Pazur, J. H., Tominaga, Y., & Forsberg, L. S. (1977) *Arch. Biochem. Biophys. 182*, 774–775.
Pazur, J. H., Tominaga, Y., de Brosse, C. W., & Jackman, L. M. (1978) *Carbohydr. Res. 61*, 279–290.
Rupley, J. A., Gates, V., & Bilbrey, R. (1969) *J. Am. Chem. Soc. 90*, 5633–5635.
Sawai, T. (1967) *Proceedings of the Amylase Symposium, 1967*, pp 111–117, Society of Amylase Researchers, Osaka, Japan.
Sawai, T., Yamaki, T., & Ohya, T. (1976) *Agric. Biol. Chem. 40*, 1293–1299.
Shibaoka, T., Suetsugu, N., Hiromi, K., & Ono, S. (1971) *FEBS Lett. 16*, 33–36.
Shibaoka, T., Ishikura, K., Hiromi, K., & Watanabe, T. (1975) *J. Biochem. (Tokyo) 77*, 1215–1222.
Still, W. C., Kahn, M., & Mitra, A. (1978) *J. Org. Chem. 43*, 2923–2925.
Suetsugu, N., Hiromi, K., & Ono, S. (1971) *J. Biochem. (Tokyo) 70*, 595–601.
Tsai, C. S., Tang, J. Y., & Subbarao, S. C. (1969) *Biochem. J. 114*, 529–534.
Wehrli, F. W., & Wirthlin, T. (1976) *Interpretation of Carbon-13 Nuclear Magnetic Resonance Spectra*, pp 53–65, Heyden, London and New York.
Wolfrom, M. L., Weisblatt, D. J., Evans, E. F., & Miller, J. B. (1957) *J. Am. Chem. Soc. 79*, 6454–6460.

Kinetics of Cytochrome *b* Oxidation in Antimycin-Treated Submitochondrial Particles†

Youssef Hatefi* and Takao Yagi

ABSTRACT: It has been shown that in bovine heart submitochondrial particles, antimycin and 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) inhibit the oxidation of NADH, succinate, and reduced ubiquinone incompletely, the uninhibited rate being about 20–40 nmol of substrate oxidized min^{-1} (mg of protein)$^{-1}$. By contrast, rotenone, cyanide, BAL (2,3-dimercaptopropanol), and 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole [Trumpower, B. L., & Haggerty, J. G. (1980) *J. Bioenerg. Biomembr. 12*, 151–164] caused essentially complete inhibition when added alone or after maximal inhibition by antimycin or HQNO. Having thus ascertained that the electron leak through the antimycin block appeared to follow the normal path through complex III (ubiquinol: cytochrome *c* oxidoreductase) and cytochrome oxidase, the reduction of the *b* cytochromes by substrates and their oxidation through the leak in the antimycin block by molecular oxygen were studied. It was shown that at normal electron flux from NADH and succinate, both cytochromes b_{562} and b_{566} were reduced in antimycin-treated submitochondrial particles. Their oxidation after substrate exhaustion was biphasic, however. At 565 minus 575 nm, 56% of the total reduced cytochrome *b* was oxidized through the leak in the antimycin block at a more rapid rate, while the remaining 44% was oxidized about 10 times slower. When electron flux from substrates to complex III was slowed down by the use of inhibitors or substrates at $\leq 0.1K_m$ concentration, then only reduced b_{562} accumulated in antimycin-treated particles. The oxidation of b_{562} after substrate exhaustion or inhibition of substrate oxidation by an appropriate inhibitor occurred at a rate comparable to that of the slower reoxidation phase described above. These results indicated, therefore, that cytochromes b_{566} and b_{562} are oxidized through the leak in the antimycin block at two different rates, the reoxidation rate of b_{566} being about 10 times faster than that of b_{562}. The implications of these findings on the kinetic relationship of these two cytochromes in the respiratory chain have been discussed.

