ELECTRON AND ENERGY REQUIREMENTS FOR CYTOCHROME b REDUCTION DURING THE OXIDATION OF TETRAMETHYL-p-PHENYLENE DIAMINE *

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The energy-dependent reversal of electron flow in the cytochrome region of the respiratory chain has been demonstrated (1-4). These and other similar reactions are conveniently studied with submitochondrial particles which are low in endogenous substrates and nucleotides. Reduction of DPN and endogenous Coenzyme Q_{10} by ascorbate in the presence of N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) (3,4) has been demonstrated in this manner. We wish to report some preliminary data on the ATP-dependent reduction of cytochrome b by ascorbate-TMPD in beef heart submitochondrial particles (BHP). These results suggest the modification of the reactivity of cytochrome b in the transition from phosphorylating preparations to non-phosphorylating preparations.

METHODS

Beef heart submitochondrial particles were prepared by sonication of beef heart mitochondria in the presence of Mg^{++} and Mn^{++} as described

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previously (5). Rat liver mitochondria were prepared in 0.25 M sucrose as described previously (6). Rat heart mitochondria were prepared by the method of Hagihara (14). All incubations were performed at 25° C, with a reaction mixture volume of 3 ml, as specified in the figure legends. Oxygen consumption and P:O ratios were measured polarographically with a Clark oxygen electrode. The difference spectra of the respiratory pigments were recorded with the techniques and instruments developed at the Johnson Foundation (7).

RESULTS

As shown in Fig. 1, the addition of ascorbate to a suspension of submitochondrial particles causes a slow oxygen consumption, as indicated by a downward deflection of the oxygen electrode trace. At the same time the spectrophotometric trace indicates a small reduction of cytochrome b and cytochromes c + c_1. When TMPD was added, the rate of oxygen uptake increased; a simultaneous increase in cytochrome c + c_1 reduction and an

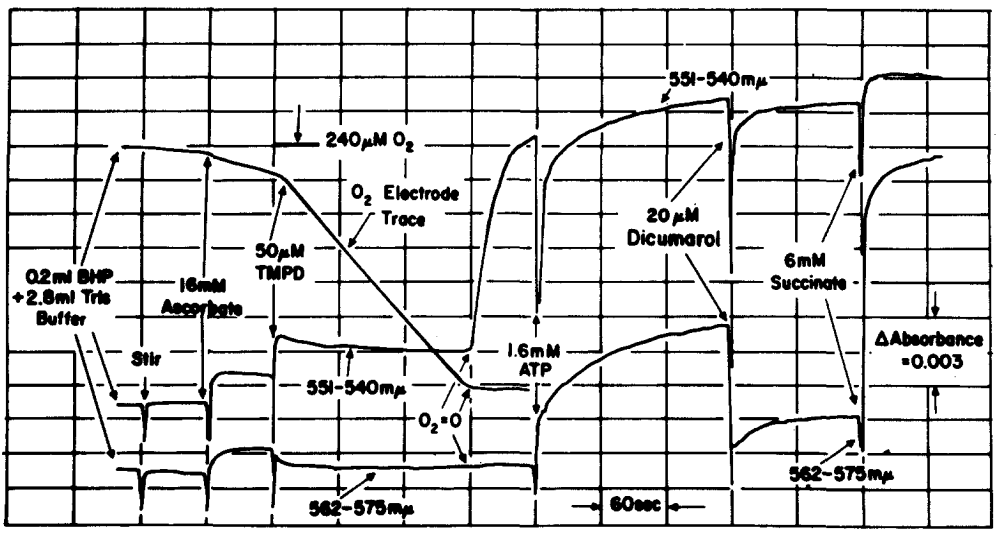

Fig. 1. Correlation of respiratory activity (O_2 electrode trace) with oxidation-reduction levels of cytochrome b (562-575 mμ) and cytochromes c + c_1 (551-540 mμ) in a suspension of beef heart particles (BHP) to which ascorbate, TMPD, ATP, dicumarol and succinate were added. The particles (2 mg protein/ml) were suspended in tris-sulphate buffer (0.05 M, pH 7.5).

