EFFECT OF ATP ON THE REDOX CYCLE OF RESPIRATORY CHAIN-LINKED DPNH DEHYDROGENASE AND THE LOCALIZATION OF COUPLING SITE I

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SUMMARY

On adding DPNH to a phosphorylating membrane preparation in the presence of O_2 the reduction of a chromophore, which has been tentatively identified as nonheme iron of DPNH dehydrogenase, may be observed by dual wavelength spectrophotometry at 470–500 mμ. With rotenone or piericidin present a part of the chromophore remains permanently bleached even after exhaustion of the DPNH. ATP, however, causes rapid and complete reoxidation of the chromophore. This effect is abolished by oligomycin and dinitrophenol. Control experiments show that under these conditions the rotenone block between DPNH dehydrogenase and cytochrome b is substantially complete. Analysis of the data suggest that coupling site I is located between the specific binding site of rotenone and the nonheme irons responsible for the g=1.94 signal, both of which appear to be constituents of DPNH dehydrogenase.

Bois and Estabrook (1) described a cycle of absorbance changes on adding DPNH to an aerobic sample of ETP, a nonphosphorylating membrane preparation from heart mitochondria, which may be monitored at 470–500 mμ with a dual wavelength spectrophotometer. The characteristic features of the cycle are illustrated in Fig. 1 and the left side of Fig. 2 for untreated and rotenone-blocked samples, respectively. Although the experiments shown were conducted with a phosphorylating preparation (ETP_H), essentially the same results are obtained with ETP. The kinetic and thermodynamic parameters of this redox cycle have been analyzed by Gutman and Singer (2, 3) with the aid of specifically and unspecifically bound rotenone (4). For the purposes of the present paper the following aspects are of importance.

On adding DPNH to an aerobic ETP or ETP_H sample and monitoring the behavior of the system at 470–500 mμ, immediate bleaching (reduction) is observed. Since O_2 is present, DPNH is rapidly exhausted at which time recolorization (reoxidation) occurs (Fig. 1). Although the rate is fast, it may be measured with the Aminco-Chance spectrophotometer. The extent of reoxidation ($\Delta A_{reox.}$) is incomplete and a residual bleaching ($\Delta\Delta_o$) remains.

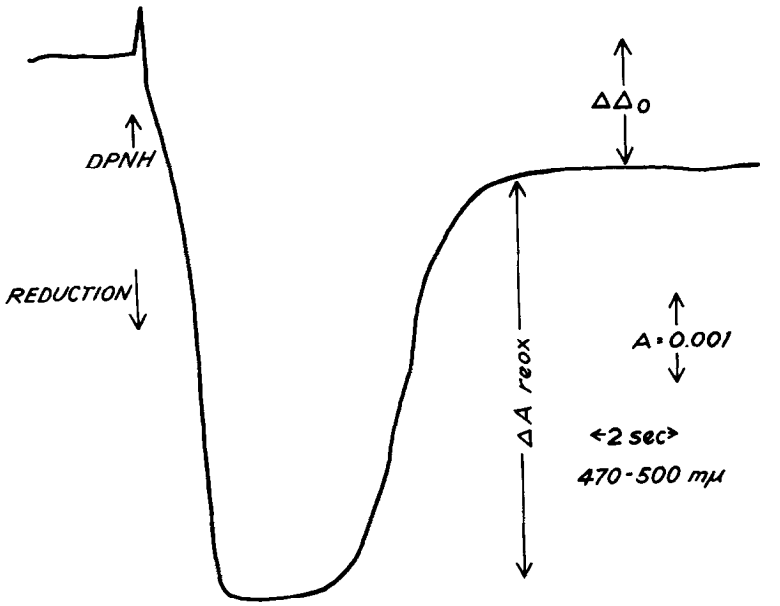

Fig. 1. Kinetics of absorbance changes induced by DPNH at 470–500 mμ. Beef heart ETP_H was suspended in 0.18 M sucrose–50 mM Tris-acetate, pH 7.4 — 5 mM $MgSO_4$ at a protein concentration of 4 mg/ml. The reaction was started by addition of 0.25 mM DPNH and absorbance was monitored at room temperature with the Aminco-Chance spectrophotometer.

