Mitochondrial Respiratory Control

EVIDENCE AGAINST THE REGULATION OF RESPIRATION BY EXTRAMITOCHONDRIAL PHOSPHORYLATION POTENTIALS OR BY \([\text{ATP}]/[\text{ADP}]\) RATIOS*

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To explore how mitochondria can respiration at high physiological, extramitochondrial phosphorylation potentials, two series of experiments were conducted. In the first, intact rat liver mitochondria were incubated in oxygraph medium containing 5 mM succinate (+rotenone), 1.0 mM ATP, 20 mM glucose, pH 7.2, at 37 °C. Yeast hexokinase (0.02 to 1.0 IU) was added to establish steady state rates of respiration. Samples were removed, assayed for ATP, ADP, and Pi, content, and ratios were calculated. As previously reported, low rates of respiration were observed at high phosphorylation potential (\([\text{ATP}]/[\text{ADP}] \times [\text{Pi}]\) or \([\text{ATP}]/[\text{ADP}]\) ratio values, and the rates of respiration increased as these values declined.

In a second series of experiments, only sufficient hexokinase was added to potentially stimulate respiration to 90% of the ADP State 3 rate. At constant hexokinase, 0.35 IU, ATP (5 μM to 10.0 mM) was titrated into the medium to establish steady state rates of oxygen consumption. Under these conditions, low rates of respiration correlated with low \([\text{ATP}]/[\text{ADP}]\) ratios and extramitochondrial phosphorylation potentials, while maximum rates of respiration were observed at high values of these ratios, the opposite of the previous experimental case. Therefore, it may be concluded that these extramitochondrial parameters per se exert little or no regulatory influence on the rates of respiration, and thus matrix ATP synthesis.

In both cases, the concentrations of ADP correlated with respiratory rates. Double reciprocal plots were used to estimate the apparent \(K_{\text{M,ADP}}\) for respiratory stimulation. The values are 56 μM for constant [ATP] and 15 μM at constant hexokinase. The value calculated from direct ADP pulses was 25 μM. Together, these results suggest that the most plausible explanation of respiratory control is the availability of ADP and the kinetics of its transport by the adenine nucleotide translocase, a hypothesis first proposed by Chance and Williams more than 25 years ago (Chance, B., and Williams, G. R. (1955) J. Biol. Chem. 217, 385-393).

In the cell, the rates of mitochondrial oxidative phosphorylation must be rigorously coordinated to meet the ATP demands of the cytoplasm. This phenomenon is called respiratory control, and how it is achieved \(in vivo\) is a cardinal issue in the field of cellular bioenergetics. Chance and Williams (1, 2) initially proposed that rates of respiration graded between State 3 and State 4 were a function of ADP availability. Klingenberg (3) subsequently postulated that the extramitochondrial phosphorylation potential, \([\text{ATP}]/[\text{ADP}] \times [\text{Pi}]\), was the parameter which determined the immediate rates of oxygen consumption. This hypothesis has received much experimental and theoretical support (4–14). In 1973, Slater et al. (15) postulated that respiratory control was simply a function of the \([\text{ATP}]/[\text{ADP}]\) ratio and somewhat independent of \([\text{Pi}]\). This theory has likewise achieved considerable attention (15–24). The former theory is based on thermodynamic considerations of ATP synthesis while the latter is founded on kinetic considerations of the adenine nucleotide translocase.

