CORRELATION OF THE STEADY-STATES OF REDUCTION OF COMPONENTS OF THE RESPIRATORY CHAIN WITH PHOSPHORYLATION STATES OF LIVER MITOCHONDRIA

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SUMMARY: Rat liver mitochondria were incubated with glutamate plus malate in the presence of 2 mM ATP and inorganic phosphate. Respiration and the steady-state ATP/ADP ratios were altered by addition of controlled amounts of purified ATPase. The redox states of NADH and of cytochromes b, c-c_1, and a-a_3 were followed at specified wavelength pairs. NADH, as well as the cytochromes, became more oxidized when electron transfer was stimulated by ATPase. In addition, all of the above components became further oxidized when the phosphorylation state ([ATP/[ADP x P_i]) was further diminished with higher levels of ATPase. The latter occurred without change in the rate of electron transfer. The data are taken to show a thermodynamic interaction between the phosphorylation steady-state and the redox state of the electron transfer carriers. It is emphasized that the state of reduction of respiratory chain carriers measured in extracts from intact tissues cannot be equated with the kinetic steady states defined by Chance and Williams (Adv. Enzymol. 17, 65-134).

The early studies of Chance and Williams (1) established important kinetic relationships between the redox states of mitochondrial electron carriers and the rate of electron transfer. These studies preceded the experimental feasibility of any detailed evaluation of the relationships between energetic- and redox states of the system. More recently, much attention has been given to energy-coupled influences on the redox characteristics of respiratory chain components (e.g. refs. 2-4 for reviews).

In this communication we report changes in the steady-states of reduction of mitochondrial respiratory chain components which accompany changes in the external phosphorylation state* generated by an ATPase in the presence of rat liver mitochondria. Although a valid mathematical treatment does not seem to us to be justified, we feel that the present data are compatible with the proposal (5,6) that, under physiological conditions, the steady-states of reduction of electron-transfer compo-

*The term 'phosphorylation state' used in this communication is defined as the ratio, [ATP]/[ADP] x [P_i] generated outside the matrix space of mitochondrial suspensions. 'Phosphorylation potential', (ΔG_p), refers to the free energy of hydrolysis of ATP present in the extra-matrix space and is numerically equal to the standard free energy of hydrolysis of ATP ($\Delta G_0'$), plus the concentration term 1.36 log [ATP]/[ADP][P_i].

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nents are in near thermodynamic equilibrium with the prevailing phosphorylation state.

Since the extent of oxidation of the cytochromes is shown to be affected by the phosphorylation state, even when the rate of electron transfer is unchanged, we would like to point out that respiration rates (or states) which are under control by the phosphorylation state cannot be equated with the resting (State 4, in which the phosphorylation state is very high) and active (State 3, in which the phosphorylation state is very low) states defined by Chance and Williams (1). Thus, it is probably erroneous to infer the respiratory states of intact tissue from measurements of the degree of oxidation of electron carriers. Some of these results have been presented in abstract form (7).

MATERIALS AND METHODS

Rat liver mitochondria were prepared according to Johnson and Lardy (8). The basic incubation medium contained 110 mM KCl, 33 mM Tris-HCl, 6 mM $MgCl_2$, 2 mM potassium phosphate and 2 mM ATP brought to a final pH of 7.4. Added substrate was potassium glutamate (10 mM) plus potassium malate (2 mM). The incubation concentration of mitochondria was always 1.0 mg of protein/ml. Reactions were carried out at 30° in an Aminco-Chance dual-wavelength spectrophotometer. The following wavelength pairs were used: nicotinamide nucleotides, 340 minus 374 nm; cytochrome b(T), 566 minus 576 nm; cytochrome b(K), 561 minus 576 nm; cytochrome c plus c_1, 552 minus 576 nm; and cytochrome(s) a-a_3, 606 minus 630 nm. In parallel incubations, reactions were terminated with perchloric acid (at the times indicated by arrows in the figures). The latter samples were neutralized in the cold and assayed for ATP and ADP. Reaction usually followed the sequence: mitochondria were added to the basic incubation medium without substrate; after 2.5 min. 500 nmoles of ADP was added in order to approximate State 2 of Chance and Williams (1); after an additional one min., substrate was added (State 4), followed by carefully controlled amounts of purified ATPase (mitochondrial F_1) to achieve the desired amount of respiratory stimulation and the desired phosphorylation state. The volume of ATPase added was constant in all incubations, regardless of the amount of ATPase added.

Parallel incubations were carried out under identical conditions in a Gilson oxygen polarograph in which the exact amount of ATPase required to obtain the desired amount of respiratory stimulation was determined. Added ATPase as indicated in the figures represent 50% (0.5) and 100% (1.0) of maximum stimulation of respiration (State 3 rate), and 500% (5.0) indicates a five-fold excess of ATPase over that required for State 3 rate of respiration. The numbers above the traces indicate the measured ATP/ADP ratios at the times indicated.