The electron transport system of bovine heart mitochondria contains three spectroscopically distinct *b* cytochromes (Davis et al., 1973). A low potential cytochrome *b* (b_{560}) fractionates into the succinate:ubiquinone oxidoreductase complex (complex II). This cytochrome has been purified in a preparation composed of two polypeptides of M_r 13 500 and 15 000 (Hatefi & Galante, 1980). The role of cytochrome b_{560} is not clear, even though the purified preparation has been shown to recombine in a 1:1 molar ratio with succinate dehydrogenase and reconstitute a highly active succinate:ubiquinone oxidoreductase system (Hatefi & Galante, 1980). In addition, it has been shown that in complex II dithionite-reduced b_{560} is rapidly oxidized by fumarate via succinate dehydrogenase or by ubiquinone. The other two *b* cytochromes (b_{562} and b_{566}) occur in the ubiquinol:cytochrome *c* oxidoreductase complex (complex III).[1] Although involved in electron transfer from substrates to cytochromes c_1 + *c*, the precise roles of cytochromes b_{562} and b_{566} in electron transfer through complex III are not clear either. Cytochrome b_{562} is reduced when submitochondrial particles are treated with substrates, and is oxidized when the reduced particles are treated with oxygen. Reduction of b_{566} is not observed under these conditions and requires treatment of the particles with antimycin, which creates an oxidation–reduction "crossover" between the *b* and the *c* cytochromes. Even in the presence of antimycin, the reduction of b_{566} by substrates requires that cytochromes c_1 + *c* and the complex III iron–sulfur protein be oxidized (this effect is referred to as oxidant-induced extra reduction of

† From the Department of Biochemistry, Scripps Clinic and Research Foundation, La Jolla, California 92037. *Received July 28, 1982.* Supported by U.S. Public Health Service Grant AM 08126.

[1] In addition to b_{562} and b_{566}, a cytochrome *b* like chromophore with an absorption peak at 558 nm at 77 K was identified in complex III by Davis et al. (1973). Recently, Briquet et al. (1981) have observed a similar component in yeast mitochondria and have designated it cytochrome b_{558}.

0006-2960/82/0421-6614$01.25/0

cytochrome *b*). In an attempt to rationalize these phenomena, and moreover to account for the observed $2H^+/e^-$ stoichiometry in the complex III region of the respiratory chain, Mitchell (1976) proposed an interesting electron transfer pathway, which is known as the Q cycle.[2] The Q cycle suggests, among other things, that the *b* cytochromes of complex III function in the sequence $\xrightarrow{e} b_{566} \xrightarrow{e} b_{562} \xrightarrow{e}$ and cycle an electron between two species of ubisemiquinone, and that the site of antimycin block is immediately on the oxidant side of b_{562} (Mitchell, 1976; Trumpower, 1981; Van Ark et al., 1981).

In the present study, the oxidation rates of b_{562} and b_{566} were studied in antimycin-treated submitochondrial particles, taking advantage of the fact that the antimycin block leaks at a rate of 20–40 nmol of substrate oxidized min^{-1} mg of $protein^{-1}$. It is shown that b_{562} and b_{566} are oxidized through the antimycin block at different rates. This finding is difficult to reconcile with a sequential $\xrightarrow{e} b_{566} \xrightarrow{e} b_{562} \xrightarrow{e}$ pathway. It also poses a problem for the assumption that antimycin inhibits the oxidation of a single obligatory component, such as b_{562}, in the respiratory chain. These considerations are discussed with regard to the electron transfer pathway through the *b* cytochromes and the mode of inhibition by antimycin.

Methods and Materials

Submitochondrial particles (ETP) were prepared from beef heart mitochondria as described by Hanstein et al. (1974), and the protein concentration was determined by the biuret method (Gornall et al., 1949) in the presence of 1 mg of potassium deoxycholate/mL. Oxygen uptake was measured at 30 °C by a Clark-type electrode. Cytochrome *b* reduction and oxidation were studied at 565 minus 575 nm at 30 °C by using the Aminco-Chance dual-wavelength spectrophotometer. The spectra at 77 K were recorded by using as before (Davis et al., 1973) a modified single-beam Cary Model 14 spectrophotometer on line to a PDP 8/I digital computer for data collection and fourth derivative analysis of the spectra. Experimental details are given in the figure legends.

NADH and NADPH were obtained from P-L Biochemicals; Tris, sodium succinate, glutathione, glutathione reductase, and HQNO were from Sigma; Seconal was from Eli Lilly and Co.; antimycin was a gift from Kanegafuchi Chemical Industrial Co., Japan, or was purchased from Calbiochem; UHDBT was a generous gift from Dr. B. L. Trumpower, Dartmouth Medical School, Hanover, NH.

