ON THE STOICHIOMETRY BETWEEN ADP AND OXYGEN CONSUMED DURING STATE 3 RESPIRATION

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Summary

The amount of oxygen consumed during state 3 respiration was plotted on the ordinate as a function of the dose of ADP or AMP added to aerobic suspensions of isolated rat-liver mitochondria. A straight line relationship was obtained. While the slope faithfully reflected theoretical values for P:O ratio, the straight line, when extrapolated to zero dose of the nucleotide, crossed the ordinate on the positive side. Thus, it is suggested that oxygen may be consumed not only for the ATP synthesis, but also for the process of mitochondrial transitions per se from state 4 to 3 and to 4.

Introduction

On the basis of the observation that the rate of respiration of coupled mitochondria differs considerably depending on the presence or absence of phosphate acceptor, Chance and Williams introduced the oxygen electrode method for the measurement of ADP:O ratio as an equivalent for P:O ratio (1). The validity of utilizing these ratios as an index of phosphorylation efficacy has since been amply supported by a wide variety of experimental evidence. One of the problems not fully explored yet is the question whether the ADP:O ratio is or is not constant irrespective of the dose of ADP employed to induce state 3 respiration. This seems important not only for defining theoretical ratios but also for understanding such phenomena as "ATP jump" (2) and "energization" of respiring mitochondria.

The results of this communication indicate that the amount of oxygen consumed in state 3 respiration is a linear function of the dose of ADP. However, scrutiny of the data disclosed that certain amount of oxygen is consumed for a process other than for ATP synthesis. It is suggested that this portion of oxygen consumption is utilized for the recovery of the energy expended in the transitions of mitochondrial state.

Methods

Male Wistar rats, weighing 100 to 150g, were used. Liver mitochondria were prepared by the procedure of Rasmussen and Ogata (3). Mitochondria were

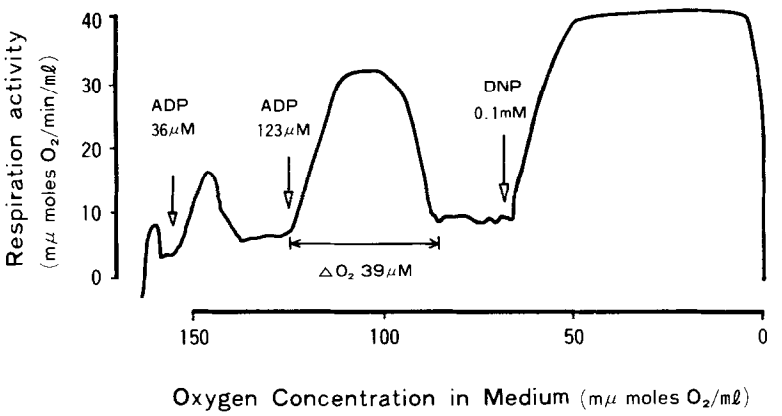

Fig. 1. A typical experiment showing respiratory responses of mitochondria to ADP and DNP.

The respiratory activity on the ordinate was recorded in relation to the medium oxygen concentration on the abscissa. because the oxygen concentration is high in the left and low in the right, the reaction proceeds from left to right of the trace, and upward deflections reflect the respiratory activation. The medium contained 5 mM succinate (+0.1 μg rotenone/ml) as a respiratory substrate. Mitochondria: 1.8 mg protein per ml.

incubated in an air-tight vessel at 22° with 3.8 ml of solutions of 225 mM mannitol, 75 mM sucrose, 3 mM $MgCl_2$, 0.5 mM EDTA, 10 mM KCl, 20 mM Tris-HCl, 3 mM Tris PO_4, pH 7.4, substrate, and other additions as indicated in the text. Oxygen concentration was monitored with a Clark type electrode (Yellow Springs Instrument Co., No. 5331) installed with a thin Teflon membrane. By means of passing through an appropriate electronic circuit (the outlines were described in Ref. 4), the output current from the electrode was transformed so that a continuous tracing was provided on an XY recorder where dO_2/dt was displayed on the ordinate in relation to the medium oxygen concentration on the abscissa. Adequate mixing was obtained by a stirring bar in the vessel driven by a magnetic stirrer. The whole system showed the response time and stability needed for the purpose of the present experiments. Additions were made with microsyringes through a tiny hole in the roof of the vessel. The oxygen concentration of the reaction medium was titrated downward with graded amounts of glucose in the presence of glucose oxidase and upward with graded amounts of H_2O_2 in the presense of catalase. Both approaches provided coincident results. Mitochondrial protein was assayed by a biuret method (5), where bovine serum albumin served as a standard. The results described in the text were reproducible in more than 3 repetition experiments.