Purified ATPase was prepared as previously reported (9) by a modification of the method described by Penefsky (10). ATP and ADP were determined by standard spectrophotometric procedures.

RESULTS AND DISCUSSION

Figure 1 (top) shows that a high phosphorylation state was maintained on addition of substrate (State 4, ATP/ADP = 90), and nicotinamide nucleotides were strongly reduced. Then on stimulating respiration half-maximally with ATPase (top trace), NADH

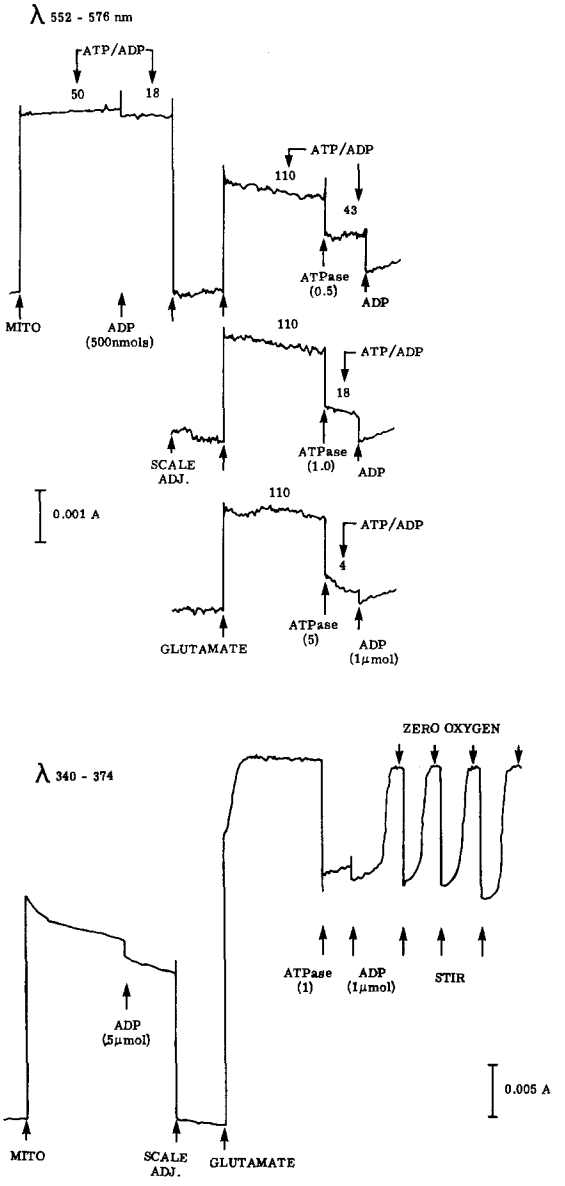

Fig. 1. The effects of ATPase-stimulated respiration and phosphorylation state on the oxidation of mitochondrial nicotinamide nucleotides.

reached a new steady-state of oxidation, and the new steady-state (see ref. 9) ATP/ADP ratio was decreased to 45. When respiration was stimulated maximally (middle trace), NADH was further oxidized, and the ATP/ADP ratio was decreased to 16. This kinetic response to oxidation resulting from an increased rate of electron transfer is, of course expected, based on the original observations of Chance and Williams (1),