Results

The purpose of this work was to study the kinetic relationship between cytochromes b_{562} and b_{566}. In most schemes these two cytochromes are placed in the sequence $\xrightarrow{e} b_{566} \xrightarrow{e} b_{562} \xrightarrow{e}$ largely because the reduction potential of b_{566} is lower than that of b_{562}. Otherwise, no kinetic information regarding this sequence is available. Eisenbach & Gutman (1975) measured the reduction rates of these cytochromes in submitochondrial particles and intact mitochondria treated with antimycin ± cyanide. The rate of electron transfer was controlled by temperature, the activation state of succinate dehydrogenase when succinate was the electron donor, or the addition of duroquinol as the substrate which delivered electrons rapidly and presumably directly to the *b* cytochromes.

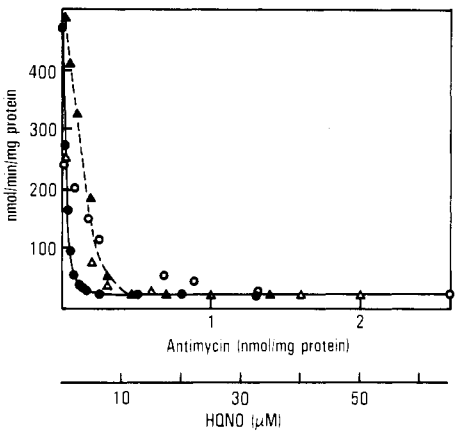

FIGURE 1: Inhibition of NADH and succinate oxidase activities of ETP by antimycin and HQNO. The reaction mixture contained 0.25 M sucrose, 50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM NADH (●, ▲), and 23 mM succinate (○, △). At low inhibitor levels, the ETP concentration was 0.29 mg/mL. At high inhibitor levels, the activities were measured both at 0.29 (●, ▲) and 1.71 mg of ETP/mL (○, △). Before addition of substrates, the indicated amounts of antimycin (▲, △) or HQNO (●, ○) were added. Activities were measured polarographically at 30 °C, with a Clark-type oxygen electrode. In the absence of inhibitors, the NADH and succinate oxidase activities were respectively 468 and 264 nmol min^{-1} (mg of $protein)^{-1}$.

None of these manipulations allowed a distinction between the reduction rates of b_{562} and b_{566}. In our studies, we concentrated on the oxidation rates of these cytochromes. However, since it was necessary to add antimycin to reduce b_{566}, we decided to examine the rates at which b_{562} and b_{566} are oxidized through the leak in the antimycin block (Slater, 1967). For this purpose, certain preliminary experiments were necessary, which are reported below.

Figure 1 shows the titration of NADH and succinate oxidase activities of ETP by antimycin or HQNO. It is seen that a fraction of the activity could not be inhibited by increasing concentrations of either inhibitor. Similar results were obtained with Q_2H_2 as the substrate. With succinate or NADH as the substrate, the uninhibited rate was relatively constant, even though in the absence of inhibitors NADH oxidase activity was nearly twice the succinate oxidase activity. While a system partially inhibited by either antimycin or HQNO could be further inhibited by addition of the other inhibitor, addition of HQNO to the system maximally inhibited by antimycin, or addition of antimycin to the system maximally inhibited by HQNO, did not produce further inhibition. However, in either case the residual uninhibited rate could be reduced to near zero by addition of rotenone, cyanide, BAL, or UHDBT.[3-5] In agreement with the conclusions of Van Ark et al. (1981), these results suggested that antimycin and HQNO inhibit electron transfer by a similar mechanism and at a site which can be saturated by either inhibitor. The fact that the inhibition by HQNO could be reversed by dilution

[2] Abbreviations: Q, ubiquinone; Q_2H_2, reduced ubiquinone 10 (coenzyme Q_2); HQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole; ETP, electron transfer particles; Tris, tris(hydroxymethyl)aminomethane; *b* and *c*, cytochromes *b* and *c*, respectively; BAL, British anti-lewisite (2,3-dimercaptopropanol).

[3] In order to obtain complete inhibition of oxygen consumption by addition of cyanide, BAL, or UHDBT, the substrate must be succinate, not NADH, because the span from NADH to Q leaks to oxygen at a rate of up to 10 nmol min^{-1} mg^{-1} (Hatefi & Bearden, 1976).

[4] These observations may be related to the results obtained by Bowyer & Trumpower (1980), who showed that the oxidant-induced reduction of cytochrome *b* in antimycin-treated preparations was inhibited by UHDBT, and by Von Jagow & Engel (1981), who found that the combinations of antimycin and myxothiazol completely inhibited *b* reduction by ubiquinol.

