THE INHIBITION BY ANTIMYCIN A OF THE CLEAVAGE OF ONE OF THE COMPLEXES OF THE RESPIRATORY CHAIN

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The QH_2-cytochrome c reductase complex (III) which contains cytochrome b and c_1 was isolated from beef heart mitochondria and characterized as to enzymic properties by Hatefi and his coworkers (1961, 1962). It is of interest that this complex contains the site of the respiratory chain that is sensitive to antimycin A. Heretofore, the only measureable effects of antimycin A on the respiratory chain have been its inhibition of electron transfer and its effects on the reducibility of cytochrome b with substrates (i.e. DPNH, Succinate and QH_2) and on the spectral properties of cytochrome b (Chance, 1958; Pumphrey, 1962). However, it has been discovered in this laboratory that antimycin A exerts a powerful and a specific inhibition of the cleavage of complex III into its component cytochromes. This communication will deal with a description of this phenomenon, its implications with regard to the structure of the complex, and its possible use as a new tool in obtaining information as to the structural and chemical nature of the antimycin-sensitive site.

Complex III can normally be split into its individual cytochrome components by exposure to bile salts (3-6%, w/v; depending on the type of bile acid and the protein concentration) in the presence of ammonium sulfate (25-

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30% saturation at 0°). Under these conditions cytochrome b separates as an insoluble precipitate whereas cytochrome c_1 and the unsplit complex remain in the supernatant solution. Variations of this procedure have been used for the separation of cytochromes b or c_1 prior to their purification (Green, can be estimated approximately by measuring the increase of turbidity due to the precipitated cytochrome b or, more exactly, by isolating the cytochrome b precipitate and determining its heme content as the pyridine hemochrome. The relative amount of cytochrome b in the sedimented precipitate can also be determined by spectral measurements on a solution of the pellet in 0.5% sodium dodecyl sulfate. However, this method is neither as sensitive nor as quantitative as the pyridine hemochrome procedure.

Fig. 1 illustrates the pronounced effect of different amounts of antimycin A_3 on the amount of cytochrome b split from complex III as a function of the incubation time. Complex III was incubated with the antimycin prior to the addition of the cholate and the ammonium sulfate. In this case the relative amounts of cytochrome b precipitated were estimated turbidimetrically, after appropriate dilution, by the increase in absorbancy at 700 mμ. The antimycin effect involves a complete blocking of the splitting reaction; even with prolonged incubation the yield of separated cytochrome b was not increased above a level determined by the amount of antimycin added.

To determine whether a relationship exists between the effect of antimycin on splitting and on the inhibition of electron transfer, a comparison was made between the inhibition of the splitting reaction by varying amounts of antimycin added to complex III and the inhibition of the QH_2-cytochrome c reductase activity in an aliquot of the complex. After addition of the cholate and ammonium sulfate to III the mixture was incubated at 20° for 40 minutes. Under these conditions the splitting reaction was essentially complete, about 75% of the cytochrome b being precipitated from the control sample which had no added antimycin. Prolonged incubations, or incubations at higher temperatures, increased the degree of splitting to 95%; however, under these conditions some loss of heme from the cytochromes occurred. The amount of