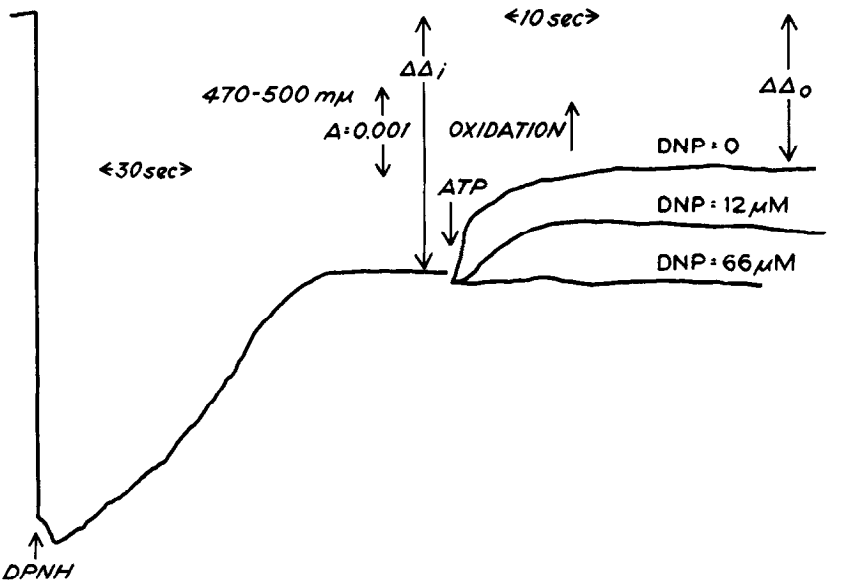

Fig. 2. Kinetics of absorbance changes observed at 470–500 mμ in rotenone-inhibited ETP_H and the effect of ATP. Experimental conditions were as in Fig. 1, except that the preparation was inhibited with 0.5 mμmole of rotenone per mg of protein. At the end of the redox cycle 3.6 mM ATP was added in the presence or absence of dinitrophenol, as indicated.

The chromophore responsible for the $\Delta\Delta_o$ value has been identified as a b-type cytochrome by its absorption spectrum (1,3) and, being the same in control and rotenone-inhibited samples, may be used to correct for the contribution of cytochrome b to the absorbance changes.

In the presence of rotenone (Fig. 2) or piericidin A, the rate and extent of bleaching by DPNH are unaffected, as expected from the fact that these inhibitors act on the O_2-side of DPNH dehydrogenase (5) but, depending on the concentration of inhibitor, the cycle is prolonged, and both the rate and the extent of reoxidation are decreased. The "permanently bleached chromophore" ($\Delta\Delta_i$), corrected for the $\Delta\Delta_o$ value contributed by the b-type cytochrome, appears to be due to nonheme iron - labile S moieties of DPNH dehydrogenase, as judged from EPR data and absorption spectrum (1-3). Although Bois and Estabrook (1) did not attempt to identify the chromophore responsible for the reversible bleaching by DPNH (ΔA_{reox}), studies in this laboratory have made it likely that the nonheme iron and perhaps the flavin components of DPNH dehydrogenase are responsible for most or all of this signal (2,3). In this connection it is important to note that in contrast to earlier attempts to follow the reduction of DPNH dehydrogenase spectrophotometrically, which were carried out in anaerobic conditions or in the absence of cytochrome oxidase activity with resulting interference by cytochromes, CoQ, and other nonheme iron and flavoproteins (5-8), the present experiments are carried out in the presence of O_2 and rotenone (or piericidin) and thus interference by other chromophores is minimized or eliminated.

The right side of Fig. 2 shows that ATP causes rapid and complete reoxidation of the "permanently bleached chromophore" provisionally identified as nonheme irons of DPNH dehydrogenase. After ATP addition the absorbance is exactly the same as in control samples not containing rotenone: thus only the $\Delta\Delta_o$ value due to the b-type cytochrome remains bleached (cf. Figs. 1 and 2). The conclusion that the effect of ATP involves the operation of coupling site I rests on the following evidence: DNP completely abolishes the effect and oligomycin (0.8µg/mg) inhibits the rate of reoxidation by 99%, and, further, ATP causes no reoxidation in nonphosphorylating preparations. The electron acceptor for reoxidation of the nonheme irons presumably involved here does not appear to be accumulated DPN, since prior treatment with Neurospora DPN-ase does not affect the reaction.