[5] We also tested the effect of salicylhydroxamic acid on the residual uninhibited rate. As expected, there was no effect.

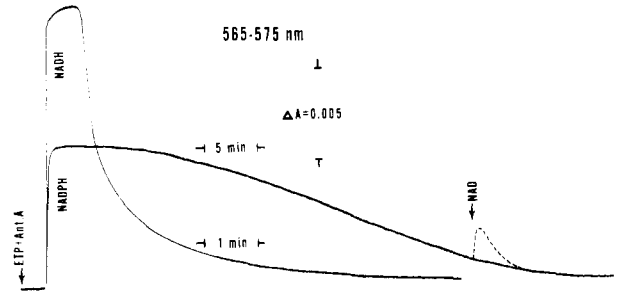

FIGURE 2: Reduction and reoxidation of *b* cytochromes in antimycin-treated ETP. The reaction mixture at 30 °C contained 1.1 μM antimycin and 1.04 mg of ETP/mL of 0.25 M sucrose–50 mM Tris–acetate, pH 7.5. Where indicated 30 μM NADH, NADPH, or NAD was added, and reduction and reoxidation of the *b* cytochromes were followed at 565 minus 575 nm. The time scale shown was 1 min for the experiment with NADH and 5 min for the experiment with NADPH.

(Yagi et al., 1982) indicated that the bc_1 region of the electron transport system was not irreversibly modified by HQNO. Furthermore, the fact that the antimycin-uninhibited rate could be completely inhibited by BAL, UHDBT, or cyanide indicated that the slow uninhibited rate still involves the normal electron transfer pathway through the respiratory chain and is not due to a bypass in the bc_1 region or a leak to oxygen prior to cytochrome oxidase.

Having thus ascertained that the electron leak through the antimycin block is not due to a gross derangement of the system, we then proceeded to study the oxidation of the substrate-reduced *b* cytochromes through the antimycin leak. As shown in Figure 2, submitochondrial particles were treated with saturating amounts of antimycin, then 30 μM NADH (upper trace) or NADPH (lower trace) was added, and reduction and oxidation of cytochrome *b* were monitored at 565 *minus* 575 nm. When NADH was the substrate, cytochrome *b* was rapidly reduced and then oxidized after about 1 min of steady-state reduction. Addition of $Na_2S_2O_4$ after steady-state reduction of cytochrome *b* with NADH caused a further slow reduction of about 10–20%, which might be in part due to reduction of cytochrome b_{560} of complex II (Davis et al., 1973; Hatefi & Galante, 1980). When the substrate was 30 μM NADPH, then only about 50% of the reduction observed with NADH took place, the steady-state reduction span was much longer (note that the time scale for the NADPH experiment is 5 times that of the NADH experiment), and the reoxidation of the partially reduced cytochrome *b* was very slow. The difference between the two substrates is the slow oxidation rate of NADPH by complex I. Not only was the pH of the experiment (pH 7.5) unfavorable for NADPH oxidation but also 30 μM NADPH was considerably below K_m (≃550 μM) (Hatefi & Hanstein, 1973). Independent polarographic measurements under the conditions of Figure 2 showed that the initial oxidation rates of NADH and NADPH by antimycin-treated ETP were respectively 42 and 2 nmol min^{-1} (mg of protein)$^{-1}$. The dashed trace in Figure 2, showing increased *b* reduction followed by rapid reoxidation upon NAD addition, indicates NADPH oxidation by way of the nicotinamide-nucleotide transhydrogenase reaction, which is more rapid at pH 7.5 for NADPH oxidation and has a much lower K_m for NADPH (Rydström, 1977). The absorption spectra of NADH- and NADPH-reduced ETP (Figure 3), arrested in the stage of steady-state reduction by freezing the mixtures at 77 K, indicated that in the NADH-treated ETP both cytochromes b_{562} and b_{566} were reduced (Figure 3, trace A), while in the NADPH-treated ETP only reduced b_{562} had accumulated (Figure 3, trace B). As seen in Figure 4, the difference

