THE INTERACTION OF REDOX MEDIATORS WITH THE 'SECOND PHOSPHORYLATION SITE'; SIGNIFICANCE FOR THE CYTOCHROME b_T HYPOTHESIS

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1. Introduction

Using a potentiometric technique, Wilson and Dutton [1] and Chance et al. [2] have found that the measured midpoint potential of part of the cytochrome *b* in animal mitochondria increases by more than 250 mV upon addition of ATP. These authors consider the E_m shift to be due to formation of a 'high-energy' form of cytochrome *b* (b_T, see refs. [2,3]) having an energy-transducing function at the so-called coupling site 2 of the respiratory chain. However, as first pointed out by DeVault [4] and Caswell [5], and subsequently by others [6–8] the technique employed to measure E_m's is vulnerable to criticism because of its dependence on added redox mediators to bring the membrane-bound cytochromes into redox equilibrium with the electrode potential. Thus, if the mediators react rapidly with cytochrome $c+c_1$, but only very slowly with cytochrome *b*, then the apparent midpoint potential shift in energized mitochondria could simply be attributed to reversed electron transport from cytochrome $c+c_1$ to cytochrome *b* without a direct involvement of the latter in energy transduction.

A key question relevant to the interpretation of the apparent E_m shift pertains to the presence or absence of mediator-induced ATPase activity. Wilson and co-workers [3,9] claim that the redox mediators are able to 'clamp' the redox potential on both sides of 'Site 2' equal to the potential of the measuring electrode. A priori such a redox clamp would be expected to cause a rapid ATPase activity due to reversed electron flow from cytochrome $c+c_1$ to cytochrome, *b*, followed by recycling of electrons from the latter cytochrome to the former via the redox mediators [3,6–8]. Experimentally, however, the ATPase activity under the conditions of the potentiometric titrations has been reported to be very slow [9] and the measured high E_m form of cytochrome *b* has been found to be stable for at least 20 min [10]. Proponents of the reversed electron transport interpretation consider this situation to reflect the sluggish interaction between the redox mediators and cytochrome *b*, with the ATPase activity being limited by the inefficiency of the redox mediators in shunting electrons from cytochrome *b* to cytochrome $c+c_1$. However, the alternate interpretation, advanced by Wilson et al. [3], is that the ATPase activity is limited by a slow reaction intrinsic to 'Site 2'. With this postulate it is possible for the defenders of the 'b_T hypothesis' to assume partial equilibrium between the redox mediators and pertinent electron transport components so that the measured ATP-induced E_m shift could still be interpreted as being due to formation of a 'high-energy' form of cytochrome *b* [3,8].

From the above considerations it is obvious that the correct interpretation of the apparent ATP-induced E_m shift depends on the relative kinetics of reversed electron transfer and the mediator interac-

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tions. Remarkably, the latter have received very little attention despite the great significance that has been attached to the potentiometric results. The present report is addressed to the interaction of the redox mediators with the electron transport components at 'Site 2' making use of the mediator-induced ATPase activity as a measure of the rate of interaction. Evidence is presented which suggests that the concentrations of redox mediators used in the potentiometric titrations are far too low to overcome the effect of reversed electron transport and that, therefore, the observed E_m shift for cytochrome *b* cannot be taken as evidence for the existence of 'high-energy' forms of cytochrome *b* with direct function in energy transduction.

2. Materials and methods

Rat liver mitochondria were isolated as described previously [11], but in 0.25 M sucrose and 1 mM EDTA, pH 6.9. The mitochondria were subsequently washed in the same medium without EDTA. ATPase activity was usually measured by the pH-technique [12] assuming the n value for the reaction ATP + $H_2O \rightarrow ADP + P_i + nH^+$ to be 0.880 in the measured pH interval (pH 7.4 to 7.5). A Beckman pH electrode plus a calomel reference electrode were coupled via a Vibron Electrometer (Electronic Instruments, Richmond, U.K.) equipped with pH unit C-33-B, to a Goerz Servogor S strip chart recorder. The reaction was carried out in an open vessel, stirred rapidly with a magnetic flea, and maintained at 28°C. ATPase activity measured in this way was virtually completely inhibited by oligomycin confirming that it was due specifically to the respiratory chain ATPase. Comparable results were obtained by direct chemical determination of release of P_i. Following each experiment the buffering power of the suspension was calibrated by additions of standard HCl and KOH solutions. Mitochondrial protein determinations were carried out with the biuret method [13].

