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REACTIONS OF *b*-CYTOCHROMES WITH ATP AND ANTIMYCIN A IN PIGEON HEART MITOCHONDRIA

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SUMMARY

In pigeon heart mitochondria antimycin A induces a red shift of less than 1 nm in the λ_{max} of ferrocytochrome b_K ($E_{m7.0}$ +40 mV; λ_{max} 561 nm) but not of ferrocytochrome b_T ($E_{m7.0}$ −30 mV; λ_{max} 565 nm). Antimycin A inhibits the ability of ATP to induce a measurable high-potential cytochrome b_T. The stoichiometry of these interactions is one antimycin A per cytochrome b_K or cytochrome b_T. Antimycin A binding to mitochondrial membranes elicits no significant alteration of the oxidation–reduction midpoint potential of either cytochrome b_K or b_T in the resting state. No spectrophotometric alterations were detected in either ferrocytochrome *b* on addition of ATP.

INTRODUCTION

The problem of spectral, kinetic and oxidation–reduction properties of the mitochondrial *b*-cytochromes has been the subject of much study and controversy, especially with respect to their interactions with adenosine triphosphate and the antibiotic, antimycin A (see ref. 1). Further characterization[2–6] of the discovery[7] of multiple forms of cytochrome *b* in mitochondria has excited further investigation into these problems.

Effects with ATP

Chance and co-workers[8–10] reported a *b*-cytochrome the properties of which were dependent on the energy state of the mitochondria. More recently, Wikström[2] described the spectrum of ATP-induced cytochrome *b* reduction; two maxima were observed, one at 557 nm and the other at 566 nm. Slater and co-workers[11] have interpreted the ATP-induced absorbance maximum at approximately 565 nm as a red shift in the spectrum of a reduced cytochrome *b* (refs 12, 13). Wilson and Dutton[3] and Chance *et al.*[14] reported the oxidation–reduction potential properties of one *b*-cytochrome of various mitochondrial preparations to be "energy dependent": in the presence of ATP its measured midpoint potential (pH 7.2) is approx. + 240 mV, while in the resting state its midpoint appears to be close to − 30 mV. In contrast to the interpretation of Slater and associates, evidence derived from studies with pigeon heart mitochondria[5, 14, 15, 36] has suggested this cytochrome *b* (designated cytochrome b_T) has a reduced absorption maximum at 565 nm under both energized

and unenergized conditions. Another cytochrome *b* (designated cytochrome b_K) with an absorption maximum at 561 nm was reported to have a midpoint potential nearer +30 mV at pH 7.2 which is not affected by the energy state of the system.

Effects with antimycin A

Cytochrome *b* in submitochondrial particles which is reduced by substrate in the presence of antimycin A has been reported as differing from that in the absence of antimycin A (refs 6, 11, 16–20, 24). Slater *et al.*[11], Wegdam *et al.*[12], Bonner and Slater[13] and Bryla *et al.*[18] recently reported that the antimycin A induces a 2–3 nm red shift in the reduced spectrum (which also generates a λ_{max} at 565 nm) of one of the *b*-cytochromes. However, unlike the antimycin red shift described by Pumphrey[17], the reported shift was very much diminished when all the *b*-cytochromes were essentially reduced in the presence of dithionite. Sato *et al.*[5,15] have verified the existence of the antimycin A-induced red shift, but reported that it is confined to cytochrome b_K, and, like that reported by Pumphrey[17], the extent of the shift is very slight.

This communication attempts to further clarify in spectrophotometric and potentiometric terms, the relationships of the mitochondrial cytochrome *b* complex with antimycin A and ATP.

MATERIALS AND METHODS

Pigeon heart mitochondria were prepared by the method of Chance and Hagihara[21]. Cytochromes were assayed by simlutaneous readout of absorbance (dual wavelength spectrophotometer) and oxidation–reduction potential (platinum electrode in conjunction with a calomel reference electrode) in an anaerobic cuvette[22] as previously described[3,4]. The oxidation–reduction potential was made more electropositive with 100 mM potassium ferricyanide and more electronegative with a freshly prepared, dilute solution of sodium dithionite. In aerobic experiments, O_2 consumption was measured polarographically with a Clark electrode. Computer analysis of oxidation–reduction potential titrations was performed with a Digital Equipment Corporation PDP6 Computer (N.I.H. Biotechnology Resources RR15). The program treated the absorbance increase due to cytochrome reduction as the dependent variable. A best fit curve was computed for one, two or three cytochrome ($n = 1$) components of unknown midpoint potentials and unknown relative contribution to the overall absorbance change encountered during the titration. A best fit value for the 100 % oxidation absorbance level was also computed because, in this case, it was not possible to obtain a clear value for the start of the titration at high potentials.