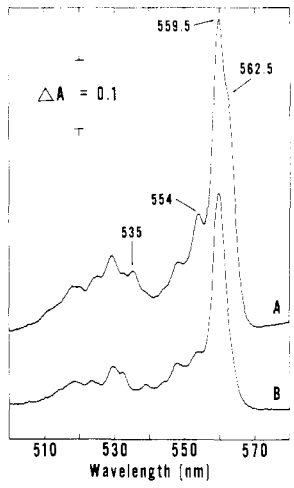

FIGURE 3: Low-temperature difference spectra of the *b* cytochromes reduced with NADH and NADPH. Reaction conditions were the same as in Figure 2. The samples were frozen in liquid nitrogen within 15 s after the addition of 30 μM NADH (trace A) or NADPH (trace B). Reference in either case was unreduced, antimycin-treated ETP. The peak at 559.5 nm (actually 560 nm) is the position of the α band of cytochrome b_{562} at 77 K, and the shoulder at 562.5 nm is due to the α band of cytochrome b_{566} at 77 K. The fourth derivative of trace B (not shown) showed no peak at 562.5 nm. For other details, see Davis et al. (1973).

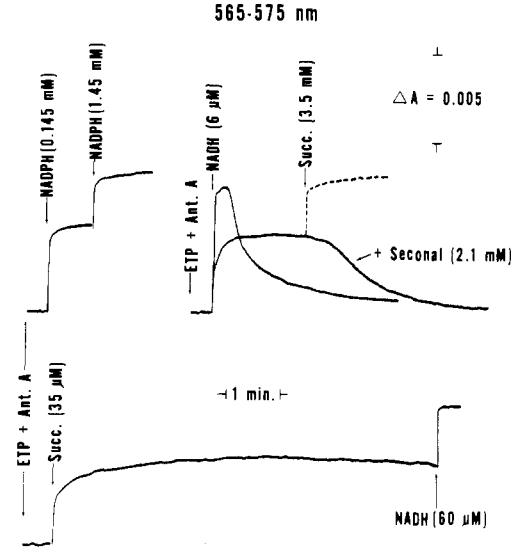

FIGURE 4: Effect of electron flux on the extent of cytochrome *b* reduction in antimycin-treated ETP. The reaction mixture at 30 °C contained 1.1 μM antimycin and 0.55 mg of ETP/mL of 0.25 M sucrose–50 mM Tris–sulfate, pH 7.4. Where indicated by vertical arrows, additions of substrates were made at the final concentrations shown. In the upper right-hand experiment, middle trace, Seconal was added prior to addition of 6 μM NADH. Cytochrome *b* reduction and reoxidation were followed as in Figure 2.

in the reduction of the *b* cytochromes by NADH and NADPH was not due to a peculiarity of the latter substrate, but rather it seemed to be associated with the rate of electron flow from complexes I and II to complex III. Thus, the upper left-hand trace in Figure 4 shows that increasing the NADPH concentration to about 2.5K_m caused further cytochrome *b* reduction. Similarly, the upper right-hand traces show that 6 μM NADH resulted in near-maximal reduction of cytochrome *b*, while the presence of 2.1 mM Seconal, which caused considerable inhibition of NADH oxidation at the level of complex I, resulted in partial *b* reduction. However, when succinate was added to the system containing Seconal, then the remainder of cytochrome *b* was reduced (dashed trace). The

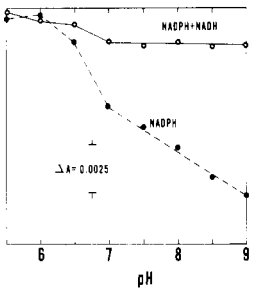

FIGURE 5: Effect of pH on the extent of cytochrome *b* reduction by NADPH in antimycin-treated ETP. The reaction mixture at 30 °C contained 0.7 μM antimycin and 0.82 mg of ETP/mL of 0.25 M sucrose–0.1 M potassium phosphate at pH 6–9 or 0.25 M sucrose–0.1 M sodium acetate at pH 5.5. At each experimental point shown 120 μM NADPH was added and the absorbance increase at 565 minus 575 nm recorded (●). Then 60 μM NADH was added, and the total absorbance increase caused by NADPH plus NADH was recorded (○).

