CONTROLLED REDUCTION OF CYTOCHROME b IN SUCCINATE-CYTOCHROME c REDUCTASE COMPLEX BY SUCCINATE IN THE PRESENCE OF ASCORBATE AND ANTIMYCIN

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Summary More than 75 percent of the cytochrome b of resolved succinate-cytochrome c reductase complex is rapidly reduced upon addition of succinate. In the presence of ascorbate and antimycin, the reduction of b by succinate is markedly inhibited such that electron transfer into b proceeds as a pseudo-first order reaction with a rate constant of 3×10^{-2}/min at 25°C. This inhibition is consistent with a model in which the total succinate-reducible b is regulated by the redox status of cytochrome c_1 or another, currently unidentified component of the cytochrome b-c_1 complex whose redox status closely parallels that of cytochrome c_1.

Introduction

Cytochrome b of the mitochondrial electron transport chain has been intensively studied during the 50 year interval since its discovery in heart muscle fragments (1). However, two of the most fundamental questions regarding the biological function of cytochrome b have yet to be answered in unequivocal fashion. These are (1) what is the sequence of events, including electron transfer, proton transport, and changes in protein structure, associated with electron transfer to and from cytochrome b, and (2) does cytochrome b participate in the primary step of energy conservation by which oxidation is coupled to phosphorylation?

Of particular interest is the phenomenon in which addition of O_2 to anaerobic mitochondria in the presence of antimycin and substrate causes an increased reduction of cytochrome b (2-5).

This "oxidant-induced reduction of b" is currently not understood, but it appears that antimycin induces a regulatory effect similar to one which exists intrinsically in the succinate-cytochrome c reductase complex (6). To date the only proposed mechanisms for the oxidant-induced reduction of b have relied on postulated changes in the midpoint potential of cytochrome b (cf 7).

We report here experiments with resolved succinate-cytochrome c reductase complex which demonstrate the inverse of the oxidant-induced reduction of b and which suggest that regulation of electron transfer into b is a kinetic event spanning the second coupling site.

Materials and Methods

Succinate-cytochrome c reductase complex was prepared from bovine heart mitochondria (8). Cytochrome c reductase activity was measured at 30°C, using 30 μM cytochrome c (8).

For measurement of cytochrome spectra and rates of b reduction, reductase complex was suspended at 1.42 mg per ml in 0.1 M sodium phosphate-0.5 mM EDTA, pH 7.4 and difference spectra recorded on a Cary 118C Spectrophotometer. Cytochrome b was measured against a reference in which cytochrome c_1 was reduced with ascorbate (9). Additions to the sample and reference cuvettes are recorded in the figure legends. Reduction of b was followed by repetetive scanning of the wavelength region 585 → 555 nm at a scan rate of 0.5 nm per sec and amounts of reduced b were calculated from the absorbance difference at 563 nm minus 577 nm (9).

Results and Discussions

Resolved succinate-cytochrome c reductase complex catalyzes reduction of cytochrome c by succinate with a zero order rate of 6-8 μmole cytochrome c reduced per min per mg and, as shown below, this activity is fully sensitive to antimycin. The reduc-

tase complex contains 2.3 nmole of cytochrome b and 1.3 nmole of cytochrome c_1 per mg protein. Less than 10 percent of the dithionite-reducible b is reactive to carbon monoxide (data not shown). Thus this preparation is characterized by high activity and very little denatured b (cf 9).

As shown in Figure 1, when succinate is added to the reductase, cytochrome b is rapidly reduced in a reaction which comes to completion before a spectrum can be obtained. This agrees with the reported half-time of less than 100 msec for reduction of b by succinate in resolved reductase complex (10). The extent of

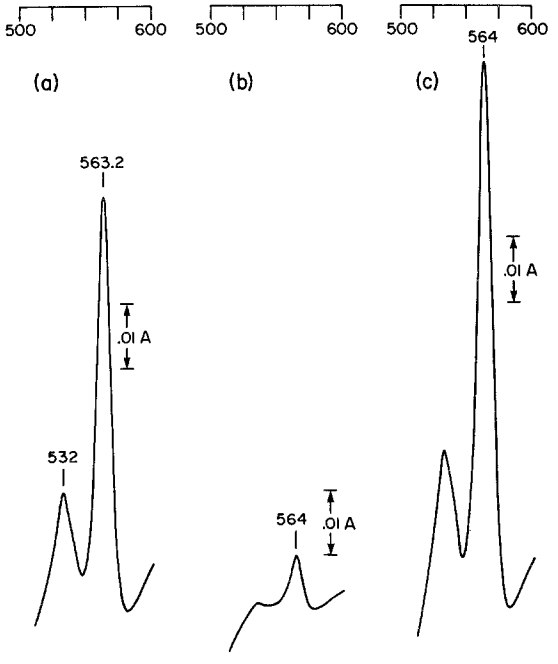

Figure 1 Absorption difference spectra of cytochrome b in resolved succinate-cytochrome c reductase complex. Spectrum (a) is of the cytochrome b reduced by succinate. Spectrum (b) is of the cytochrome b reduced by succinate in the presence of 1 mM ascorbate and 2 µg antimycin. Spectrum (c) is of the cytochrome b reduced by dithionite, which was identical in the presence or absence of ascorbate and antimycin.