RESULTS

In situ oxidation–reduction potentials of the b-cytochromes at pH 7.0

Fig. 1A is a computer printout of the actual experimental data obtained for the course of oxidation and reduction of the *b*-cytochromes in pigeon heart mitochondria. The absorbance increase at 562 nm with respect to 575 nm is plotted against increasing electronegative potentials. Points from two titrations are presented. Fig. 1B shows the absorbance data presented as the logarithm of the ratio of oxidized

to reduced cytochrome. The slight sigmoidicity suggests the presence of two cytochrome components with fairly close midpoint potentials. In the past (see refs 3, 4, 23) we have used the inflexion point as a guide to separate the two components by assuming that at this point (in this case at about 0 mV) the lower component was 100 % oxidized and the upper component was 100 % reduced. For components separated in midpoint potentials by, for example, only 60 mV this is clearly not the case; Fig. 1C shows that the points as separated in this manner deviate from the expected $n = 1$ curves as the inflexion point is approached. Fig. 1D shows the best fit for full resolution of two cytochrome components determined by the computer, programmed as given in the Materials and Methods section. The points fit well to the theoretical lines drawn. They are computed for two one-electron acceptors with oxidation–reduction midpoint potentials (pH 7.0) of +43 mV (cytochrome b_K) and −27 mV (cytochrome b_T) which contribute 75 % and 25 %, respectively, to the overall absorbance change recorded at 562–575 nm. The sum of the squares deviation for the points from the two titrations was 18.4, showing the fit to be good. Very poor or nonsense fits were obtained when the curve was analyzed for one or three components.

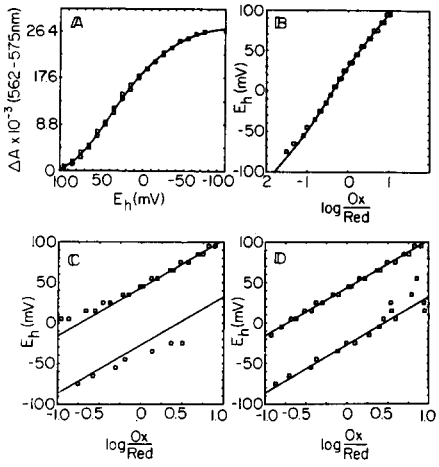

Fig. 1. Computer analysis of the course of oxidation–reduction of *b*-cytochromes in pigeon heart mitochondria. The mitochondria (2.4 mg protein/ml) were stirred under an Ar atmosphere (less than 1 ppm O_2) in 0.2 M mannitol, 0.05 M sucrose, 0.05 M morpholino-propanesulphonate (MOPS) buffer pH 7.0. The following redox mediators were added: 20 μM diaminodurol (DAD), 40 μM each of phenazine methosulphate (PMS), phenazine ethosulphate (PES) and duroquinone, 6 μM pyrocyanine and 15 μM 2-hydroxy-1,4-naphthaquinone. The measuring wavelengths were 562–575 nm. (A) Absorbance change as a function of measured oxidation–reduction potential. (B) Absorbance data replotted as the logarithm of the ratio of oxidized to reduced cytochrome. (C) Resolution of Curve B into two components by simple arithmetic after estimating that the inflexion point of Curve B was 0 mV and approximating that it represented the 100 % oxidized or reduced points of the more negative and positive components, respectively. (D) Full resolution of the two components.