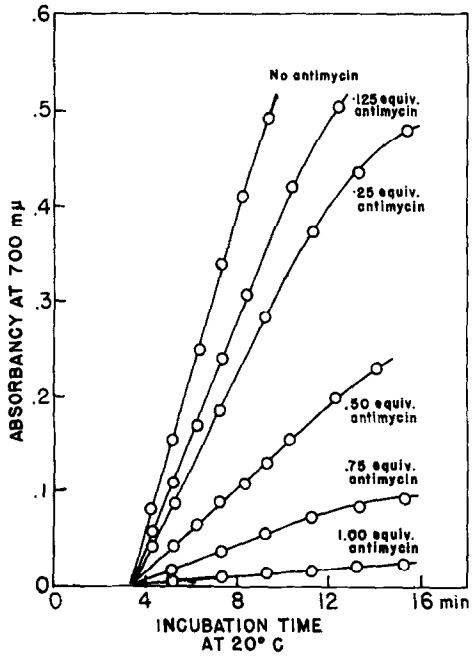

Fig. 1. Effect of antimycin A on the rate of separation of cytochrome b from the QH_2-cytochrome c reductase complex. The amount of antimycin added is expressed as cytochrome c_1 equivalents. One ml portions of the complex [protein (biuret) = 17 mg/ml; cyt c_1 = 65 mμmoles/ml] were preincubated with the antimycin for 15 minutes at 0°, then treated with 0.22 ml of 20% K cholate and 0.25 ml of sat. $(NH_4)_2SO_4$ and incubated at 20°C. At the desired time, a 0.1 ml aliquot was withdrawn and added to 0.4 ml of a 0.66 M sucrose -.01 M Tris solution (pH 8). The turbidity of the precipitated cytochrome b was determined by the absorbancy of the suspension at 700 mμ read against a "zero time" blank. The cytochrome c_1 content of the reaction mixture was determined spectrophotometrically at 0° after reduction with ascorbate. A molar absorbancy value of 17,100 was used for the reduced-oxidized spectrum at 554 mμ (Green, et al., 1958).

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cytochrome b precipitated was determined both by the turbidimetric and the pyridine hemochrome methods. Essentially all of the cytochrome c_1 remained in the supernatant solution. The results of this experiment are shown in Fig. 2. The inhibition curve calculated from the turbidimetric measurements is in very good agreement with that of the QH_2-cytochrome c reductase. However, the curve obtained from the direct analysis of the sedimented cytochrome b is displaced from, although parallel to, the enzyme inhibition curve. The reason for this displacement is unknown at present; however, it is significant that the slope of the linear portion of this curve corresponds to that

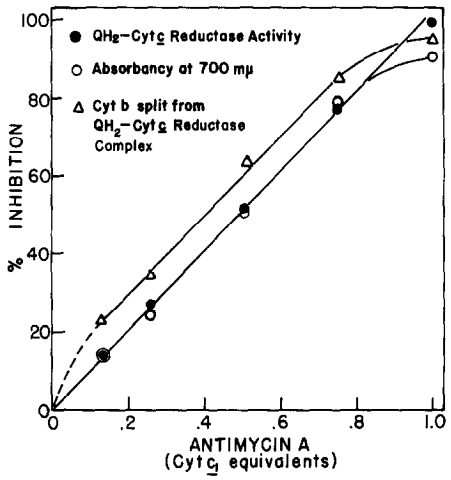

Fig. 2. Comparison between the effect of antimycin A on the degree of splitting of the QH_2-cytochrome c reductase complex and on the inhibition of QH_2-cytochrome c reductase activity. Conditions were the same as those described in the legend of Fig. 1 except that the incubation time (splitting) was 40 minutes. For the pyridine hemochrome determinations, the cytochrome b pellet was dissolved in 1 ml of a mixture (v/v) of 12.5 parts water, 1.5 parts of M KOH and 6 parts pyridine. The QH_2-cytochrome c reductase activities were determined by a slight modification of the procedure described by Hatefi, et al., (1962).

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of the enzyme inhibition curve. This is good evidence that the stoichmetry of the inhibition of splitting and that of the inhibition of enzyme activity by antimycin are identical. It is also significant that the molar ratio of antimycin to the cytochrome c_1 content of complex III required for complete inhibition of both the enzymic activity and the splitting reaction is close to unity. This ratio has remained invariant, being independent of the concentration or the amount of the complex present in the sample.

When antimycin A, in excess of the cytochrome c_1 content of complex III, was added to a "splitting" mixture of cholate, ammonium sulfate and the complex, at increasing time periods after the cholate addition, it was found that the ability of antimycin to block the splitting reaction was lost by approximately a first order kinetic process. Also, this loss of antimycin-sensitivity preceeded the actual separation of the cytochrome b. This is illustrated in Fig. 3. The first order nature of the antimycin-sensitive reaction is demonstrated in Fig. 4.