b reduction by succinate varied from 72 to 80 percent of the dithionite-reducible b, assuming a homogenous extinction coefficient for the total b pool.

When the reductase is treated with antimycin and ascorbate, subsequent addition of succinate resulted in virtually no reduction of b within 2 min. Thus, ascorbate completely inhibits the reduction of b by succinate in the presence of antimycin and there is no distinction between b_{562} and b_{566}. When added prior to succinate, ascorbate and antimycin could be added in either sequence and the same inhibition was manifested. If added after succinate, ascorbate and antimycin caused no detectable reoxidation of the rapidly reduced b. Ascorbate minus antimycin caused no inhibition of b reduction; antimycin minus ascorbate caused a slight inhibition of b reduction by succinate as shown below.

The amounts of antimycin required for the ascorbate-dependent inhibition of b reduction and for inhibition of electron transfer through the b-c_1 complex were found to be identical as shown in Figure 2. In agreement with the results of Slater and coworkers (11), inhibition of cytochrome c reductase by antimycin reflects a stoichiometry of one antimycin binding site per molecule of c_1. It seems likely that the same binding site is involved in the ascorbate-dependent inhibition of b reduction by succinate. Figure 2 also shows the slight inhibition of b reduction which is caused by antimycin in the absence of ascorbate. This effect of antimycin was not eliminated by adding excess ferricyanide.

It was of particular interest to establish if the ascorbate-dependent inhibition represents a change in the midpoint potential of a b species or a change in the kinetics of b reduction. As shown in Figure 3, although ascorbate plus antimycin completely inhibits the rapid reduction of b by succinate, there is a slow

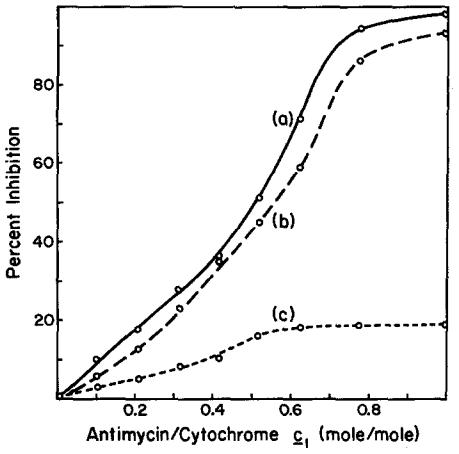

Figure 2 Effect of antimycin on cytochrome c reductase activity and reduction of cytochrome b by succinate in the presence of ascorbate. Curve (a) depicts the extent of inhibition of succinate-cytochrome c reductase activity. In the absence of antimycin the reductase complex had activity of 6.9 μmole cytochrome c reduced/min-mg. Curve (b) depicts the extent to which antimycin inhibits reduction of cytochrome b by succinate in the presence of 250 μM ascorbate. In the absence of antimycin, 2.8 nmole of b was reduced by succinate. Curve (c) shows the effect of antimycin on reduction of cytochrome b by succinate in the absence of ascorbate. Prior to addition of succinate, 500 μM ferricyanide was added to the sample cuvette.

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reduction of b such that all of the succinate-reducible b is eventually reduced. The inhibited b reduction proceeds as a pseudo-first order reaction, as shown in Figure 4, with a rate constant of 2.96×10^{-2}/min, which corresponds to a half-time of 23 min. The small portion of succinate-reducible b whose inhibition is caused by antimycin minus ascorbate also goes slowly reduced with the same rate constant as the fully inhibited b (data not shown).