oxidation of cytochrome b was observed. When the suspension attained anaerobiosis, a large reduction of cytochrome c + c_1 occurred, whereas cytochrome b remained oxidized. The subsequent addition of ATP to the anaerobic suspension caused a reduction of cytochrome b which became reoxidized rapidly on the further addition of oxygen and an uncoupling concentration of dicumarol. The final addition made in the experiment showed that the cytochrome b reduced by succinate amounted to more than twice that reduced by ascorbate-TMPD in the presence of ATP.

Similar experiments with tightly coupled rat heart mitochondria showed that the addition of ascorbate and TMPD produced a rapid reduction of cytochrome b. This reduction of cytochrome b was independent of the presence of rotenone and malonate, thereby eliminating the possible contribution of endogenous substrates. The addition of ATP was not necessary in this case, since high energy intermediates produced during the oxidation of TMPD in the cytochrome oxidase region could be efficiently utilized for the reversed electron flow. Consistent with this explanation, subsequent additions of uncoupling agents or ADP led to a reoxidation of the reduced cytochrome b. In the anaerobic state, succinate caused very little further reduction of cytochrome b when added to the ascorbate-TMPD treated system.

In other experiments similar to that shown in Fig. 1, the influence of antimycin A and oligomycin on the energy linked reduction of cytochrome b of submitochondrial particles was investigated. The addition of 0.04 μg antimycin A/mg protein, instead of dicumarol, caused the rapid reoxidation of reduced cytochrome b. This concentration of antimycin A was found to be the concentration required to inhibit succinate oxidation by more than 95 per cent. When 0.3 μg oligomycin/mg protein was added during the aerobic steady state, a slow and relatively small reduction of cytochrome b occurred. The oligomycin presumably prevents loss of the high energy intermediates produced during the oxidation of ascorbate-TMPD, permitting their utilization for energy linked cytochrome b reduction. The addition

of ATP to the oligomycin-treated particles did not promote any further reduction of cytochrome b.

In order to confirm the identity of the pigments responsible for the absorbancy changes at the pairs of wavelengths used in these experiments, difference spectra in the visible region were recorded at liquid nitrogen temperatures, using the trapped steady state technique described by Chance and Spencer (8). The insert in Fig. 2 shows an experiment similar to that seen in Fig. 1. The lower curve records the response of a system preincubated with ATP while the upper curve illustrates one with no ATP added. As seen in Fig. 2, the difference spectrum of ascorbate-TMPD treated particles contained absorption maxima at 549 and 553 mμ, indicative of reduced