3. Results

Phenazine methosulphate (PMS) is considered to be one of the key mediators in the potentiometric titrations of *b* cytochromes. Fig. 1 shows an experiment examining the effect of PMS on the ATPase activity of rat liver mitochondria. The experiment is initiated by suspending the mitochondria in a weakly buffered medium containing ATP; rotenone and cyanide are also present to trap electrons in the respiratory chain, and the ATPase activity is measured by following the release of protons which accompanies ATP hydrolysis (see Materials and methods). The most important result is that addition of 1 mM PMS causes a striking stimulation of the latent ATPase activity, and that this activity can be inhibited by addition of antimycin at concentrations which block electron transfer between cytochromes *b* and c_1. In subsequent experiments it was found that the ability of PMS to stimulate the ATPase activity was dependent upon its redox state such that reduction of PMS by NADH or ascorbate abolished the stimulation reversibly. These findings show that the PMS-induced ATPase activity is due to reversed electron transport across 'Site 2' followed by recycling of electrons to cytochrome $c+c_1$ via a PMS-catalyzed redox shunt.

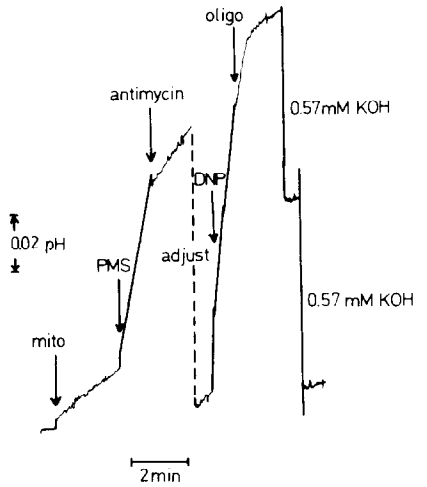

Fig. 1. Effect of phenazine methosulphate on latent ATPase activity. Rat liver mitochondria (1.2 mg protein per ml) were incubated for 2 min, in a medium containing 0.2 M sucrose, 20 mM KCl, 1 mM Tris–HCl, 3 μM rotenone, 1.5 mM KCN, and 3.8 mM ATP. The pH was adjusted to 7.5 with small aliquots of HCl and KOH. The reaction volume was 2.7 ml. Concentrations of other reagents used in the experiment were: PMS, 1 mM; antimycin, 0.2 μg per mg protein; 2,4-dinotrophenol, 0.13 mM; oligomycin, 5 μg per mg protein.