However, \(^{31}\text{P}\) nuclear magnetic resonance data on the \(in vivo\) rat brain (25), our own studies of the perfused heart, computer calculations of heart nucleotide content (26), and a recent re-estimation of tissue phosphorylation potentials (27) all cast considerable doubt on both of these theories. These data (25-27) suggest that the concentrations of free ADP in tissue are much lower than that estimated from the values of ADP measured in cellular acid extracts. As a consequence, the phosphorylation potentials and \([\text{ATP}]/[\text{ADP}]\) ratios may be at least an order of magnitude higher than previously estimated. In fact, the new values fall in control ranges where only very low rates of mitochondrial respiration were measured. Thus, the new information creates a paradox as to how mitochondria can actively respiration in tissues under the presumably inhibitory conditions of high phosphorylation potentials or high \([\text{ATP}]/[\text{ADP}]\) ratios. The design of our experiments was different from previous studies, in that we initiated steady state respiration under conditions of a constant concentration of hexokinase, an ADP-generating enzyme. Various concentrations of ATP were used to titrate constant respiratory rates. These results were compared to data obtained at constant [ATP] and variable hexokinase, the more traditional experimental approach. The results clearly show that under steady state conditions, respiration is directly controlled by the concentrations of extramitochondrial ADP, with little or no absolute correlation with either the extramitochondrial phosphorylation potentials or the extramitochondrial \([\text{ATP}]/[\text{ADP}]\) ratios.

EXPERIMENTAL PROCEDURES

Materials—The adenine and pyridine nucleotides, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, ethylene glycol bis(β-amino-
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In experiments designed to define the regulation of mitochondrial respiration, three experimental approaches have classically been employed. In the first, samples are removed from the oxygraph medium at different points during the State 3 to State 4 transition, the period of “dynamic control" (4, 6). The second approach has been to monitor the effects of translocase inhibitors (9, 20, 22) upon a series of complex mitochondrial reactions. The third method has been to preincubate mitochondria in fixed concentrations of ATP and P, and stimulate respiration by initiating ADP regeneration, either by ATPase or hexokinase titrations (15-18). A nonexperimental method involved computer simulation of respiratory control (7, 8, 13, 19, 24). From these combined approaches, arguments have been generated as to the degree to which phosphorylation potential, oxygen, substrate availability, [P,], or the adenine nucleotide translocase regulate the rates of oxidative phosphorylation.

In Fig. 1 we show our respiratory data for one of these

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7 The abbreviations used are: EGTA, ethylene glycol bis(aminomethyl)ether N,N,N',N'-tetraacetic acid, phosphoenolpyruvate, and the coupling enzymes were purchased from Sigma. The adenine nucleotides were the substantially vanadium-free grade. The enzymes were the following types: hexokinase, sulfate-free type F-300 from yeast; glucose-6-phosphate dehydrogenase, sulfate-free type IX from yeast; lactate dehydrogenase, Type XI from rabbit muscle; and pyruvate kinase, Type III from rabbit muscle. Sucrose and glucose were obtained from J. T. Baker, and all other reagents were of the highest purity commercially available. All solutions were prepared in deionized water.

Mitochondrial Isolation—The mitochondrial fraction was isolated from the livers of retired Sprague-Dawley breeder rats according to a modified method of Vecsei et al. (28). The isolation medium contained 0.5 mM K-EGTA, 3.0 mM K-HEPES (pH 7.4), 0.25 mM sucrose, and no protease. Standard methods of differential centrifugation were employed (29), and care was taken to maintain solutions at 2 °C. The respiratory control ratios of these preparations varied from 6 to 10, and the ADP/O ratios were in the range of 1.4-1.9 (5 mM succinate and 5 μM rotenone). The final pellet was resuspended at 25 mg of protein/ml, and stored at 2 to 4 °C for use within 4 h. Protein concentrations were determined by the method of Bradford (30) with nitrogen-calibrated, crystallized bovine serum albumin as the protein standard.