The present experiments permit a closer localization of coupling site I than has been possible in previous work. The following considerations are relevant in this connection. Hinkle et al. (9) suggested that coupling site I is between cytochrome b and an unidentified, autooxidizable component which is located on the substrate side of the rotenone sensitive site. In their studies with submitochondrial particles in the presence of succinate, malonate, sulfide, and antimycin A an ATP-dependent oxidation of cytochrome b was observed. The reaction was inhibited by rotenone and uncoupling agents.

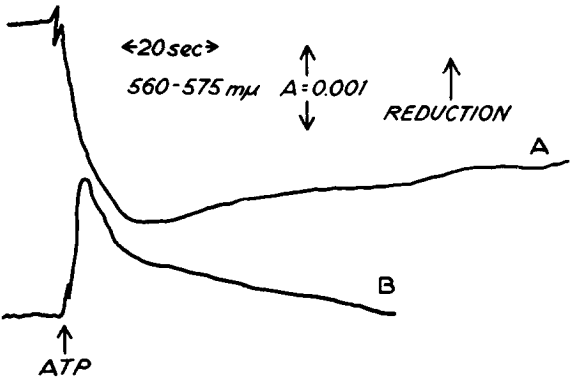

Fig. 3. Kinetics of absorbance changes of cytochrome b monitored at 560–575 mμ. Curve A, no rotenone; curve B, with 0.5 mμmole of rotenone /mg of protein. Experimental conditions were as in Figs. 1 and 2.

In our simpler system, with uninhibited ETP_H, at a time when all DPNH is exhausted relatively fast oxidation of cytochrome b occurs on addition of ATP (Fig. 3, curve A). Under these conditions electrons probably flow backward to the CoQ pool and perhaps via DPNH dehydrogenase to DPN. As ATP is exhausted by ATP-ase action (25% hydrolysis/min of the added ATP) b becomes slowly reduced again, perhaps by reversal of the process. As shown in curve B, when rotenone is present the ATP-dependent oxidation of cytochrome b is not only prevented (9), but an ATP-dependent reduction of b is observed. Since the rotenone block is complete under these conditions, electron flux is expected to come from the O_2-side of cytochrome b. The rapid reduction is again transient and the energy redistribution consequent on hydrolysis of the added ATP permits gradual reoxidation of cytochrome b.

Since comparison of curves A and B of Fig. 3 indicate that the rotenone block between DPNH dehydrogenase and cytochrome b is very efficient under the experimental conditions, the conclusion that coupling site I is on the substrate side of the rotenone block (9) is confirmed. Under these conditions the energy for the reduction of cytochrome b comes from coupling sites II or III, while that for the reoxidation of the 470–500 mμ chromophore from site I. A closer localization is possible, however, on the basis of the following facts. There are two specific binding (and inhibition) sites of rotenone per mole of DPNH dehydrogenase in membrane preparations (10). Both seem to involve noncovalent bonds to protein and lipid (3, 11). The protein involved in at least one of the two specific binding sites (probably both (3)) appears to be DPNH dehydrogenase itself, since mercaptide formation with Type V -SH group of the enzyme (12) abolishes one of the two specific binding sites and consequently prevents inhibition by

rotenone or piericidin. The -SH-dependent binding site is the one responsible for most of the inhibition of electron transport by rotenone and piericidin.

Another fact is that ferricyanide reoxidizes extremely rapidly the nonheme irons responsible for the DPNH-reduced EPR signal at g=1.94 (13) but cannot serve as electron acceptor for phosphorylation site I (14). Thus, assuming a linear sequence of the electron carriers in question, it appears that energy coupling site I is associated with DPNH dehydrogenase, located between the g=1.94 iron and the specific binding site of rotenone.

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