Figure 3 also shows the rapid reduction of b which is induced when the ascorbate-dependent inhibition is reversed by addition of ferricyanide. This response is analogous to the oxidant-induced b reduction (2-5). The small amount of b whose rapid

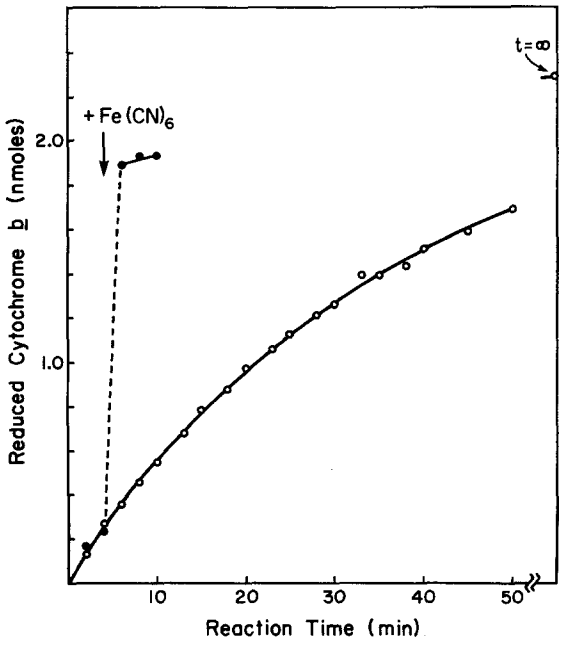

Figure 3 Time-course of the reduction of cytochrome b by succinate in the presence of ascorbate and antimycin. Succinate-cytochrome c reductase complex, containing 3 nmole of dithionite reducible b, was preincubated 5 min with 1.5 nmole of antimycin and 1 μmole of ascorbate in a volume of 1 ml. The reaction was initiated by addition of 20 μmole of succinate. Each point indicates the amount of reduced cytochrome b at the indicated time intervals after addition of succinate. The rapid reduction of b resulting from addition of 1.5 μmole of ferricyanide is indicated by the dashed line.

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reduction is not induced by ferricyanide corresponds to the b whose inhibition occurs with antimycin minus ascorbate, as can be seen by comparing Figures 2 and 3.

We conclude that the ascorbate-dependent inhibitory effect caused by antimycin is related to the oxidant-induced reduction of b (2-5). Our findings support the hypothesis (4) that the activity of cytochrome b is regulated by the redox status of cytochrome c_1 or another ascorbate-reducible component of the cytochrome b-c_1 complex. Mechanisms previously proposed to explain the oxidant-induced reduction of b have invoked a change

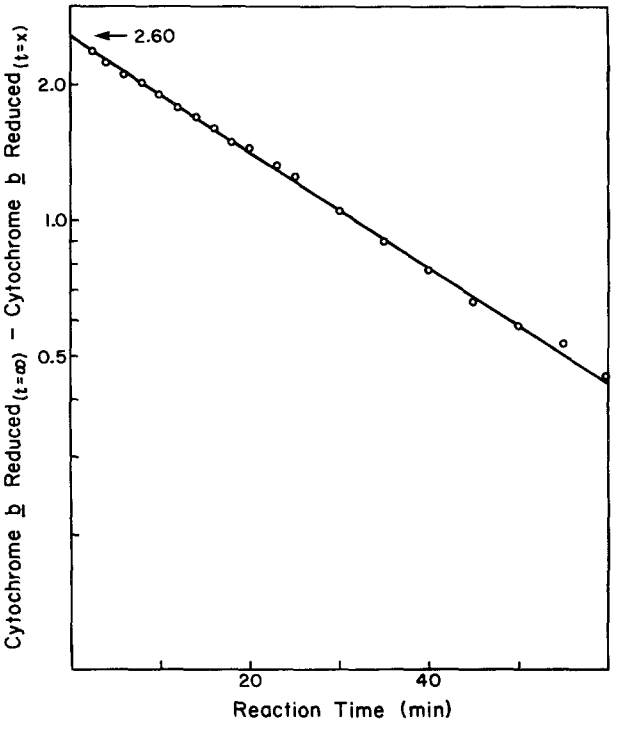

Figure 4 Semilogarithmic plot of the reduction of cytochrome b by succinate in the presence of antimycin and ascorbate. Each point represents the amount of succinate-reducible b remaining unreduced at the indicated time. A total of 2.60 μmole of b was reduced by succinate at "infinite time." The first order rate constant for the reaction shown is 2.96×10^{-2}/min.

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in the midpoint potential of cytochrome b and have restricted this regulation to the postulated energy transducing species, cytochrome b_T. The inhibition which we observe involves all of the succinate-reducible b, including b_{562} and b_{566}, and our results demonstrate that the regulation of b reduction can be explained as a change in the kinetics of electron transfer into cytochrome b. This does not exclude the possibility of a transient change in the reduction potential of cytochrome b, but such a change would have to occur in the continuous presence of ascorbate. Since this inhibition results in a 14,000 fold

increase in the half-time for b reduction, there is little practical difference between kinetic regulation and a thermodynamic event, except that only the latter fulfills the criteria whereby energy might be conserved in cytochrome b.

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