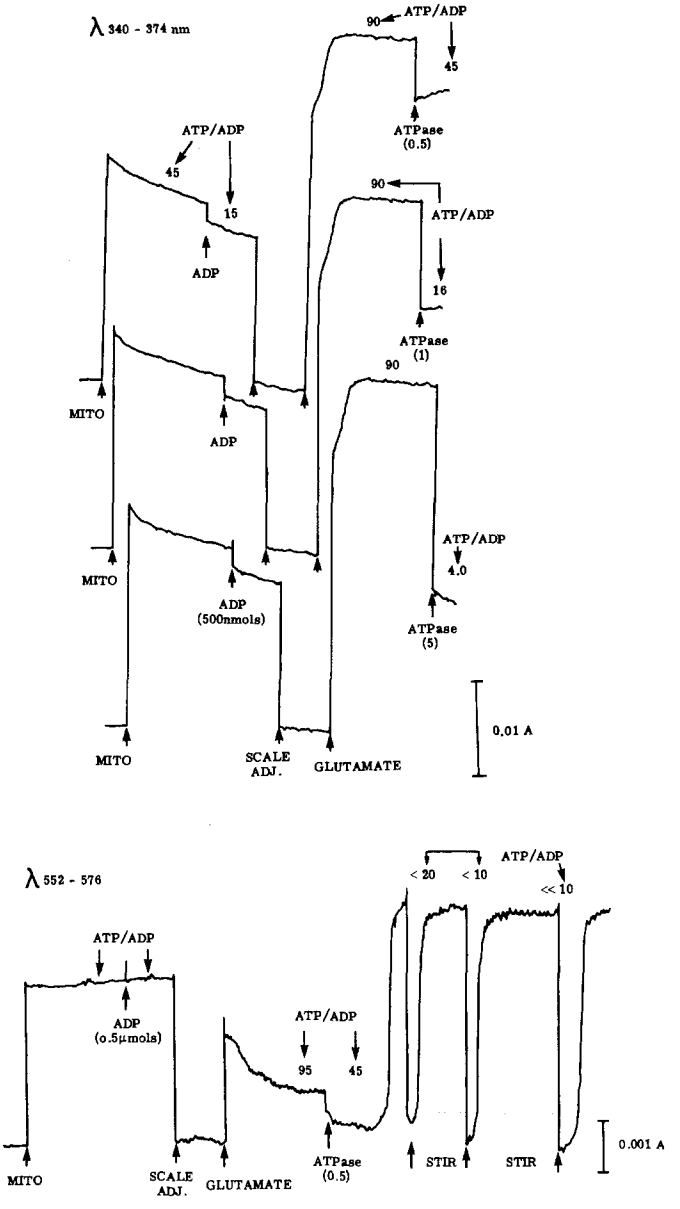

Fig. 2. The effects of ATPase-stimulated respiration and phosphorylation state on the redox of cytochrome c plus c_1.

and by many other workers. However, on further increasing the amount of ATPase to a large excess of that required for maximum respiration, mitochondrial NADH became still more oxidized. (It is unlikely that NADPH contributes appreciably to the changes observed, since it remains essentially completely reduced under these conditions [see

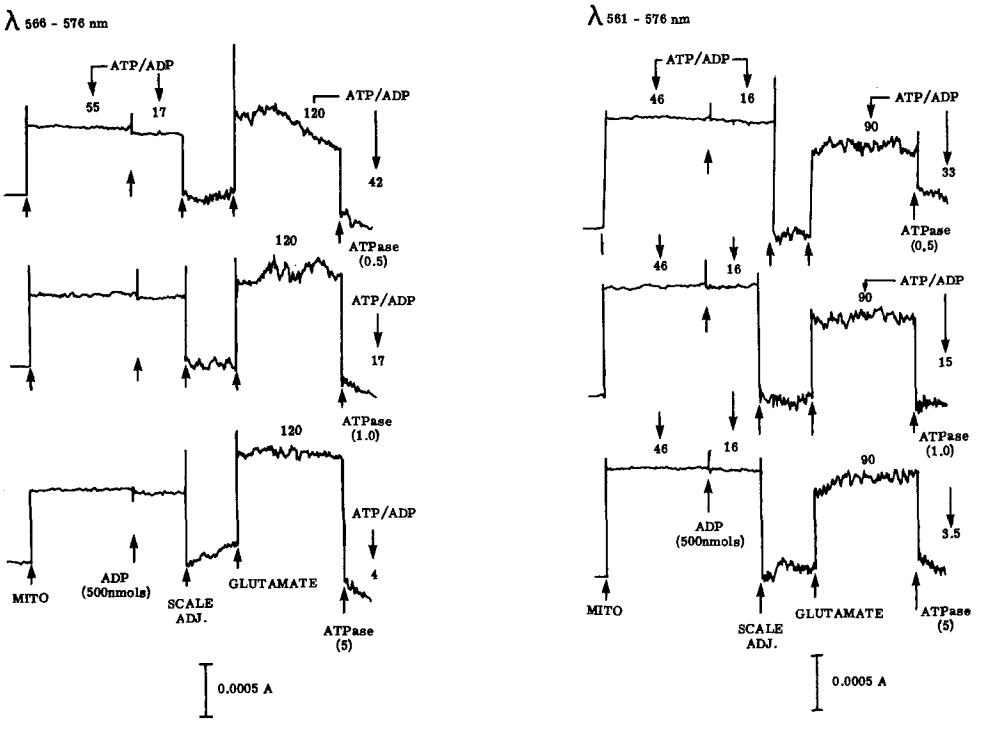

Fig. 3. The effects of ATPase-stimulated respiration and phosphorylation state on the redox of cytochrome(s) b. Fig. 3A, 566 minus 576 nm; Fig. 3B, 561 - 576 nm.

e.g. ref. 1]). It is important to emphasize that the rate of electron transfer was identical in the latter two cases (200 to 225 natoms oxygen per mg protein in the series here reported), whereas the substantial oxidation of NADH coincided with a decrease from 16 to 4 of the steady-state ATP/ADP ratio. This point is further elaborated in an experiment in which a State 3 rate of respiration is brought about with ATPase (Fig. 1, bottom trace). On addition of ADP (giving an ATP/ADP ratio of approximately 6), NADH became slightly more oxidized. Now, after a series of cycles of anaerobiosis, NADH became progressively more oxidized during intervening periods of respiration. This result would be predicted, since the ATP/ADP ratio is being continuously decreased during the anaerobic periods, owing to the action of added ATPase.