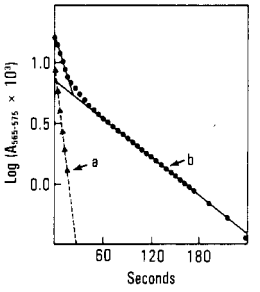

FIGURE 6: Double-exponential analysis of the reoxidation kinetics of the NADH-reduced *b* cytochromes shown in Figure 2. The rapid phase constitutes 56% of the total absorbance at 565 minus 575 nm and has a rate constant (calculated from trace a) of $\Delta A = 0.116\ s^{-1}$. The slow phase constitutes 44% of the total absorbance and has a rate constant (calculated from trace b) of $\Delta A = 0.012\ s^{-1}$. These rate constants were essentially the same in several different ETP preparations that were examined.

lower trace of Figure 4 shows the opposite experiment. Addition of $0.1K_m$ succinate resulted in partial cytochrome *b* reduction, while further addition of NADH caused maximal reduction. As shown in Figure 5, the extent of cytochrome *b* reduction by NADPH could also be altered by changing the pH of the medium and thereby altering the rate of NADPH oxidation, which is highly pH dependent and increases by 40-fold as the pH is lowered from 9 to 6 (Hatefi & Hanstein, 1973). In addition to these experiments, we have shown that modulation of the rate of electron flow through complexes I and II alters the extent of cytochrome *b* reduction between the low and high levels shown in Figures 2 and 4, and that addition of KCN to the system in which cytochrome *b* is partially reduced results in additional *b* reduction. These results indicated, therefore, that depending on the rate of electron flow to complex III, cytochromes b_{562} and b_{566} could be reduced to various extents in antimycin-treated particles. However, at low electron flux b_{562} could be maintained completely reduced while b_{566} appeared to be completely oxidized (Figure 3). An obvious possibility consistent with these results was that the oxidation rate of b_{562} in antimycin-treated particles was slower than that of b_{566}, thus allowing reduced b_{562}, but not reduced b_{566}, to accumulate at low electron flux. This conclusion agreed with the decay kinetics of the NADH-reduced *b* cytochromes shown in Figure 2, because the decay curve is biphasic.[6] A semilogarithmic plot of this curve is

[6] A similar biphasic oxidation of cytochromes *b* was observed when high levels of NADH were added and, after the cytochromes were reduced, NADH oxidation was inhibited by addition of rotenone.

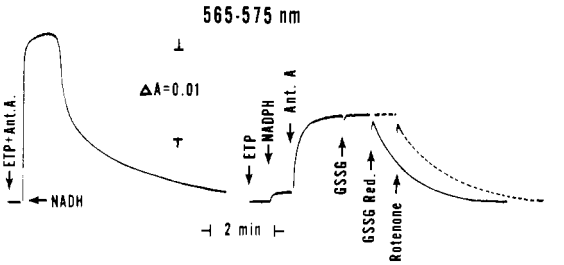

FIGURE 7: Reoxidation kinetics of cytochrome b_{562} in antimycin-treated ETP. The reaction mixture at 30 °C contained 1.07 μM antimycin and 1.32 mg of ETP/mL of 0.25 M sucrose–50 mM Tris–sulfate, pH 7.5. Where indicated, 60 μM NADH or NADPH, 10 μM rotenone, 360 μM oxidized glutathione, and 2.3 units of glutathione reductase (Sigma, type III) were added. Cytochrome *b* reduction and reoxidation were monitored as in Figure 2. In a parallel experiment, the NADPH-reduced spectrum of cytochrome *b* was examined at 77 K and was shown to be qualitatively the same as trace B of Figure 3.

given in Figure 6, which shows the two phases. Analysis of this plot indicated that at 565 *minus* 575 nm 56% of reduced cytochrome *b* (presumably b_{566}) was oxidized with a ΔA rate constant of $0.116\ s^{-1}$ and the remainder (presumably b_{562}) with a rate constant of $0.012\ s^{-1}$. That the second, slower rate is due to reoxidation of b_{562} is further supported by the experiments of Figure 7. The left-hand trace is the control, showing maximal *b* reduction upon addition of NADH to antimycin-treated ETP and a biphasic reoxidation. In the right-hand experiment, cytochrome *b* reduction was effected at low electron flux (30 μM NADPH at pH 7.5) under conditions that only reduced b_{562} accumulated and then NADPH oxidation was inhibited by addition of rotenone or the supply of NADPH was exhausted by addition of oxidized glutathione plus glutathione reductase. It is seen that the reoxidation of reduced b_{562} occurred in a manner similar to the slow phase of reoxidation of cytochrome *b* maximally reduced with NADH (compare the absorbance decay curves in the right-hand experiment of Figure 7 with the lower half of the decay curve in the left-hand experiment of the same figure; see also similar results in Figure 4, upper right-hand experiment). The implications of these results which indicate that cytochromes b_{562} and b_{566} are oxidized at different rates through the leak in the antimycin block are considered below.