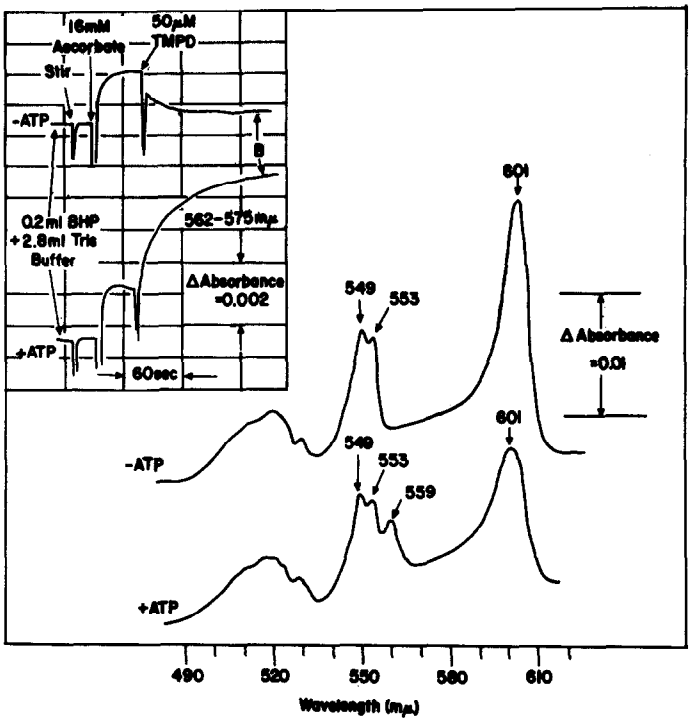

Fig. 2. Spectrophotometric recordings illustrating the influence of preincubation of beef heart particles (BHP) with ATP on the response of cytochrome b when ascorbate and TMPD were added. The inset of the figure shows the response of cytochrome b as measured in a dual wavelength spectrophotometer at 562-575 mμ. At the time indicated by the letter B, samples were withdrawn and spectra representing the difference in absorption between the treated samples and that of the preincubated suspensions containing no added ascorbate-TMPD were recorded at the temperature of liquid nitrogen. The peak appearing at 559 mμ in the difference spectrum of the ATP-treated particles is indicative of the reduction of cytochrome b. The system also contained potassium cyanide (1.5 mM).

cytochromes c and c_1. The peak of reduced cytochrome b at 559 mμ was seen only in the spectrum of ATP-treated particles.

These results provide direct evidence for the energy-linked reduction of cytochrome b and show that in submitochondrial particles only a portion of the cytochrome b may be on the energy linked pathway. In submitochondrial particles which have lost the capacity for tightly coupled respiration, by far the greater amount of cytochrome b is reducible by succinate and less in an energy linked reversed reaction. This is in contrast to results with tightly coupled heart mitochondria where most of the cytochrome b is reducible via an energy linked reversed reaction. These results suggest the hypothesis that the modification of mitochondrial structure during disruption may alter the accessibility or form of cytochrome b as it becomes displaced from the phosphorylating respiratory chain. Phosphorylating submitochondrial particles may represent an intermediate stage in the conversion of cytochrome b, which is linked obligatorily to electron transport and the phosphorylating respiratory chain, to a non-energy linked form which is present but not fully operative during electron transfer reactions. This hypothesis is consistent with the differences in kinetics of cytochrome b oxidation-reduction reactions observed by Chance and Williams (9) in mitochondria and in submitochondrial particles. The data also present an explanation for some earlier results of Chance (10) and Hommes (11), who found that the addition of ATP to sulfide inhibited mitochondria or submitochondrial particles, in which cytochrome b had been partially reduced by preincubation with succinate, produced additional reduction of cytochrome b. It would seem possible that in their experiments the reducing equivalents required for the ATP-driven reduction arose from the cytochrome c-cytochrome oxidase region by reversal of electron flow.

The present results are also pertinent to studies on the coupled oxidation of the ascorbate-TMPD system. The interpretation of the data obtained in these experiments depends in large part upon the correct identification of the site of entry of electrons into the respiratory chain.

The present studies identify the locus of entry as lying on the oxygen side of cytochrome b. Since concentrations of antimycin A sufficient to block succinate oxidation do not prevent oxygen uptake supported by ascorbate-TMPD, the locus can be more closely defined as lying on the oxygen side of the antimycin A block. Extending the conclusion of Howland (12), our experiments define the site of entry more precisely as lying between the antimycin A sensitive site and cytochrome c_1 (cf. Fig. 2). In recent experiments with rat liver mitochondria, we have confirmed that P:O ratios of greater than 1.0 are obtained during the coupled oxidation of ascorbate (15 mMolar)-TMPD (50 μMolar). However, when either rotenone or antimycin A was added to inhibit the oxidation of endogenous substrates, the P:O values were decreased to less than 1.0. Our data therefore do not support the hypothesis presented elsewhere (12,13), that more than one phosphorylation site is located between the antimycin A-sensitive site of the respiratory chain and oxygen.

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