Figures 2-4 summarize the results of experiments carried out in a manner essentially identical to those described in Figure 1, except that the wavelength pairs were chosen to estimate the responses of cytochromes c-c_1 (Fig. 2), b_T and b_K (Fig. 3, top

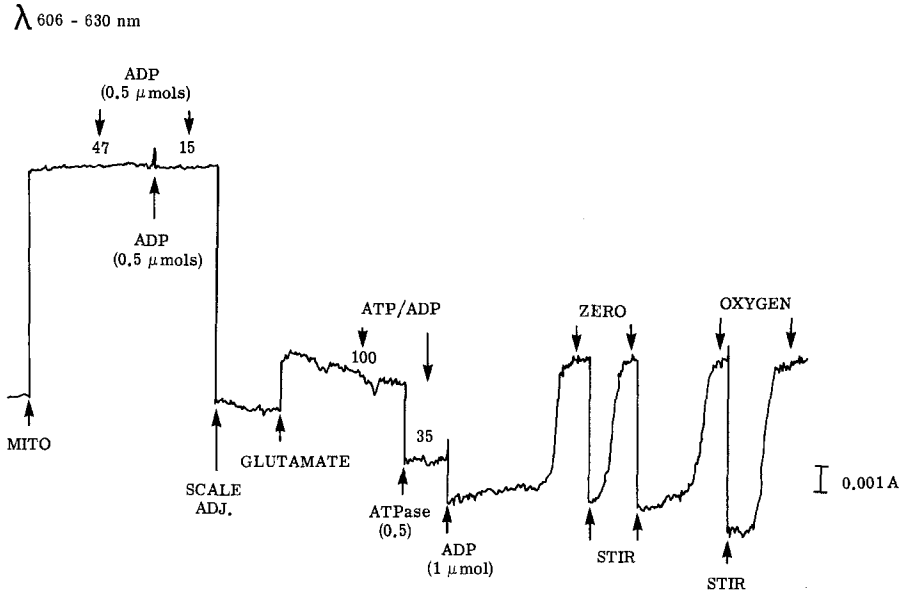

Fig. 4. The effects of ATPase-stimulated respiration and phosphorylation state on the redox of cytochrome c oxidase (606 minus 630 nm).

and bottom, respectively), and a-a_3 (Fig. 4). It is clear from these data that there was a tendency toward oxidation of all of the above cytochromes as measured when the phosphorylation state was decreased. Unexpectedly, no cross-over (ref. 1) (i.e., reduction of a-a_3) was observed on stimulation of respiration by ADP or by ATPase (Fig. 4). Indeed, the behavior of a-a_3 was in all cases similar to the response of the other cytochromes, as well as NADH. Furthermore, some difficulty was encountered in the case of a-a_3 measurements by an apparent shift in the baseline due to a dilution effect on addition of ATPase. Nevertheless, cycles of aerobic-anaerobic transition clearly show a response (oxidation) to progressively diminished phosphorylation states. There are a number of ambiguities with respect to measurements of the reduction state of cytochromes a and a_3: (a) there is disagreement as to the relative contributions of a and a_3 to the 606 minus 630 absorbance changes (see ref. 4); (b) the half-reduction potential of cytochrome a has been reported to undergo marked change as a function of the phosphorylation state (3); and (c) the point at which a cross-over occurs (or indeed whether or not one does occur) depends on a number of factors (for discussion see refs. 3 and 11).

The present data show directly an interaction between the phosphorylation state and the steady-states of reduction of several of the respiratory carriers - an interaction which is observed in the absence of changes in the rate of electron flux through the carriers. These results are interpreted to be compatible with, but do not prove, the suggestion that the redox state of the respiratory components are in near thermodynamic equilibrium with the phosphorylation state. These data suggest, in addition, that the above relationship may also obtain in respiratory steady-states higher (more rapid) than energetic steady-State 4. As previously noted (12), there is no simple relationship between the redox state of the mitochondrial NAD pool and the respiratory state imposed by a limiting ATPase. Rather, the 'reducing pressure' imposed by the primary substrate couple present tends to determine the degree of reduction of NAD. Thus, if a thermodynamic quasi-equilibrium between the phosphorylation system and the respiratory-chain components does indeed exist, the relationship most likely portrays a close relationship between the phosphorylation potential and the redox spans across portions of the electron transfer chain which are responsible for furnishing enough redox potential to allow energy transduction and subsequent ATP synthesis (13).

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