HEXOKINASE INDUCED, STEADY STATE COUPLED MITOCHONDRIA STUDIED BY INJECTION CALORIMETRY AND RESPIROMETRY

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INTRODUCTION

Adenylate concentrations in cells are at steady state under most physiological conditions. However, classical mitochondrial oxygraph experiments are carried out under non-steady state conditions, involving abrupt transitions from State 3 to State 4 (addition and depletion of ADP, respectively; Chance and Williams, 1955). During continuous mitochondrial ADP consumption and ATP production, the ATP/ADP ratio can be maintained at steady state by a metabolic load induced by the graded addition of an ATP utilizing enzyme such as hexokinase. The views on kinetic and thermodynamic mitochondrial respiratory control were substantially refined by studies under near-steady state conditions (for reviews see Brand and Murphy, 1987; Gnaiger, 1989; McCormack, Halestrap and Denton, 1990; Westerhoff and van Dam, 1987). The dynamics of such steady state coupled mitochondria have not yet been studied by direct calorimetry (compare Chien and Burkhard, 1975; Nakamura and Matsuoka, 1978; Tamura et al., 1989). Therefore, we investigated the relation between heat and oxygen flux in steady state coupled mitochondria as a function of the load imposed by the addition of various hexokinase levels.

METHODS

Rat liver mitochondria were prepared as described by Haller (1990). The mitochondria were stored in ice-cold isolation buffer and the experiments were completed within 8 hours after preparation.

The microcalorimeter used was the Thermal Activity Monitor (TAM, ThermoMetric AB, Sweden) equipped with a perfusion/titration system of 3.5 cm^3 stainless steel chamber containing 2.7 cm^3 buffer and air as the gas phase. A turbine stirrer (modification after Schön and Wadsö, 1986) was run at c. 130 rpm continuously during experimental and baseline experiments. The static reference chamber contained 4 cm^3 tap water. The TAM was operated at 25 °C in a constant temperature room set at experimental temperature. The respirometric measurements were carried out with the CYCLOBIOS Oxygraph (pp. 13-16) adjusted at 5 cm^3 chamber volume without gas phase.

The incubation buffer contained 0.22 $mol.dm^{-3}$ sucrose, 3 $mmol.dm^{-3}$ HEPES (pH 7.3), 11 $mmol.dm^{-3}$ $MgCl_2$, 2 $mmol.dm^{-3}$ KH_2PO_4, 0.5 $mmol.dm^{-3}$ EGTA, 1 $mmol.dm^{-3}$ ATP, 20 $mmol.dm^{-3}$ glucose and variable (0-11 $IU.dm^{-3}$) activities of yeast hexokinase. In the calorimetric and respirometric experiments, the mitochondrial protein concentration was 0.09 $mg.cm^{-3}$ air-saturated buffer. After stability of the calorimeter signal was attained, steady state activities were initiated by adding 54 mm^3 of a 250 $mmol.dm^{-3}$ K^+ succinate solution into the slowly stirred titration

chamber by means of an electronically controlled micro-syringe. Steady state heat flux was observed a few minutes after succinate titration and remained nearly constant for >1 h (Fig. 1). Total heat production of dynamically corrected data was calculated during a 10 min reaction period.

THEORY

Thermochemical calculations are required to interpret coordinated calorimetric and respirometric flux measurements, J_Q and J_{O_2}, in terms of an enthalpy balance. The experimental heat/O_2 flux ratio, Y_{Q/O_2} [kJ.mol^{-1} O_2],

$$Y_{Q/O_2} = \frac{J_Q}{J_{O_2}} \qquad (1)$$

is the calorimetric/respirometric ratio (CR ratio; Gnaiger and Kemp, 1990). The molar enthalpy of the catabolic half reaction (k) of succinate oxidation is the oxycaloric equivalent, $\Delta_k H_{O_2}$ (Gnaiger, 1983), specific for this substrate and HEPES buffer with a buffer enthalpy of neutralization, $\Delta_b H_{H^+}$ = 21.7 kJ.mol^{-1} H^+ (Schön and Wadsö, 1986). It is assumed that most of the steady state oxygen and carbon dioxide fluxes involve the exchange with the gas phase in the stirred chamber of the calorimeter.

$$\text{k: } 0 = -C_4H_4O_4^{2-}(aq) - 2\,H^+(aq) - 3.5\,O_2(g) + 4\,CO_2(g) + 3\,H_2O(l) \qquad (2)$$

$$\Delta_k H_{O_2} = \frac{1}{|\nu_{O_2}|} \sum_i \nu_i \Delta_f H_i + \nu_{H^+} \Delta_b H_{H^+} \qquad (3)$$

Inserting the enthalpies of formation of -908.7, -393.51 and -285.83 kJ.mol^{-1} for succinate dipolar ion, gaseous CO_2 and water, respectively, yields the molar enthalpy of catabolism of succinate in HEPES buffer of -422.7 kJ.mol^{-1} O_2.