Oxygraph Procedure—Respiratory traces were obtained using a Clark oxygen electrode (Yellow Springs Instrument Co.) in a 3.0-ml water-jacketed oxygraph chamber maintained at 37 °C by a Haake E12 circulator-heater. Voltage changes were recorded with a Heath-Schumberger Model SR-2558 recorder. The oxygraph medium contained 0.25 mM succrose, 3.0 mM HEPES, 2.0 mM KHP, 5.0 mM K-succinate, 1.5 mM EGTA, 11 mM MgCl₂, 5 μM rotenone at pH 7.2. Stock solutions were maintained at 37 °C. A value of 390 mg atoms of oxygen/ml was used for the solubility of oxygen at 37 °C. In all experiments, ATP was added to the chamber 1 min prior to the addition of hexokinase in order to allow the mitochondria to phosphorylate any ADP present as a contaminant of the ATP solution. Mitochondrial protein concentration was 0.5 mg/ml in all experiments.

Sample Extractions—After the addition of hexokinase, steady state rates of respiration were achieved by 10 s and remained linear for an additional 2.0 min. At 1.0-1.5 min, a 2.5-ml sample of the oxygraph medium was removed and added to 0.75 ml of 18% HCO₃⁻. The mixture was immediately filtered through 0.45-μm Millipore filters and neutralized with 0.25 ml of 5 M K₂CO₃. The crystals of potassium perchlorate were removed by centrifugation at 3000 × g for 15 min. The supernatant solution was decanted and frozen for nucleotide and P i analysis.

Quantitation of ATP, ADP, and P i—The spectrophotometric assay mix for ATP analysis contained 10 mM HEPES, 0.6 mM NADP, 20 mM glucose, 3 mM MgCl₂, 2 IU/ml hexokinase, 2 IU/ml glucose-6-phosphate dehydrogenase, 1 mM AMP. The assay was linear up to 200 nmol of ATP. A 0.1-ml sample of the neutralized extract was added to 2.9 ml of assay mix which lacked glucose-6-phosphate dehydrogenase. The sample was zeroed on a Gilford 2400 recording spectrophotometer, the reaction was started by the addition of glucose-6-phosphate dehydrogenase, and the reduction of NADP was recorded. The mixture was immediately filtered through a Whatman no. 1 filter and the optical density was adjusted to 0.2 A at 340 nm. The reaction was started by the addition of pyruvate kinase. This system was also linear to an ADP content of 200 nmol; samples routinely contained less than 100 nmol of ADP.

Inorganic phosphate was measured by a colorimetric method (31). A 0.1-ml sample of the extract was added to 2.1 ml of the above assay mix (minus pyruvate kinase), and the optical density was adjusted to 0.2 A at 340 nm. The reaction was started by the addition of pyruvate kinase. This system also gave a linear ADP content of 200 nmol; samples routinely contained less than 100 nmol of ADP.

Inorganic phosphate was measured by a colorimetric method (31). A 0.1-ml sample of the extract was added to the molybdate mix, and the color was allowed to develop for 10 min. The optical density was measured at 660 nm. A standard curve of P i was prepared daily for calibration. Changes in optical density were linear up to a P i content of 600 nmol; experimental samples normally contained 150 to 200 nmol of P i.
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classical approaches, hexokinase-induced steady state respiration. Rat liver mitochondria were added to normal oxygraph medium containing 20 mM glucose. ATP was then added to a concentration of 1.0 mM, followed by the pulsed additions of hexokinase. Within 5 to 10 s after the addition of hexokinase, graded steady state rates of respiration were established. These rates were a function of the concentration of added enzyme. Even at the highest concentrations of hexokinase, the rates of respiration were linear for more than 2.0 min. Anaerobiosis then occurred, and the mitochondria were in State 5. The complete hexokinase titration data for our 15 experimental conditions are shown in Fig. 2. As seen in Fig. 2, State 4 was approximately 15% of the ADP State 3 rate, for a respiratory control ratio of 6.67. At high concentrations of hexokinase, respiratory rates slightly greater than ADP State 3 were observed. At intermediate levels of hexokinase, rates of respiration between State 4 and State 3 were achieved. At 1.0 min after the addition of hexokinase, samples were removed, perchloric acid-extracted, assayed for [ATP], [ADP], and [P] as outlined under "Experimental Procedures." From these data, the indicated ratios were calculated. Phosphorylation potential data are expressed as M⁻¹.

