the first turnover of bc_1 complex, their relatively slow reduction driven by succinate would be balanced by their reoxidation for reducing cytochrome c_1, until the terminal acceptor becomes fully reduced. A transient reduction of cytochromes *b*, followed by rapid reoxidation concomitant to cytochrome c_1 reduction, has been observed [19]. In support of such a view, we have found that cytochrome *b* reduction by ubiquinol-1 in S_1 is faster than that of cytochrome c_1.

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References

[1] Rieske, J. S. (1980) Pharmac. Ther. 11, 415–450.
[2] Chance, B. (1952) Nature 169, 215–221.
[3] Wikström, M. and Berden, J. A. (1972) Biochim. Biophys. Acta 283, 403–420.
[4] Trumpower, B. L. and Katki, A. (1979) in: Membrane Proteins in Energy Transduction (Capaldi, R. A. ed) pp. 89–206, Dekker, New York.
[5] Mitchell, P. (1975) FEBS Lett. 56, 1–6.
[6] Bowyer, J. R. and Trumpower, B. L. (1981) in: Chemiosmotic Proton Circuits in Biological Membranes (Skulachev, V. P. and Hinkle, P. C. eds) pp. 105–122, Addison-Wesley, New York.
[7] Van Ark, G., Raap, A. K., Berden, J. A. and Slater, E. C. (1981) Biochim. Biophys. Acta 637, 34–42.
[8] Wikström, M. and Krab, K. (1980) Curr. Topics Bioenerg. 10, 51–101.
[9] Trumpower, B. L. (1981) Biochim. Biophys. Acta 639, 129–155.
[10] Smith, A. L. (1967) Methods Enzymol. 10, 81–86.
[11] Hatefi, Y. and Stiggall, L. D. (1978) Methods Enzymol. 53, 5–10.
[12] Rieske, J. S. (1967) Methods Enzymol. 10, 239–245.
[13] Hatefi, Y. and Stiggall, L. D. (1978) Methods Enzymol. 53, 21–27.
[14] Bowyer, J. R. and Trumpower, B. L. (1981) J. Biol. Chem. 256, 2245–2251.
[15] Strong, F. M., Dickie, J. P., Loomans, M. E., Van Tamelen, E. E. and Dewey, R. S. (1960) J. Am. Chem. Soc. 82, 1513–1514.
[16] Eisenbach, M. and Gutman, M. (1975) Eur. J. Biochem. 52, 107–116.
[17] Slater, E. C. (1973) Biochim. Biophys. Acta 301, 129–154.
[18] Rieske, J. S. and Das Gupta, U. D. (1972) FEBS Lett. 20, 316–320.
[19] Jin, Y. Z., Tang, H. L., Li, S. L. and Tsou, C. L. (1981) Biochim. Biophys. Acta 637, 551–554.