How does Oxygen Pressure Control Oxygen Flux in Isolated Mitochondria? A Methodological Approach by High-Resolution Respirometry and Digital Data Analysis

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Introduction

Little quantitative information is available on the oxygen dependence of mitochondrial respiration when compared to the extensive studies on adenylate control. This is due to the limited number of methods available to measure oxygen flux and oxygen pressure simultaneously at extremely low oxygen concentrations. The substantial methodological difficulties may in part explain why results on the O_2 dependence of mitochondrial respiration are controversial [1-4]. Complementary to this, a controversy exists as to the magnitude of intracellular diffusion gradients of O_2, the heterogeneity of the O_2 microenvironment for mitochondria, and the extent of hypoxia encountered under normal physiological conditions [5-6]. Oxygen may play an important part in the control of cellular respiration, particularly in the brain [7] and heart [8], and undoubtedly low O_2 exerts predominant control under environmental hypoxia and under ischemia [9,10]. We developed the approach of high-resolution respirometry as a sensitive and accurate method for the measurement of oxygen flux at extremely low p_{O_2} as relevant for mitochondrial [11] and cellular [12] respiration.

Methods

Respirometry: High-resolution respirometry is based on an optimized application of polarographic oxygen sensors [12] and instrumental design of the Oroboros® *Oxygraph* (Anton Paar, Graz, Austria [13]) in combination with digital data acquisition and analysis (Oroboros® Software DatGraf). Despite minimization of instrumental errors, such as a 10-fold lower oxygen back-diffusion as compared to typically used oxygraph systems, corrections of the raw data are necessary for maximum accuracy, particularly at oxygen levels <0.1% air saturation. Elimination of drift of the zero electrode current is achieved by internal calibration of zero p_{O_2} which extends the range of resolution below that specified for the polarographic oxygen sensor [14]. Measurements were carried out at 25 °C. Every second 1 data point was sampled for each oxygen sensor of the dual chamber respirometer. Noise and correction terms for O_2 consumption of the POS and back-diffusion of O_2 were quantified in experiments containing medium without any biological material (blank controls). Self-consumption of the POS at air-saturation levels averaged 3 pmol $O_2 \cdot s^{-1} \cdot ml^{-1}$. Maximum back-diffusion amounted to an average value of 0.5 pmol $O_2 \cdot s^{-1} \cdot ml^{-1}$. Blank control oxygen flux was a linear function of oxygen pressure, p_{O_2}, and was continuously corrected for. The response time of the oxygen sensor was experimentally determined and corrected, using a first order exponential time constant, τ, of 4 s. Maximum

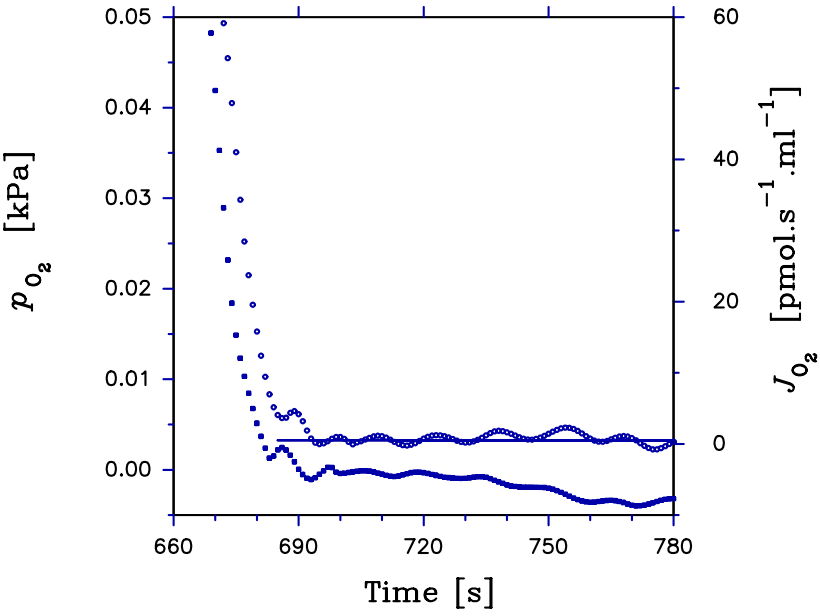

Fig. 1. Trace of oxygen partial pressure, p_{O_2} [kPa] (lower left trace), and the first time derivative of the signal, oxygen flux, J_{O_2} [pmol O_2.s^{-1}.ml^{-1}] (upper right trace), taken from a representative experiment with a suspension of isolated liver mitochondria (0.47 mg·ml^{-1}). Each symbol represents a data point. The straight line represents the linearly interpolated J_{O_2}.

normoxic flux, J_{max}, and oxygen pressure at half maximal flux, p_{50}, were calculated by a hyperbolic fit to the data smoothed with a 5-point polynomial function which retains the time resolution of the measurements.

Mitochondrial respiration: Tightly coupled (RCR > 8) rat liver mitochondria were isolated by conventional procedures. Mitochondria respired down to zero p_{O_2} and remained anaerobic for c. 10 min for internal calibration of zero drift. For methodological reasons volume-specific oxygen flux is shown, J_{O_2} [pmol O_2·s^{-1}·ml^{-1}]. The *Oxygraph* chamber volume was set at 3.0 ml. The medium in the chamber was stirred at 500 rpm with a magnetic bar sealed into glass. Mitochondrial protein concentrations ranged from 0.2 to 0.5 mg·ml^{-1}. The composition of the incubation medium was as follows: 0.23 M sucrose, 0.5 mM EGTA, 3.0 mM K^+HEPES, 11 mM $MgCl_2$, 5.0 mM K^+succinate, 5 µM rotenone, 1.5 mg/ml fatty acid free BSA, 1.94 mM ATP, and 10 mM KH_2PO_4; pH = 7.35.