Results and Discussion

A typical experiment is shown in Fig. 1. As recommended by Chance and Williams (1), a small amount of ADP (approximately 30 μM, in final) was added

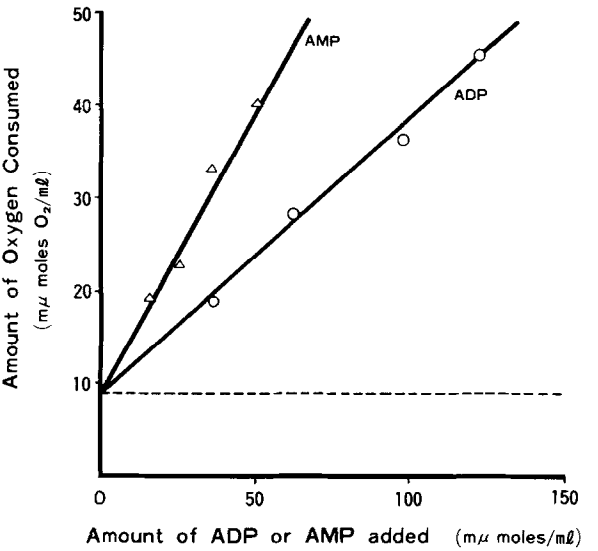

Fig. 2. Amount of oxygen consumed during state 3 respiration as a function of the dose of adenine nucleotide.
Experiments were performed as in Fig. 1., except for various amounts of AMP or ADP being employed to induce state 3 respiration.
Mitochondria: 0.7 mg protein/ml.

to attain an initial respiratory burst and subsequent state 4 condition. Then addition of ADP was made to concentrations as required for the experiment and the cycle of respiratory activation was recorded. In the present mode of presentation, determination of the transition point of respiratory state could be done relatively easily and moreover the amount of oxygen consumed during state 3 respiration was read directly from the figure (ΔO_2, in Fig. 1.). These technical advantages allowed us to examine the respiratory reaction evolved in response to lower concentrations of ADP. DNP (2,4-dinitrophenol) was added lastly for obtaining the point of zero oxygen.

The amount of oxygen consumed during state 3 respiration is plotted in Fig 2 as a function of the dose of ADP or AMP. A salient feature of the results is the fact that, though a straight line relation was obtained, the straight lines cut the ordinate on the positive side. It is remarkable that the same point was crossed on the ordinate by the line for ADP and that for AMP. These results indicate that, when the dose of the nucleotide was low, the ADP:O or the AMP:O ratio as calculated by the conventional method differs considerably depending on the dose of ADP or AMP.

On the basis of the following reasons, it is considered that the slope of the straight line should be used for calculation of the phosphorylation efficacy. In the first place, when the infinite dose of nucleotide was employ-

ed, the ADP:O ratio as calculated by the conventional method approaches to the reciprocal number of the slope. This value is fixed for a given system and may well represent the efficacy in an ideal situation. Moreover, the value should be close to the P:O ratio obtained by the Warburg method which utilizes considerable amounts of ADP. Secondly, if one accepts this value as an index of phosphorylation efficacy, the relationship between the AMP:O ratio and the ADP:O ratio fits excellently well the theoretical stoichiometry. For example, 2 was the numeral actually obtained from the data of Fig. 2. Theoretical value was similarly approximated in the relationship between ADP:O ratio of succinate oxidation and that of oxidation of NAD substrates. An example of the relationship between the ratio for succinate and that for β-hydroxybutyrate was 1.7 to 3.1.