Discussion

It has been shown that antimycin (also HQNO) does not completely inhibit electron transfer in the respiratory chain and that antimycin-treated particles can be used to study in the presence of limited amounts of substrate both the reduction and reoxidation of the *b* cytochromes. Eisenback & Gutman (1975) studied the reduction of *b* cytochromes in particles treated with antimycin ± cyanide and concluded that b_{562} and b_{566} were reduced at the same rate by substrates. In our studies, we found that these two cytochromes could be reduced in antimycin-treated submitochondrial particles to different extents by NAD(P)H or succinate by altering the rate of electron flux through complexes I and II. At high electron flux, both cytochromes were maximally reduced. At low electron flux, only reduced b_{562} accumulated, and at intermediate flux rates apparently b_{562} and different extents of b_{566} were reduced (Figure 5). The reason for these results appeared to be associated with the finding that reduced b_{566} and b_{562} were oxidized through the leak in the antimycin block at rates differing by about 10-fold. Thus at high electron flux from complex I or II to complex III, both *b* cytochromes were maintained in a highly reduced state. However, when the rate

of electron flux to complex III was lowered, then the steady-state reduction level of b_{566} diminished, presumably because of its relatively rapid reoxidation rate, while b_{562} could be maintained in a high state of reduction because of its relatively slow reoxidation rate. Of course, at very low electron flux rates [e.g., NADPH at pH 9.0 (see Figure 5)], then the steady-state reduction level of b_{562} was also diminished.

If it is assumed that antimycin does not alter the kinetic sequence of the carriers in the respiratory chain and does not produce a bypass in the system as our results with the use of inhibitors acting at other loci seem to indicate, then the different rates of b_{562} and b_{566} oxidation through the leak in the antimycin block do not agree with the sequence $\overset{e}{\rightarrow} b_{566} \overset{e}{\rightarrow} b_{562} \overset{e}{\rightarrow} |\text{antimycin}| \overset{e}{\rightarrow}$ as is assumed by other workers in the field (Mitchell, 1976; Trumpower, 1981; Van Ark et al., 1981). Nor would the reverse sequence, $\overset{e}{\rightarrow} b_{562} \overset{e}{\rightarrow} b_{566} \overset{e}{\rightarrow} |\text{antimycin}| \overset{e}{\rightarrow}$, work because in each case there would have to be a single rate of cytochrome *b* oxidation determined by the magnitude of the leak through the antimycin block. A way around this dilemma, which would keep the two *b* cytochromes in kinetic sequence, is reoxidation of the *b* cytochromes in opposite directions, e.g.

$$\overset{e}{\leftarrow} b_{566} \overset{e}{\rightleftarrows} b_{562} \overset{e}{\rightarrow} |\text{antimycin}| \overset{e}{\rightarrow}$$

In this scheme b_{566} is back-oxidized at a slow rate and b_{562} is oxidized through the antimycin block at a slower rate, provided we assume that back-oxidation of b_{562} by b_{566} (broken arrow in the above scheme) is much slower still. The possibility of very slow reoxidation of b_{562} by b_{566} agrees with the fact that the reduction potential of b_{562} is much more positive than that of b_{566}. However, it does not agree with the results of Eisenback & Gutman (1975), who studied the reduction of the *b* cytochromes in antimycin-treated particles and reached the conclusion that b_{562} and b_{566} are reduced concomitantly and at the same rate. This is because in order to have the two cytochromes in the sequence $b_{566} \overset{e}{\rightarrow} b_{562}$ and obtain the results published by Eisenback & Gutman (1975), not only should b_{566} reduction be rate-limiting but also back flow of electrons from b_{562} to b_{566} should not be slow. Otherwise, sequential cytochrome *b* reduction would be observed with reduced b_{562} accumulating prior to reduced b_{566}.