This is as far as seems reasonable to take such analysis at this time, since it was necessary for the computer to choose the 100 % oxidized absorbance position because experimentally this is not attained. This is because with intact pigeon heart mitochondria a slight net absorbance decrease is often encountered as the potential is made more electronegative in the 100 mV region indicating the existence of another

component with a negative contribution. When this additional component is not taken into account (as in Fig. 1 of ref. 23) the points deviate from the expected $n = 1$ line at the positive end of the titration. Further, in phosphorylating submitochondrial particles of pigeon heart[23] where this absorbance decrease is not apparent (possibly due to the removal of the component in question), a third *b*-cytochrome is revealed [λ_{max} 558 nm, E_m approx. +120 mV].

In comparison with our earlier analyses (see Introduction) the values obtained by computer are not very different. The midpoint potential of cytochrome b_K is some 10 mV higher; this arises from the computed absorption correction for the 100 % oxidized value (0.0044 absorbance unit added at the positive end of the titration of overall experimental absorbance changes 0.054 unit).

There is a difference in the computed relative contributions of the two cytochromes to the overall absorbance change when compared to the simple arithmetic treatment which reveals 55–65 % cytochrome b_K contribution to the total absorbance change at 562–575 nm. The computer analysis which suggests a cytochrome b_K contribution of 75 % arises from the added correction.

Potentiometric and spectrophotometric analysis of the b-cytochromes in the absence and presence of ATP

In the absence of ATP, the spectrum (Fig. 2) of the absorbance changes generated from oxidation–reduction potential +300 mV to +130 mV is typical of the reduced *minus* oxidized cytochrome $c + c_1$ only; from +130 mV to −180 mV the spectrum is comprised of both reduced *b*-cytochromes (b_K and b_T).

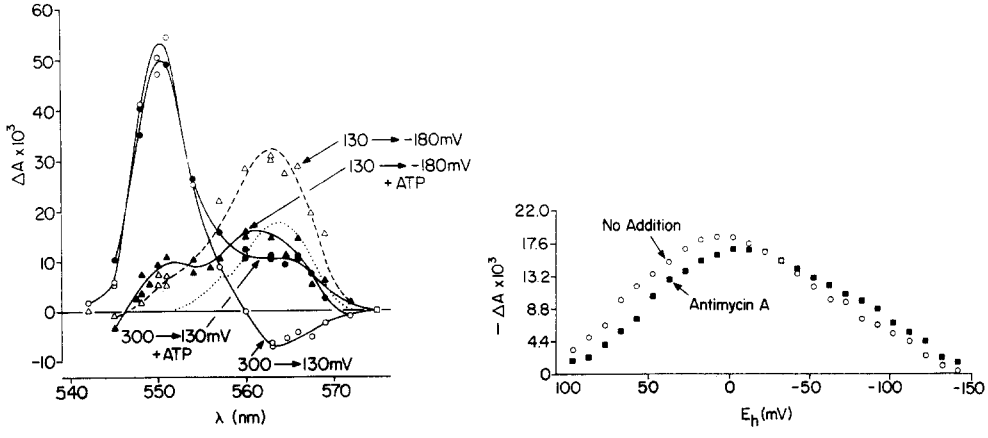

Fig. 2. Spectra of the cytochromes of pigeon heart mitochondria in the absence and presence of ATP. The mitochondria (3.1 mg protein/ml) were under similar conditions described in Fig. 1. The reference wavelength was 575 nm. In the absence of ATP: ○, absorbance change from +300 mV to +130 mV (cy ɔchrome $c + c_1$); △, absorbance change from +130 mV to −180 mV (*b*-cytochromes). In the presence of 6 mM ATP: ●, absorbance change from +300 mV to +130 mV (cytochromes $c + c_1$, and b_T); ▲, absorbance change from +130 mV to −180 mV (cytochrome b_K). ·····, subtraction of (●) values from (○) values, thus producing high-potential cytochrome b_T; – – –, sum of cytochrome b_K (▲) and the high-potential cytochrome b_T (·····). – – – follows very closely to the points (△) derived for cytochromes b_T and b_K in the absence of ATP.

Fig. 3. The course of oxidation–reduction of the *b*-cytochromes of pigeon heart mitochondria employing 566–560 nm as the measuring wavelength pair. Other conditions were as described in Fig. 1. Where indicated 1.0 nmole/mg protein antimycin A was present.