### Table I

**Comparison of measured and calculated values for tissue phosphorylation potentials and [ATP]/[ADP] ratios**

Data are taken from: (a) Ref. 26; (b) Ref. 24; (c) Ref. 32; and (d) Ref. 25.

<table>
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<th>Measured</th>
<th>Calculated</th>
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<td>Log cytoplasmic phosphorylation potentials</td>
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<td>Muscle</td>
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<td>Liver</td>
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<td>Heart</td>
<td>2.59°</td>
<td>4.88°</td>
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<tr>
<td>Cytoplasmic [ATP]/[ADP] ratios</td>
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<td></td>
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<tr>
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<td>82°</td>
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As these parameters decline, there is an increase in the rates of respiration. Maximum rates are observed at log phosphorylation potentials less than 3.0 or at [ATP]/[ADP] ratios less than 5.

However, from the standpoint of cell bioenergetics, there is a clear problem with the data of Fig. 3. Table I summarizes the best current estimates of these parameters in tissues. The measured values were derived from acid extracts of whole tissue (27, 33), while the calculated values were determined by 31P NMR (25), the computer analysis of heart adenine

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**Fig. 4.** Titration curve for respiratory stimulation at constant hexokinase and variable [ATP]. The amount of hexokinase added was limited so that sufficient to stimulate respiration to approximately 90% ADP State 3. The indicated concentration of ATP was added first, then followed by the addition of hexokinase. The inset presents data at low concentrations of ATP, between zero and the first data point in the full figure.

**Fig. 5.** Correlation of respiratory rates to phosphorylation potentials (A) and [ATP]/[ADP] ratios (B) at constant hexokinase and variable [ATP]. Respiratory rates were taken from Fig. 4, and samples were processed as described in Fig. 3.
nucleotides (26), or from enzyme equilibria data (27). In Table I, we see that in all cases the calculated values for these parameters are more than a order of magnitude higher than the measured values. This results from the fact that the concentration of free ADP in cells is quite low (25-27). It appears that most of the measured ADP is actually tightly bound and not in a soluble form. In heart and muscle, this binding is presumably to the G-monomers of actin. Thus, the data of Table I show that the calculated tissue values of these parameters fall at the low control range, where one would expect only minimal rates for tissue oxygen consumption (see arrow, Fig. 3A). This, however, contradicts the known physiology of these tissues. In heart, for example, the normal rate of respiration is approximately 50% of its maximal capacity (33). In order to resolve this apparent and important paradox, a second series of experiments was conducted.

In the experiment presented in Fig. 4, the amount of hexokinase added to the oxygraph medium was limited. Only the amount of hexokinase which stimulated respiration to 90% ADP State 3, in the presence of 20 mM glucose and 1.0 mM ATP, was used. In other words, we did not want to “overdrive” the capacity of mitochondrial respiration to phosphorylate ADP to ATP. This amount of hexokinase varied from 0.25 to 0.50 IU/3.0-ml system. At constant hexokinase, graded rates of respiration were achieved by variations in the initial concentrations of ATP (Fig. 4). Data at low ATP concentrations are presented in the inset. Fig. 4 shows that by varying [ATP] we again achieved rates of respiration from 16 to 110% of ADP State 3. At 1.0 min following enzyme addition, samples were removed and processed as previously described (Fig. 2). The data correlating respiratory rates with measured phosphorylation potentials and [ATP]/[ADP] ratios are shown in Fig. 5. It is immediately apparent that there are significant differences between the data of Fig. 3 and Fig. 5. We now see that under conditions of constant enzyme and variable [ATP], low rates of respiration correlate with low phosphorylation potentials (Fig. 5A) and low [ATP]/[ADP] ratios (Fig. 5B). Maximum rates of respiration are observed at high values of these indices. In control experiments conducted at 1 mM ATP and 20 mM glucose, the phosphorylation potentials and [ATP]/[ADP] ratios remained constant during 2.0 min of steady state respiration. Since our samples were taken at 1.0 min, the results of Fig. 5 cannot be related to sampling errors. While the lines in Fig. 5 were drawn to suggest the trends of these data, one can also see that at a constant phosphorylation potential value of 3.7 (Fig. 5A) respiratory rates vary from 35 to 110% of the ADP State 3 rate. In a similar manner, at an [ATP]/[ADP] ratio of 10 (Fig. 5B), respiratory rates also increase across the range of 35 to 110%. Therefore, these data alone (Fig. 5) suggest that there is almost no meaningful relationship between these indices and respiratory control.