Another possibility suggested by the different oxidation rates of b_{562} and b_{566} is that in antimycin-treated systems these two cytochromes are not in kinetic sequence. This possibility introduces a complication with regard to the site of action of antimycin, since there can be only a single antimycin-binding site equimolar to one cytochrome *b* or cytochrome c_1 (Rieske, 1976). Thus, one would have to assume that antimycin reacts with a component which oxidizes both b_{566} and b_{562}. This assumption does not agree with genetic evidence in yeast regarding the association of the antimycin binding site with cytochrome *b* (Roberts et al., 1980). However, regardless of whether a sequential b_{566}–b_{562} pathway or a nonsequential arrangement in which b_{562} and b_{566} are oxidized independently of one another is the case, we feel that the mechanism of antimycin inhibition may not be analogous to the mechanism of action of most other respiratory chain inhibitors. These inhibitors (e.g., cyanide, mercurials, rotenone, BAL, UHDBT, and oxaloacetate) react with an essential group or a substrate-binding site. In these situations complete modification of the essential group or saturation of the site at high inhibitor concentration results in complete inhibition. This, as pointed out above, is not the case with antimycin and HQNO. They do not cause complete inhibition, while other inhibitors acting in the bc_1 region but at different sites, such as BAL and UHDBT, can cause complete inhibition even when added on top of saturating levels of antimycin or HQNO. On the other hand, antimycin and HQNO make b_{566} reducible, while other complex III inhibitors, including BAL, UHDBT, ethoxyformic anhydride (Yagi et al., 1982), and the inhibitors described by Becker et al. (1981), do not. Another important property of antimycin is that it makes complex III structurally more stable and resistant to cleavage by guanidine (Rieske et al., 1967; Hatefi and Hanstein, 1974). Thus, it is possible that the ability of antimycin to make b_{566} reducible is associated with the fact that it causes certain conformation changes in complex III which lead, among other things, to a more stable structure. We suggest that the inhibition by antimycin may be a consequence of this structural change. In such a situation the binding of antimycin to a single site (e.g., cytochrome *b* apoprotein) could alter the kinetics of both b_{562} and b_{566}. Moreover, saturation of the site with antimycin and the consequent structural modification could restrict electron flow but not completely inhibit it.

Acknowledgments

We thank Dr. K. A. Davis for recording the low-temperature spectra and C. Munoz for the preparation of mitochondria.

References

Becker, W. F., Von Jagow, G., Anke, T., & Steglich, W. (1981) *FEBS Lett. 132*, 329–333.

Bowyer, J. R., & Trumpower, B. L. (1980) *FEBS Lett. 115*, 171–174.

Briquet, M., Purnelle, B., Faher, A., & Goffeau, A. (1981) *Biochim. Biophys. Acta 638*, 116–119.

Davis, K. A., Hatefi, Y., Poff, K. L., & Butler, W. L. (1973) *Biochim. Biophys. Acta 325*, 341–356.

Eisenbach, M., & Gutman, M. (1975) *Eur. J. Biochem. 52*, 107–116.

Gornall, A. G., Bardawill, C. J., & Davis, M. M. (1949) *J. Biol. Chem. 177*, 751–766.

Hanstein, W. G., Davis, K. A., & Hatefi, Y. (1974) *Arch. Biochem. Biophys. 163*, 482–490.

Hatefi, Y., & Hanstein, W. G. (1973) *Biochemistry 12*, 3515–3522.

Hatefi, Y., & Hanstein, W.G. (1974) *Methods Enzymol. 31*, 770–790.

Hatefi, Y., & Bearden, A. J. (1976) *Biochem. Biophys. Res. Commun. 69*, 1032–1038.

Hatefi, Y., & Galante, Y. M. (1980) *J. Biol. Chem. 255*, 5530–5537.

Mitchell, P. (1976) *J. Theor. Biol. 62*, 327–367.

Rieske, J. S. (1976) *Biochim. Biophys. Acta 456*, 195–247.

Rieske, J. S., Baum, H., Stoner, C. D., & Lipton, S. H. (1967) *J. Biol. Chem. 242*, 4854–4866.

Roberts, H. R., Smith, S. C., Marzuki, S., & Linnane, A. W. (1980) *Arch. Biochem. Biophys. 200*, 387–395.

Rydström, J. (1977) *Biochim. Biophys. Acta 463*, 155–184.

Slater, E. C. (1967) *Methods Enzymol. 10*, 48–57.

Trumpower, B. L. (1981) *Biochim. Biophys. Acta 639*, 129–155.

Van Ark, G., Raap, A. K., Berden, J. A., & Slater, E. C. (1981) *Biochim. Biophys. Acta 637*, 34–43.

Von Jagow, G., & Engel, W. D. (1981) *FEBS Lett. 136*, 19–24.

Yagi, T., Vik, S. B., & Hatefi, Y. (1982) *Biochemistry 21*, 4777–4782.