In steady state coupled mitochondria, the concentrations of ATP and ADP are held constant, whereas the concentration of Glu-6-P increases when hexokinase is used, and the catabolic reaction is complemented by the endergonic (anabolic, a) net reaction,

$$\text{a: } 0 = -\Sigma\text{Glu-6-P}^{2-}(aq) - H_2O(l) + \text{Glu}(aq) + \Sigma P_i + \nu_{H^+} \qquad (4)$$

The enthalpy change of reaction (4) was calculated according to standard values listed by Tewari et al. (1988). Under the present reaction conditions, the fraction of Glu-6-P^{2-} and P_i^{2-} were 0.241 and 0.147, the other fractions appearing as protonized and Mg-complexed forms. From this the molar enthalpy of the phosphorylation reaction was calculated as -0.47 kJ.mol^{-1}, with a stoichiometric coefficient of proton production of 0.086. This yields the molar enthalpy of the anabolic reaction (4) of -2.34 kJ.mol^{-1}, which therefore is a slightly exothermic but strongly endergonic reaction.

At fully coupled succinate respiration, the ATP/O_2 ratio is maximally 5.7. Therefore, maximally -2.34 x 5.7 = -13.3 kJ.mol^{-1} O_2 are added to the oxycaloric equivalent of -422.7 kJ.mol^{-1} O_2, yielding a molar enthalpy change of -436 kJ.mol^{-1} O_2 for the catabolic-anabolic coupled reaction. For establishing the energy balance, this value is to be compared with the experimental heat/O_2 flux ratio, Y_{Q/O_2} (CR ratio; eq. 1).

RESULTS

Steady state coupled mitochondrial respiration was routinely initiated in the oxygraph experiments by injecting hexo-

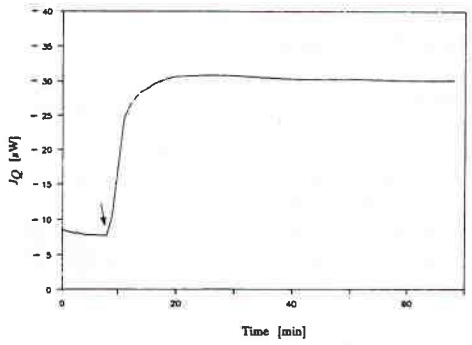

Fig. 1. Calorimetric signal of mitochondrial heat flux. Steady state was initiated by automatic succinate injection (arrow) into the incubation medium containing mitochondria and the hexokinase. Despite the dynamic time correction, the transition to constant heat flux was much slower than the transition to constant oxygen flux (compare Fig. 3).

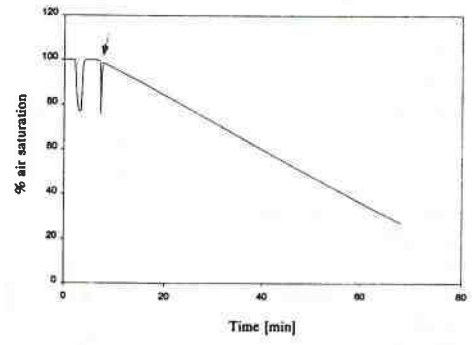

Fig. 2. Recorder trace of the POS signal from the CYCLOBIOS Oxygraph. Mitochondrial concentrations and all other incubation conditions were essentially equal as in the calorimetric experiments. 100% air saturation is equal to 242 nmol $O_2.cm^{-3}$. At the indicated point (arrow), 100 mm^3 of a 250 $mmol.dm^{-3}$ K^+ succinate solution was injected into the stirred measuring chamber, leading instantaneously to a steady state respiratory flux.

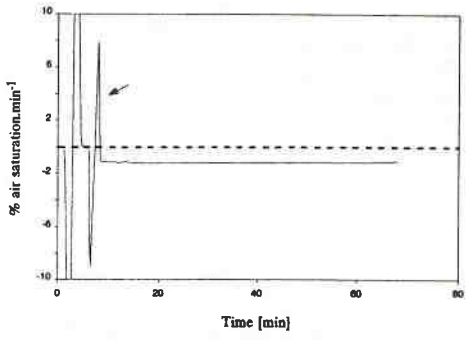

Fig. 3. First time derivative of the POS signal, shown in Fig. 2, yielding oxygen flux as a function of time.

kinase into the mitochondrial suspension, and a steady state was obtained immediately (Haller and Gnaiger, 1990). In the calorimeter, however, hexokinase-titrations always lead to unexpected high and long-lasting heat fluxes, superimposed on the steady state level. This hexokinase induced heat burst cannot yet be explained. When succinate was injected to initiate mitochondrial activity, this heat burst was absent and steady state heat flux was obtained relatively quickly (Fig. 1). Therefore, succinate injections were chosen in the coordinated calorimetric and respirometric experiments (Fig. 1 to 3).