RESULTS AND DISCUSSION

p_{O_2} and J_{O_2} of a typical experiment are shown as a function of time in the low-oxygen range when oxygen flux is already severely limited to <0.5 of maximum (Fig. 1). Even when mitochondrial respiration had dropped effectively to zero, p_{O_2} declined further at a slow and constant rate of 0.18 pmol·s^{-1}·ml^{-1}, compared to 98 pmol·s^{-1}·ml^{-1} determined for maximal mitochondrial flux, J_{max}. Accurate determination of and correction for the drift of the zero oxygen signal is crucial for quantification of the p_{50}. Over a 10 min period, the drift of the zero signal amounted to a change of 0.01 kPa. This uncertainty of ± 0.01 kPa for the zero position would be an additive component in the estimation of the p_{50}. A tenfold higher accuracy of the absolute zero position of O_2 is achieved by application of an additive "internal calibration", taking into account back-diffusion of O_2 and the stability of the zero current of the polarographic oxygen

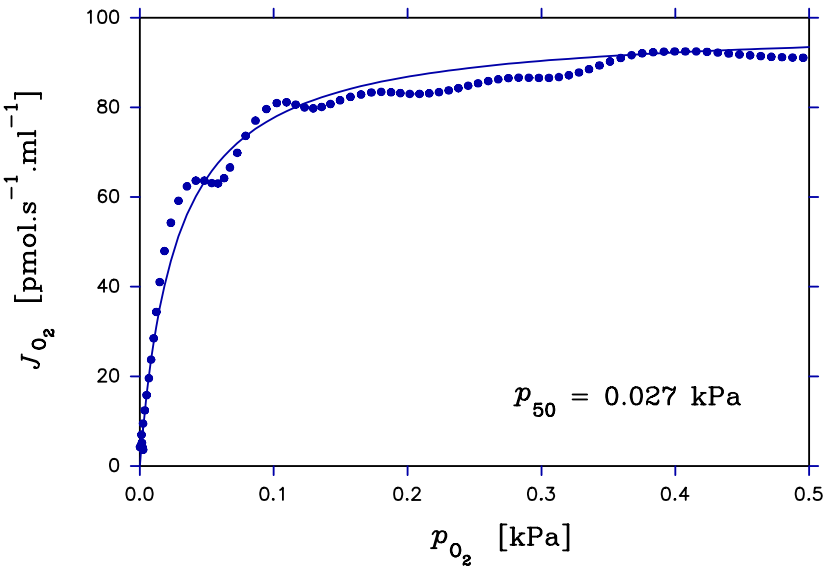

Fig. 2. Data taken from the experiment shown in Fig. 1. The solid line represents the best fit to a hyperbolic equation of the data (circles). The estimated p_{50} was 0.027 kPa (± 3.3% CV), fitted over the range of 1.1 kPa which corresponds to a 40-fold range of the p_{50}. J_{max} was 98.4 pmol $O_2 \cdot s^{-1} \cdot ml^{-1}$ ± 0.4% CV, that is 9% below the flux measured at 4-6 kPa. The goodness of fit was >0.98.

sensor. The actual value of the p_{O_2} at the compensation point, p^*, at which mitochondrial respiration is compensated by the diffusion of O_2 into the chamber, is a function of *(1)* the rate of back-diffusion relative to maximum mitochondrial respiration, and *(2)* the affinity of the mitochondria for O_2. If the actual p^* were on the order of 0.01 kPa, then mitochondrial respiration at this point, J^*, would amount to 25 pmol·s^{-1}·ml^{-1}. This estimate of J^* is 50 times higher than the experimentally de- termined back-diffusion. The estimate of p^* is therefore applicable for an accurate internal zero calibration.

Based on internal O_2 calibration, J_{O_2} is shown as a hyperbolic function of p_{O_2} (Fig. 2). The p_{50} of ADP- and oxygen-limited respiration of rat liver mitochondria was 0.027 kPa (corresponding to a concentration of 0.31 μM O_2). The major contribution of error in calibrating the absolute zero O_2 stems from signal noise (± 0.0013 kPa). The p_{50} was independent of mitochondrial concentration which serves as an important methodological test. Our results agree well with those obtained by an optical method based on the quenching of phosphorescence arising from palladium coproporphyrin [3]. Previously, the time resolution and stability of the zero oxygen signal of membrane covered polarographic oxygen sensors have been considered to be inadequate for accurately quantifying oxygen limitation in isolated mitochondria [15]. These limitations are overcome by the combination of optimized instrumentation and digital data analysis.

Recently we showed that P/O ratios of isolated mitochondria from different sources are maintained high under severe hypoxia when oxygen flux is depressed below that of state 4, using oxygen-injection microcalorimetry [11]. Thus not only respiratory flux but also mitochondrial function are amenable for the study under severely hypoxic conditions. The importance of the methodological refinements can be deduced from a comparison between the apparent K_m for ADP (20-300 μM) and the p_{50} corresponding to a 10-fold lower concentration range.

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