The parameter which unifies the data of Figs. 3 and 5 is shown in Fig. 6. In Fig. 6, respiratory rates are plotted as a function of the measured concentrations of ADP. The data of Fig. 6A were taken from experiments presented in Fig. 3, while Fig. 6B was derived from the data of Fig. 5. In both cases, there is a direct correlation between respiratory rates and [ADP]. To estimate the apparent $K_{ADP}$ for respiratory stimulation, the data for Fig. 6 are replotted in double reciprocal form (Fig. 7). The abscissa intercepts are equivalent to $-1/\text{K}_{ADP}$. The values derived from Fig. 7, as well as data obtained from direct, pulsed additions of ADP into normal oxygraph media, are presented in Table II. The value for the direct ADP addition, 25.3 $\mu$M, is well within the range initially reported by Chance and Williams (1). The higher value observed at constant [ATP]-variable hexokinase (56 $\mu$M) reflects the influence of 1.0 mM ATP at the lower rates of respiration. The third value of 14.6 $\mu$M is somewhat less than that observed by direct ADP additions but is still within the kinetic range for the calculated concentrations of free ADP in the tissue (26, 27).
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DISCUSSION

For a considerable number of years now, one of the goals of bioenergetics has been to quantitatively describe the control of mitochondrial oxidative phosphorylation. Two major indices of adenine nucleotide regulation have been formulated, the phosphorylation potential ratio and the [ATP]/[ADP] ratio (3-24). The data presented in this paper substantially undermine the validity of both of these theories of respiratory control. This view is based on a comparison of the data of Fig. 3 to those of Fig. 5.

In Fig. 3, very low rates of respiration correlate with log phosphorylation potential values of 4.75 to 4.85, and [ATP]/[ADP] ratios of 100 to 150, while in Fig. 5 maximum rates of respiration are noted at these exact values. The converse is also true. In Fig. 3, the highest rates of respiration are observed at [ATP]/[ADP] ratios of 5 and less, and at log phosphorylation potential values less than 3.1. In Fig. 5, however, these are precisely the values where we observe the lowest respiratory rates. And finally, in Fig. 5, we note that at a constant phosphorylation potential value of 3.7, or at an [ATP]/[ADP] ratio of 10, the measured rates of respiration vary from 35 to 110% of the ADP State 3 rate.

It is important to understand how two such remarkably divergent sets of data could be obtained. Let us first consider the experimental design of Figs. 1 to 3. In these experiments, we begin with a high and presumably constant concentration of ATP (1.0 mM). The rates of respiration are regulated by the amount of hexokinase added to the system. At low concentrations of enzyme, the rates of ADP production will be marginal, and the concentrations of free ADP will be quite small. When these values are inserted into the equations, it is apparent that high values of phosphorylation potential and [ATP]/[ADP] will be obtained. At the other end, high concentrations of hexokinase, the rates of ADP production by hexokinase will equal, if not exceed, the rate of adenine nucleotide translocation. In other words, ADP will accumulate. Thus, the steady state concentrations of ATP will decline, ADP will increase, and lower ratio values will be calculated. These statements are confirmed by our analytical experimental data. At intermediate rates of respiration, intermediate steady state ratios of ATP and ADP will be obtained, but always overlaid with a high starting background of [ATP].