In the presence of ATP, the spectrum obtained from +300 mV to +130 mV now includes, besides the cytochromes $c + c_1$ contribution, high-potential cytochrome b_T. By subtracting the contribution from cytochrome $c + c_1$ obtained in the absence of ATP we obtain the spectrum (dotted line) of the ATP-induced high-potential cytochrome b_T; its λ_{max} is 565 nm and has a slight shoulder at 557. The midpoint of b_K is unaffected by the ATP and hence becomes reduced in the +130 mV to −180 mV potential range; it has a λ_{max} at 561 nm.

Summating the spectra of cytochromes b_K and b_T, separated in the presence of ATP, produces the dashed line which fits the experimental points (△) obtained by reduction of both these *b*-cytochromes in the absence of ATP. This suggests that ATP induces no major shifts to longer wavelengths of the α-bands of either ferrocytochromes b_K or b_T.

That cytochrome b_T is intrinsically a "longwavelength" *b*-cytochrome and hence is not a manifestation of an ATP-induced red-shift (*cf.* refs 11–13) is more clearly demonstrated in Fig. 3. The absorbance changes are measured with no additions using the wavelength pair 566 nm *minus* 560 nm and plotted as a function of oxidation–reduction potential. As the potential is made more negative from +100 mV a net absorbance decrease is revealed. At +5 mV the absorbance decrease now becomes a net increase as the potential is made more negative; below −150 mV no further changes are observed. This behavior is entirely that expected from the above described properties of cytochromes b_K and b_T. From +100 to +5 mV the reduction of cytochrome b_K ($E_{m7.0}$ +40 mV; λ_{max} 561 nm) is dominant and thus there is a net absorbance decrease measured at 566 nm with respect to 560 nm. However, as 5 mV is approached, the reduction of cytochrome b_T ($E_{m7.0}$ −30 mV; λ_{max} 565 nm) becomes dominant resulting in the observed net absorbance increase. The fact that there is little overall absorbance difference between +100 and −150 mV is consistent with the two cytochromes providing similar reduced *minus* oxidized absorbance contributions to the changes at 560 nm (mainly cytochrome b_K) and 566 nm (mainly cytochrome b_T). It would therefore be expected that the maximum net absorbance change in the titration be at approximately +5 mV, midway between the two measured midpoint potentials.

The effect of antimycin A on the properties of the b-cytochromes in the absence and presence of ATP

Fig. 3 also shows the oxidation–reduction potential titration done at 566 nm *minus* 560 nm in the presence of antimycin A. These measuring wavelengths are sensitive to any cytochrome *b* spectral or potential alterations. If, for instance, in the presence of antimycin A the λ_{max} of cytochrome b_K shifted 2 nm to 563 nm, its contribution to the 566 *minus* 560 nm absorbance difference on oxidation–reduction would be zero (assuming asymmetrical absorbance band); thus a very different pattern of absorbance change would be observed on lowering the potential from +100 mV to −150 mV; there would only be an absorbance increase observed as cytochrome b_T was reduced. Also if the midpoints of either cytochrome changed more than a few mV this would be immediately apparent. For example, in the special event of the two cytochromes assuming the same midpoint potential essentially no absorbance changes would be observed during the titration.

Since a very similar curve is generated as obtained in the absence of antimycin

A it may be concluded that antimycin A induces no major modifications in either the spectrophotometric or oxidation–reduction potential properties of either *b*-cytochrome. A difference does exist, however, in a slight but consistent diminution of the absorbance increase which occurs from +100 mV to 0 mV when absorbance changes due to cytochrome b_K reduction are dominant. Such an effect may originate from the antimycin A-induced spectrophotometric red-shift which has recently been attributed uniquely to a minor shift in the ferrocytochrome b_K (refs 5, 15). The actual extent of this shift appears to be less than 1 nm (see Discussion).

The antimycin A dependence of the minor red shift of the ferrocytochrome b_K has been examined to determine the relationship of the interaction. This is shown in Fig. 4. The course of appearance of the red shift throughout the titration is linear with respect to the added antimycin A. This is indicative (see refs 17, 28) of the high affinity of the antimycin A with its site of binding. The stoichiometry of the interaction given by the break in the curve indicates the shift is induced on the basis of one mole of antimycin A per mole of cytochrome b_K heme (assuming 0.2–0.25 nmole cytochrome b_K heme per mg protein).