If one admits the above considerations, the most intriguing question is as to the nature of the oxygen utilized extraneously other than for phosphorylation. In a given set of experiments where ADP was the only variable, the amount of "extra-oxygen" was constant irrespective of the dose of ADP or of the time spent during state 3 respiration. Therefore, such possibility is excluded that the "extra-oxygen consumption" represents state 4 respiration or some basic respiration which continues at a constant rate in state 3 respiration but without participating in the phosphorylation reaction. If such basic respiration actually took place, the magnitude of the "extra-oxygen" should alter in relation to the time spent during state 3 respiration. We propose as an alternative that this "extra-oxygen" is somehow related to the process of transitions of mitochondrial state per se.

If the number of ADP molecules is more than sufficient to saturate the mitochondrial sites for ADP, the respiratory state of whole mitochondrial population should shift from state 4 to 3 and then to 4 in the course of reactions such as in Fig. 1. In such situation, the over-all process of transitions themselves is not dependent on how much ADP was consumed. If one assumes that these transitions take place at the expense of some energy supplied by oxygen consumption, then this part of oxygen consumption should depend not on the dose of ADP or the time spent for state 3 respiration but on the number of mitochondria, the extent of energy difference between the states or on the efficiency of energization. Actually in a given set of mitochondrial preparation the magnitude of the "extra-oxygen consumption" was demonstrated to alter roughly in paralled with the concentration of mitochondria. Thus in an experiment where 3 graded amounts of mitochondria were employed the value, 5.4 ± 0.4 (mean ± S.E., n=3) μmoles O_2/g protein, was obtained when succinate served as substrate. It should be noted here that the amount of "extra-oxygen" was different from one mitochondrial preparation to

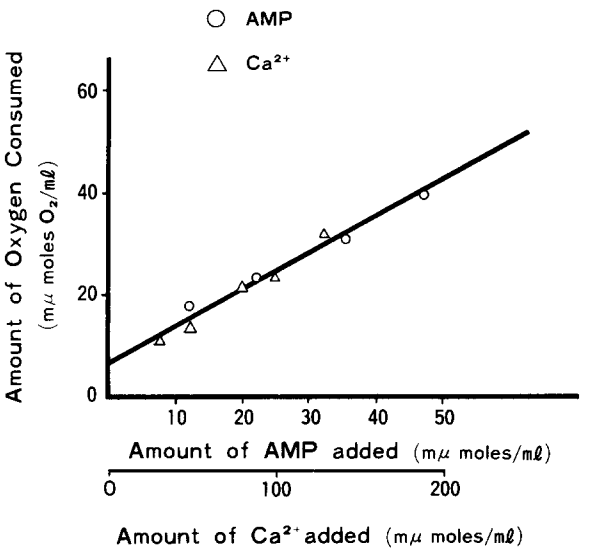

Fig. 3. Amount of oxygen consumed during activated respiration in relation to the dose of AMP or Ca^{++}.

Experiments were perfomed as in Fig. 2, except that the medium contained 5 mM succinate, 5 mM glutamate, and 1 mg per ml of bovine serum albumin, and that in one set of experiments oligomycin (1 μg/ml) replaced EGTA and graded doses of $CaCl_2$ were used to activate the respiration. Mitochondria: 0.8 mg protein/ml.

another and in a given set of preparation it was constantly lower when a NAD-dependent substrate was employed than when succinate was employed as an electron donor. IN one set of experiments the value of 0.7 μmoles O_2/g protein was obtained for β-hydroxybutyrate as compared to that of 1.5 μmoles O_2 for succinate. The results of Fig. 3 disclose that, when oxygen consumption owing to the Ca^{++}-induced respiratory burst was plotted as a function of the dose of Ca^{++}, again the linear function was obtained which is exactly identical to that obtained in the AMP-induced respiration. The outstanding point here is the fact that not only the magnitude of "extra-oxygen consumption" was identical but also the straight line itself was identical when Ca^{++} and AMP were scaled on the abscissa at 4 to 1 proportion. These results support the contention that the energy needed for phosphorylation and that for Ca^{++} accumulation is a stoichiometric relationship of 1 to 2 (6,7), and indicate that the energy lost during the mitochondrial transitions is identical in these two energy consuming reactions.