Now let us consider the events occurring in the experimental design of Figs. 4 and 5. To our knowledge, this approach has not been reported to date. In these experiments, we specifically limited the amount of hexokinase. Under maximum conditions for hexokinase, high [ATP], and high [glucose], we did not want to produce ADP at a rate faster than could be rephosphorylated by the mitochondria. Arbitrarily, we used a concentration of hexokinase which stimulated respiration to 90% of the ADP State 3 rate. In spite of this, however, under some conditions, the rates of respiration exceeded State 3. Nevertheless, in this experimental design, respiration was not limited by [hexokinase], but by the [ATP] and the kinetics of hexokinase. Low respiratory rates were therefore observed at low concentrations of ATP. However, because of the kinetics of the adenine nucleotide translocase, a substantial concentration of ADP must accumulate to initiate respiration. For example, at an ATP concentration of 14 μM, the measured concentration of ADP was 5.3 μM, for a calculated [ATP]/[ADP] ratio of 2.64 and a log phosphorylation potential value of 3.04. In spite of these ratios, respiration was occurring at only 16.7% of State 3 (State 4 = 15.5%). At the other end of the scale, ATP is saturating and hexokinase becomes rate limiting. This usually occurred at [ATP] = 1.0 mM. Above 1.0 mM ATP, respiratory rates could not be further stimulated, so the numerator of these ratios increased while the denominator remained constant. Thus, the ratios continued to increase while respiratory rates plateaued, as clearly seen in Fig. 5. Therefore, the observed results are quite explainable on the basis of differences within our experimental protocols.

We believe that the best interpretation of these data is that respiratory control is simply a function of the rate of transport of ADP into the mitochondrial matrix (Figs. 6 and 7), mediated by the kinetic properties of the adenine nucleotide translocase (22). However, when we compare our data to the reported kinetic properties of the translocase, some discrepancies become apparent. Paff and Klingenberg (34) first suggested that ATP interacts at the translocase in a manner competitive with ADP exchange. Souverijn et al. (35) confirmed these results and estimated that the Ki for ATP inhibition was in the 100 to 200 μM range. It must be noted that these latter data were obtained at 0 °C, with oxidative phosphorylation inhibited by oligomycin. When we substitute our data into the equation of Souverijn et al. (35), using our value for the apparent Ki, 14.5 μM, and their values for Ki, 100 to 200 μM, we calculate that, at 10 mM ATP, respiration would only be occurring at a rate of 6% of State 3. However, this clearly contradicts the data of Fig. 4, where respiration at 10 mM ATP is occurring at 80% of State 3. We have therefore redetermined the Ki for ATP inhibition of respiration under phosphorylating conditions at 37 °C (data not shown). These data suggest that the Ki atp is not in the 100–200 μM range (35), but is actually closer to 30 to 40 mM. This very low ability of ATP to inhibit State 3 respiration is additional evidence why theories of respiratory control formulated on a simple [ATP]/[ADP] ratio are invalid. Such a ratio is only valid if ATP is a strong, competitive inhibitor of ADP binding. Together with more recent data (36), it appears that our knowledge of the kinetics and mechanistic properties of the adenine nucleotide translocase is incomplete.

We recognize that the results presented in this communication speak only to issues of the adenine nucleotide control of respiration. While our data are consistent with the view that the adenine nucleotide translocase rate limits oxidative phosphorylation (22), it is also clear that other factors play important roles within the cell. These factors include oxygen and the concentration and transport of phosphate (6, 15, 22), substrate availability (4, 5, 7), and matrix [ATP]/[ADP] ratios (37, 38). Defining the interactions of each of these factors under physiological conditions will provide a more complete picture of the cellular control mechanisms involved in the obligate regulation of oxidative phosphorylation.

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