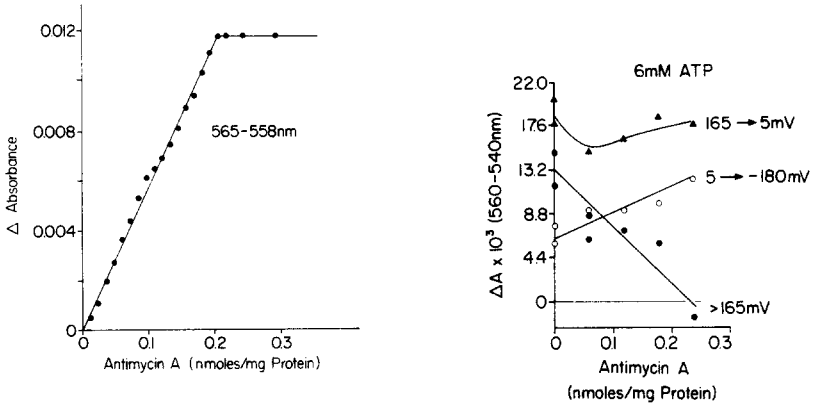

Fig. 4. The effect of antimycin A on the magnitude of the red shift of cytochrome b_K in pigeon heart mitochondria reduced by dithionite. Mitochondria (12.5 mg protein/ml) were suspended in 0.22 M mannitol, 0.075 M sucrose, 0.2 M EDTA, 50 mM Tris–HCl, pH 7.4 and supplemented with 5 mM succinate and 5 mM glutamate. The experiment was performed in a split-beam spectrophotometer using open cuvettes (1 nm light path). Excess sodium dithionite was added to both cuvettes which maintained all the cytochromes in a reduced state during the experiment. Varying quantities of antimycin A were added to the sample cuvette and the induced absorbance difference between 558 and 565 nm recorded.

Fig. 5. The course of inhibition by antimycin A of the ATP-induced high-potential cytochrome b_T in pigeon heart mitochondria. The mitochondria (2.8 mg protein/ml) were under similar conditions as described in Fig. 1. The suspension was set at +165 mV and then 6 mM ATP was added. The ATP induced reduction observed here was due to the high-potential cytochrome b_T (●). The oxidation–reduction potential of the same sample was lowered from +165 mV to +5 mV to give an absorbance change resulting mainly from the reduction of cytochrome b_K (▲). The absorbance increase from +5 mV to −180 mV (○) resulted from the reduction of any low-potential cytochrome b_T plus some cytochrome b_K. A separate run was performed for each level of antimycin A examined. Antimycin A was preincubated for 6 min before commencement of the experiment. Each run took approx. 20 min; this limited the number of points taken in any experiment. To avoid a systematic error resulting from any deterioration of the mitochondria over the 3–4-h experimental period the runs at different antimycin concentration were performed out of numerical sequence.

The effect of antimycin A on the ability of ATP to generate a measured high-potential form of cytochrome b_T is described in Fig. 5. In this experiment the oxidation–reduction potential was initially poised at +165 mV. The extent of cytochrome *b* reduction observed at this potential on addition of ATP gives the amount of high-potential cytochrome b_T formed. Subsequent lowering of the potential to 5 mV gives absorbance changes which are predominantly cytochrome b_K (and some of any existing low-potential cytochrome b_T). The absorbance change from 5 mV to −180 mV, gives a measure of the amount of cytochrome b_T existing in the low-potential form (*plus* a fixed contribution from cytochrome b_K, since its midpoint is unaffected by added ATP). Increasing amounts of antimycin A progressively inhibit the formation of the ATP-induced measured high-potential cytochrome b_T with an appropriate increase in amount of the low-potential "unenergized" form of cytochrome b_T measured between 5 mV and −180 mV. The amplitude of absorbance increase generated from +165 mV to 5 mV (mainly cytochrome b_K) appears only slightly affected. Thus, antimycin A prevents the formation of the measured ATP-induced high-potential form of cytochrome b_T. The endpoint of the titration (approx. 0.25 nmole antimycin A per mg protein) suggests that the inhibition is affected with approximate stoichiometry of one molecule of antimycin A per cytochrome b_T heme, which in this case is the cytochrome assayed.