Oxygen flux was linear for >1 h, indicating that the critical p_{O_2} was never

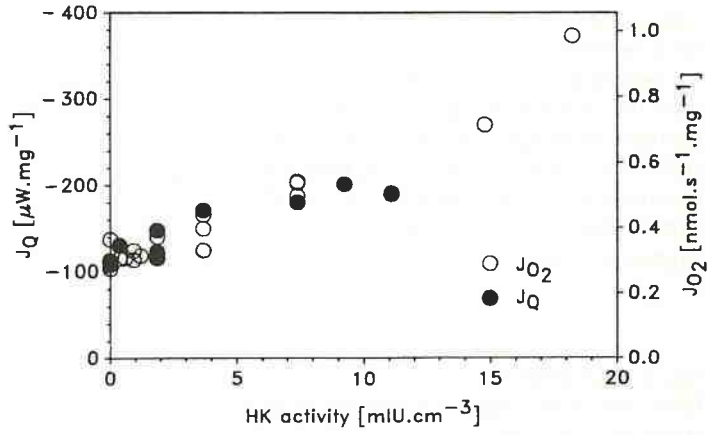

Fig. 4. Oxygen and heat flux of steady state coupled mitochondria at various hexokinase activities. The calorimetric measurements were consistent with the heat flux calculated from the oxygen flux measurements, assuming an oxycaloric equivalent of -379.0 kJ.mol^{-1} O_2.

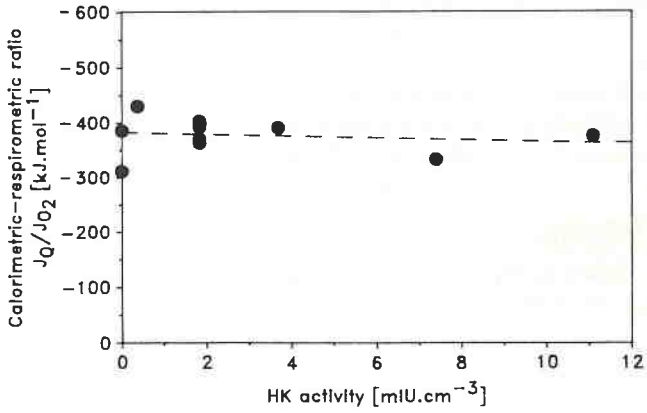

Fig. 5. The calorimetric-respirometric heat/oxygen flux ratio (CR ratio) was independent of the rate of phosphorylation induced by various activities of hexokinase. The average CR ratio amounted to -379 kJ.mol^{-1} O_2.

reached in the respirometer (Fig. 2) and less so in the calorimeter where oxygen exchange took place between the stirred sample and the water-saturated gas phase. In contrast to the calorimetric signal, the steady state observed in the respirometer stabilized almost instantaneously after succinate injection (Fig. 3). The discrepancy between the time behavior of oxygen and heat flux may be ascribed to the slower time response of the calorimeter (Poe, 1969), but the calorimetric signal was corrected for the time constant (second order) in our experiments.

At constant ATP concentrations, heat and oxygen flux were a function of the activity of added enzyme (Fig. 4). Fluxes were low at zero hexokinase concentration, and increased nearly linearly with enzyme activity until saturation was reached at hexokinase activities >40

mIU.cm^{-3}. Apparent respiratory control ratios (RCR; State 3/State 4 ratio), were approximately 3 in these experiments, that is significantly lower than the separate RCR determinations of 5 to 6, obtained by pulsed additions of ADP to the mitochondria containing 1 mmol.dm^{-3} ATP without addition of hexokinase.

Calorimetric-respirometric ratios (J_Q/J_{O_2}) were calculated from the respirometric and calorimetric data of Fig. 1. The CR ratios were independent of hexokinase stimulated mitochondrial activity (Fig. 5), averaging -379.0 ± 32.8 kJ.mol^{-1} O_2. This is slightly less exothermic than the theoretically expected value of -436 kJ.mol^{-1} O_2.

DISCUSSION

Further studies are required for explanation of the small discrepancy between the measured and calculated heat changes (Fig. 4). Specific binding effects induced by the buffer must be considered (Tewari et al., 1988). No enthalpy change is associated with potassium ion transport between mitochondria and buffer (Poe, 1968), but a heat absorbing endergonic reaction due to the formation of a phosphorylated compound in the presence of phosphate buffer is suggested by Poe et al. (1967). The >50% discrepancy due to an insufficiently exothermic heat/oxygen flux ratio during State 4 respiration (Nakamura and Matsuoka, 1978) cannot be explained, whereas the further reduction of the calorimetric/respirometric flux ratio in State 3 respiration is due to the endothermic phosphorylation of ADP to ATP (Gajewksi et al., 1986). An opposite effect of excessively exothermic effects in intact mitochondria compared to sonically disrupted mitochondria was noted by Dohm et al. (1970).

The high respiratory control ratios obtained in oxygraph experiments containing high mitochondrial concentrations indicated well coupled mitochondria (see also Haller and Gnaiger, 1990). To avoid oxygen limitation in the prolonged calorimetric steady state mitochondrial experiments, the mitochondrial concentration was reduced c. 10-fold in the coordinated calorimetric-respirometric study. It must be noted that the mitochondrial concentration possibly exerts an unexpected but significant effect on the respiratory control ratio and on the flux control kinetics of hexokinase activity (compare Fig. 4, p. 70 and Fig. 1, p. 64 in Haller and Gnaiger, 1990). This potentially important aspect is currently under investigation.

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