The addition of DNP to low concentrations (less than 5 μM; Experiments with higher concentrations of DNP were not feasible because of ambiguity in the determination of the point of transition.) caused an upward but parallel shift of the straight line (Fig. 4), implying that the phosphorylation efficiency in state 3 is not altered, but the energy lost during the transitions

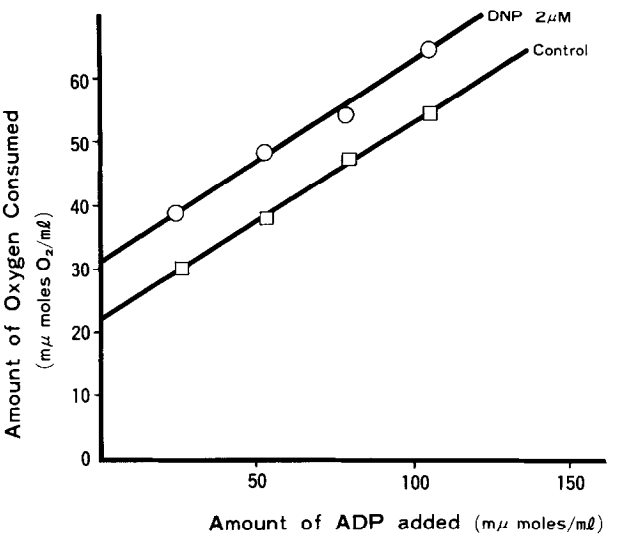

Fig. 4. Effect of small concentration of DNP on the stoichiometric relationship between oxygen and ADP consumed during state 3 respiration.
Experiments were performed as in Fig. 2, except that the medium contained 5 mM succinate and 5 mM glutamate and that in one set of experiments DNP was included to 2 μM. Mitochondria: 1.1 mg protein per ml.

is enlarged. This appears to have some bearing on so-called loosely coupled phosphorylation.

From the above findings it is concluded that at least in the present in vitro system the mitochondrial transitions are an energy requiring phenomenon. The results of Eisenhardt and Rosenthal clearly showed that certain amount of utilizable energy is actually stored in mitochondria of state 4, which is consumed on addition of ADP for the synthesis of ATP ("ATP jump", Ref. 2). It should be stressed here that according to this concept the ATP synthesis linked with electron transport occurs mostly after the depletion of the stored energy. After the completion of phosphorylation of added ADP, an extraneous electron transport should take place in order to supplement the energy stored in state 4 mitochondria. If the efficiency is comparable among the electron-transport dependent ATP synthesis, the electron-transport dependent energization to state 4 and the ATP synthesis elicitable at the expense of the stored energy, no "extraneous oxygen consumption" should occur. The fact, this was demonstrated, indicates that the efficiency is not comparable, and some extent of energy loss occurs which can be compensated for by "extraneous" election transport. The data of Fig. 4 suggest that the energy loss may occur in a manner similar to that occurring under the influence of a low concentration of classic uncouplers. Therefore the possibility can not be excluded that it reflects uncoupling occurring as an artifact in the in

vitro system. Though further work is needed to elucidate the nature of this phenomenon and such work will shed more light on the detail of oxidative phosphorylation, the present study indicates that when ADP:O ratio is measured as an index of phosphorylation efficacy, due care should be paid in terms of mitochondrial concentrations and the dose of ADP.

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References

1) Chance, B., and Williams, G. R., J. Biol. Chem. 217, 383 (1955).
2) Eisenhardt, R. H., and Rosenthal, O., Biochemistry 7, 1327 (1968).
3) Rasmussen, H., and Ogata, E., Biochemistry 5, 733 (1966).
4) Kinebuchi, Y., Nishiki, K., Kobayashi, S., Ogata, E., and Yoshitoshi, Y., Proc. Jap. Soc. Med. Electronics, 1972, P. 437.
5) Gornall, A. G., Bardawill, C.J., and David, M. M., J. Biol. Chem.177, 751 (1949).
6) Rasmussen, H., Chance, B., and Ogata, E., Proc. Nat. Acad. Sci. U. S., 53, 1069 (1965).
7) Chance, B., J. Biol. Chem., 240, 2729 (1965).