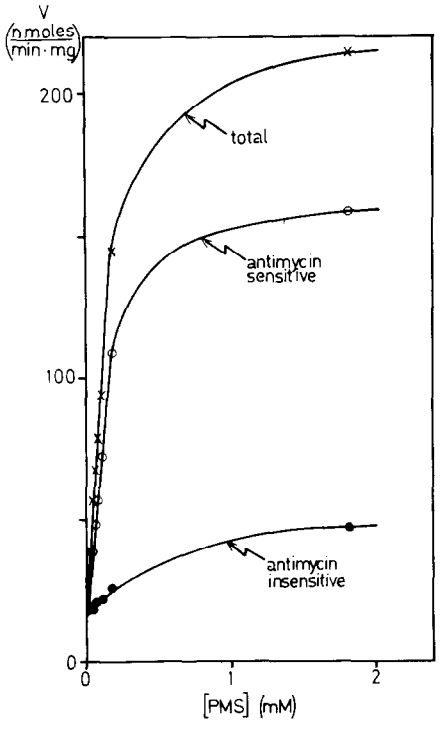

Fig. 2. ATPase activity as a function of PMS concentration. The experiment was carried out as in fig. 1. Rates of ATP hydrolysis were calculated from the number of moles of H^+ released as described under Materials and methods. A separate experiment was performed for each PMS concentration. 'Total' refers to the initial rate of ATP hydrolysis following addition of PMS. In each experiment, antimycin (0.2 μg/mg protein) was added after the PMS for determination of antimycin-sensitive and antimycin-insensitive ATPase activities.

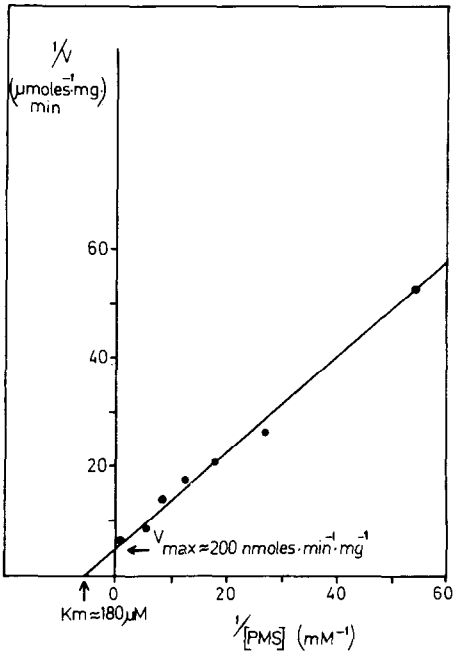

Fig. 3. Double reciprocal plot of antimycin-sensitive component of the ATPase activity in fig. 2.

The shunt requires the presence of oxidized PMS to accept electrons from the substrate side of 'Site 2' and reduced PMS to donate electrons to cytochrome $c+c_1$. Since the reaction of oxidized PMS with the substrate side of 'Site 2' is much slower than that of the reduced mediator with cytochrome $c+c_1$ (M.K.F. Wikstrom, unpublished results), the rate of the PMS shunt should be maximal at redox potentials higher than the E_m for PMS ($E_{m,7}$ = 80 mV, ref. [14]). This also was confirmed experimentally.

Having demonstrated a PMS-induced ATPase activity which is sensitive to antimycin, the key question pertinent to the interpretation of the potentiometric titrations is whether this activity is limited by a reaction step intrinsic to 'Site 2' as proposed by Wilson and coworkers [3] or, alternatively, whether it is limited by the concentration of PMS as expected from the reversed electron transport interpretation (see above). The concentration dependence for the PMS-induced ATPase activity is shown in fig. 2. At low concentrations of PMS, comparable to those used in the potentiometric titrations (approx. 50 μM), the ATPase activity is very low (cf. refs. [9,10], while at higher concentrations of PMS, the ATPase activity increases rapidly as expected from the reversed electron transport interpretation. The double reciprocal plot of these data (fig. 3) shows that the V_{max} of the antimycin-sensitive PMS-induced ATPase activity is 200 nmoles ATP hydrolyzed per min per mg protein, about half the activity induced by the subsequent addition of uncoupler (2,4-dinitrophenol). Incidentally, it should be noted that if the efficiency of energy transduction at 'Site 2' under the above conditions is assumed to be 'theoretical', i.e. 2 electrons transferred per ATP hydrolyzed, the maximal rate of reversed electron transport (400 ng electrons per mg protein per min) is similar to the State 3 rate of active phosphorylating respiration. The experimental conditions of very high phosphate potential and very high mediator concentration are indeed likely to be optimal for observation of fast reversed electron transfer.