Fig. 6 describes the influence of antimycin A on the state of reduction of the *b*-cytochromes and the rate of O_2 consumption performed in an open cuvette. The results are in accord with similar previously reported steady state experiments[11–13,16,17,24,25]. These also revealed sigmoid curves of antimycin inhibition of O_2 consumption in States 3 and 4 and the steady state level of cytochrome *b* reduction in the uncoupled state. Much emphasis with regard to energy conservation mechanisms has been placed on the curve shapes of antimycin A interactions with the respiratory chain under steady state conditions[18]. Kröger[33] (see also refs 34,35) has pointed out

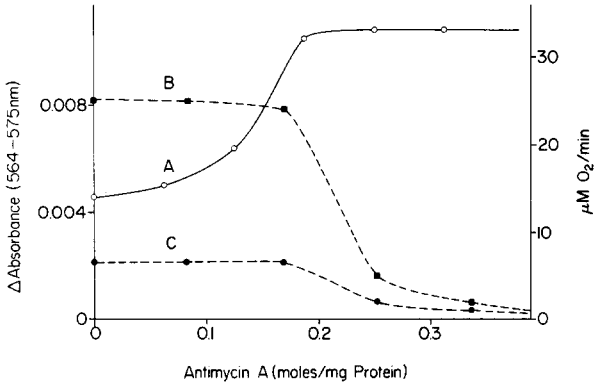

Fig. 6. The effect of antimycin A on the extent of steady state cytochrome *b* reduction (○) and the rate of oxygen uptake (■ and ●) in pigeon heart mitochondria. The mitochondria (1.1 mg protein/ml) were suspended in 0.22 M mannitol, 0.075 M sucrose, 0.2 mM EDTA, 50 mM Tris–HCl, pH 7.4. (A) The medium contained 5 mM cyanide, 5 mM ascorbate, 80 μM tetramethyl-*p*-phenylenediamine, 4 μM rotenone and 5 mM succinate; under these conditions cytochrome b_K is mainly reduced and cytochrome b_T is mainly oxidized. Antimycin A was then added and the absorbance change recorded. (B) Antimycin A was added to mitochondria in State 3; 5 mM succinate, 5 mM glutamate, 1 mM ADP and 2 mM phosphate were present. (C) Antimycin A was added to mitochondria in State 4; 5 mM succinate and 5 mM glutamate were present.

that the variation between linear, hyperbolic and sigmoidal curves could rest on kinetic parameters associated closely with the site of antimycin A binding with which the antibiotic has a high affinity as shown in Fig. 4.

DISCUSSION

Directives which emerge from this study on the interaction of the *b*-cytochromes with the antibiotic antimycin A and ATP lead to the following conclusions. Two forms of cytochrome *b* (designated b_K and b_T) are present in similar quantities in the mitochondrial respiratory chain. They can be readily differentiated by their physical properties in mitochondria in the resting state.

In functional terms, it has previously been reported[3,14] that the midpoint potential of cytochrome b_T in the presence of ATP assumes a measured value some 270 mV more electropositive; that of cytochrome b_K apparently remains unaltered. The "energy dependence" of the measured midpoint potential of cytochrome b_T has been the basis for the consideration that this cytochrome acts as a transducer of electrochemical energy into chemical energy at Site II in the mitochondrial respiratory chain[3,14,36] (but see refs 37–40). There appears to be no significant ATP-induced modification of the spectrum of the reduced α-band of cytochrome b_T (or b_K). This conflicts with other reports[11–13].

In concert with a recent report by Sato *et al.*[5,15], antimycin A does appear to influence the reduced absorption spectrum of cytochrome b_K; the red-shift so affected was reported by these workers as representing an absorbance difference between the trough at 558 nm and a peak at 565 nm which was less than 8% of that obtained for the reduced *minus* oxidized difference spectrum of cytochrome b_K. It can readily be estimated from these data with the reasonable assumption that the full band width at half-height of the cytochrome is 12 nm (ref. 26) that the actual extent of the observed spectrophotometric red-shift is markedly less than 1 nm. It seems reasonable to consider that this shift is the same as that observed by Pumphrey[17] in dithionite-reduced submitochondrial particles.