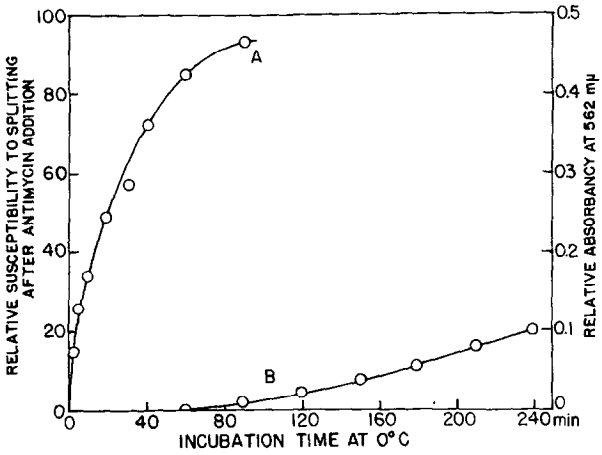

Fig. 3. Curve A: The effect of time of incubation of QH_2-cytochrome c reductase complex under "splitting" conditions on the subsequent inhibition of splitting by antimycin A. A mixture containing 2.0 ml of the enzyme complex (cytochrome c_1 = 100 mμmoles/ml), 1.0 ml sat. $(NH_4)_2SO_4$, and 1 ml 20% K cholate was incubated at 0°. At the desired times, 0.2 ml aliquots were removed and treated with 40 mμmoles antimycin A_3. After the antimycin had completely reacted (5 min. at 0°) the aliquot was incubated for 75 minutes at 20° to complete the splitting of the cytochromes. The sedimented precipitates of cytochrome b were dissolved in 0.5 ml of 0.5% sodium dodecyl sulfate and the relative cytochrome b content estimated spectrophotometrically. The yield of cytochrome b from each aliquot was normalized to that obtained from a control aliquot with no antimycin treatment.

Curve B: The release of cytochrome b as a function of incubation time at 0° for the control preparation as described for curve A, showing the consecutive nature of the cytochrome b release to the initial loss of antimycin sensitivity.

From the preceeding results it appears that the antimycin site consists of a chemically reactive group which also functions as an actual supporting link between cytochrome b and cytochrome c_1. The splitting phenomenon apparently occurs in two steps; a primary cleavage at the antimycin site (with a concurrent loss of sensitivity to antimycin) which then allows a slower consecutive separation and/or polymerization of the cytochrome b. When antimycin A is bound to the intact antimycin site, this site can no longer react with the as yet undetermined reagent (possibly water) which is responsible for the primary reaction in the splitting process. If this hypothesis is correct, a detailed study of the splitting process in the QH_2-

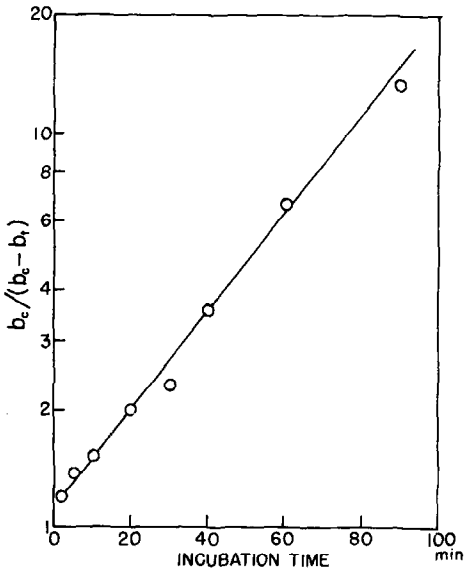

Fig. 4. A plot of the data given in curve A, Fig. 3 on semi-logarithmic coordinates showing the 1st order nature of the antimycin-sensitive reaction; b_c = relative amount of cytochrome b split from control aliquot of the "splitting" mixture with no added antimycin and b_t = relative amount of cytochrome b split from an aliquot of the mixture following the addition of antimycin after the stated preincubation period.

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cytochrome c reductase complex should provide useful information as to the organization of the cytochrome components as well as to the nature and function of the antimycin site in this particular complex.

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