The K_m for PMS is 180 μM under the above condi-

tions (PMS about 25% reduced) although the exact value is a function of the redox potential of PMS and much higher values for K_m are found when the redox mediator is highly reduced or oxidized. It should also be noted from fig. 2 that the PMS-induced ATPase activity remains largely sensitive to antimycin at all concentrations of the mediator, and thus PMS must continue to act by the 'shunt' mechanism outlined above. Only at very high concentrations does PMS appear to act as a weak uncoupling agent of the 'classical' type (i.e. the antimycin-insensitive activity). Note that in fig. 3 only antimycin sensitive activity has been plotted.

Among the other redox mediators used in the potentiometric titrations, phenazine ethosulphate (PES) gave data similar to that in fig. 2. However, *N,N,N',N'*,-tetramethyl-*p*-phenylenediamine (TMPD), duroquinone, pyocyanine, and 2-OH-1,4,-naphtoquinone at maximal concentrations of 2.0 mM, 30 μM and 0.15 mM, respectively, did not stimulate the ATPase activity in the presence or absence of PMS. The lack of stimulation by these mediators can be attributed to the ineffectiveness of their oxidized forms in accepting reducing equivalents from the substrate side of 'Site 2' (see ref. [15] for duroquinone), or, in the case of TMPD, which has a very high midpoint potential ($E_{m,7}$ = 260 mV, ref. [14], to the virtually complete reduction of the mediator under the present experimental conditions.

4. Discussion

The results presented indicate that very high concentrations of redox mediators (PMS and PES) are required to react efficiently on both sides of 'Site 2' (and particularly on the substrate side). Although such interaction is required for equilibrium during the potentiometric titrations, we conclude that for all previous titrations carried out in the presence of ATP, it appears that the concentration of redox mediators was far too low to overcome the effect of reversed electron transport so that redox equilibrium was not established. Thus, the ATP-induced E_m shift observed using such methods is likely to be due to reversed electron transport and cannot be considered evidence for an energy-transducing function of part of cytochrome *b*. It should be emphasized that our results directly contradict the postulate of Wilson et al. [3] that the mediator-induced ATPase activity is limited by a reaction intrinsic to 'Site 2' and show instead that the mediator-catalyzed redox 'shunt' across 'Site 2' is rate-limiting. Moreover, our data show that reversed electron transport across 'Site 2' is a fast reaction. A corollary of these findings is that it would be difficult, if not impossible, to measure the true midpoint potential of cytochrome *b* in the presence of ATP since the approach to redox equilibrium using high concentrations of redox mediators will always induce a large ATPase activity and effectively 'uncouple' the system.

The conclusion that the large apparent E_m shift in cytochrome *b* is due to reversed electron transport is supported by two lines of evidence other than that reported here. First, the effect is abolished by antimycin at concentrations which inhibit reversed electron transport between cytochromes $c+c_1$ and *b* [7,16,17], and second, the effect is missing in plant mitochondria [18] even though plant mitochondria contain a set of *b* cytochromes very similar to those of animal mitochondria [18–20]. Indeed, the lack of apparent midpoint potential shift for cytochrome *b* in plant mitochondria has recently been attributed to the fact that the redox mediators can react more effectively on the substrate side of 'Site 2' and short-circuit reversed electron transport more effectively than they do in animal mitochondria ([21] cf. data reported here).

Finally, it should be added that although the reversed electron transport interpretation has so far been tested and discussed only in relation to 'Site 2', the main considerations do apply equally well to components at other coupling sites. Thus ATP-induced apparent midpoint potential shifts in iron-sulphur Centre 1a [22] at 'Site 1' and cytochrome a_3 at 'Site 3' ([23] contrast [24,25]) has been proposed to reflect a direct role in energy transduction for these components. In neither case, however, has it been shown that the component can react effectively with the employed redox mediators. It is striking that all three E_m shifts are in exactly the direction predicted for reversed electron transport with cytochromes *b* and a_3 equilibrating with the electrode potential via cytochrome $c+c_1$, and with iron-sulfur Centre 1a equilibrating through the pyridine nucleotide pool.

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