In view of the measured properties of cytochromes b_T and b_K some doubt therefore may be cast on the reliability of the wavelength pairs similar to 566 nm *minus* 560 nm (Fig. 3) as a unique measure of spectrophotometric red-shifts (*cf.* refs 11–13, 18).

Fig. 3 also confirms the finding measured directly[27] that antimycin A elicits no detectable midpoint potential alteration in cytochrome b_K or b_T. This implies that whatever is the ultimate effect of antimycin A, it is equal with respect to both the oxidized and reduced forms of the *b*-cytochromes; this applies at least to the experimental conditions used; the measurements were made under conditions where no net electron flux through the *b*-cytochromes is evident (*cf.* below).

It still cannot be stated that antimycin A reacts directly with a *b*-type cytochrome (see also refs 28, 30). However, the reaction with the mitochondrial component in question must account for the observed red-shift in the reduced spectrum of cytochrome b_K and also the prevention of the ATP induction of the measured high-potential cytochrome b_T. The titration of these two phenomena yielded an approximate 1:1 relationship between the component which reacts with the antibiotic and either cytochrome b_K or b_T, and hence an overall relationship of one antimycin A molecule

per two *b*-cytochrome hemes which is in agreement with previous findings[28,29].

Two facts seem important in the consideration of the mode of action of antimycin A at Site II of the respiratory chain. The first is that the ATP induction of a measured high potential form of cytochrome b_T is inhibited by antimycin A. The second pertains to the "anomalous" cytochrome *b* oxidation–reduction reactions in the presence of antimycin A first observed by Chance[31]. Cytochrome *b* in the presence of antimycin A was shown to undergo rapid and essentially complete reduction on addition of oxidants to mitochondrial preparations. Wikström[2] showed that the spectrum generated on addition of O_2 to anaerobic rat liver mitochondria was the same as that he demonstrated was generated by ATP addition to coupled preparations. Wilson *et al.*[27] similarly reported with solubilized succinate–cytochrome *c* reductase from chicken heart that the λ_{max} of the reduced cytochrome *b* was 565 nm and hence was consistent with the properties of cytochrome b_T and not cytochrome b_K. Furthermore, Wilson *et al.*[27] demonstrated that cytochrome b_T ($E_{m7.0}$ −30 mV) became fully reduced on addition of ferricyanide even though the succinate–fumarate ratio was adjusted to confer a "clamping" potential of +65 mV on the system before the addition. The interpretation given to the oxidant-induced reduction by both Rieske[30] and Wilson *et al.*[27] was that the midpoint potential of cytochrome *b* had assumed a more positive value during the electron flux through the site. Antimycin A was considered to serve in some way in stabilizing the high-potential cytochrome *b* oxidation–reduction couple when this form is generated by electron transport. The mode of action of antimycin A has been likened to that of respiratory control under conditions of high phosphate potential[11,30]. From the foregoing it seems reasonable to consider that antimycin A acts as an inhibitor of energy transfer as depicted in Fig. 7. Its relationship to uncouplers and oligomycin is shown. All three types of chemicals prevent the ATP induction of a measured high-potential cytochrome b_T (uncouplers, refs 3, 14, 32; oligomycin, ref. 32). Unlike the uncouplers which activate rapid dissipation of high energy states, oligomycin prevents energy transfer between X ~ I and ATP. Antimycin A may be regarded as acting in a similar way to oligomycin but preventing energy transfer between cytochrome b_T and X ~ I.

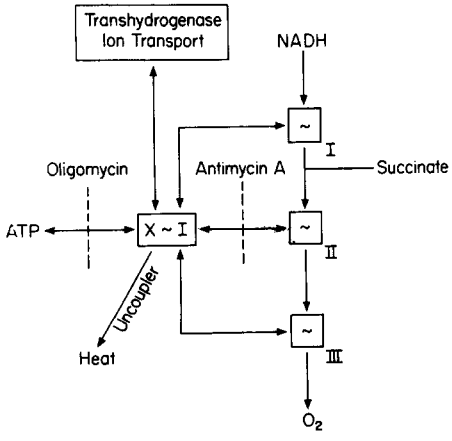

Fig. 7. Working scheme for the location of antimycin A with respect to electron transport and energy transfer in mitochondrial respiration.

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