High $K_m$ of oxidative phosphorylation for ADP in skinned muscle fibers: where does it stem from?

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Kongas, Olav, Tai L. Yuen, Marijke J. Wagner, Johannes H. G. M. van Beek, and Klaas Krab. High $K_m$ of oxidative phosphorylation for ADP in skinned muscle fibers: where does it stem from? Am J Physiol Cell Physiol 283: C743–C751, 2002. First published May 1, 2002; 10.1152/ajpcell.00101.2002.—Mitochondria in saponin-skinned cardiac fiber bundles were reported to have an order of magnitude lower apparent affinity to ADP than isolated mitochondria. Although ADP was measured outside the bundles, it was thought that the low affinity was not caused by diffusion gradients because of relatively short diffusion distances. Here we test the hypothesis that considerable ADP diffusion gradients exist and can be diminished by increasing the intrafiber ADP production rate. We increased the ADP-producing activity in rat heart skinned fiber bundles by incubating with 100 IU/ml yeast hexokinase and glucose. Consequently, we observed a significant decrease of the apparent Michaelis constant ($K_m$) to ADP of the respiration rate of bundles from 216 ± 59 to 50 ± 9 μM. Fitting the results with a mathematical model, we estimated the $K_m$ of mitochondria in the bundles to be 25 μM. We conclude that the affinity to ADP in situ mitochondria in heart is of the same order of magnitude as that of isolated mitochondria.

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The large difference between mitochondrial affinities in situ and in vitro was explained as follows: the diffusion gradients of ADP between oxygraph medium and mitochondria are negligible, but the mitochondrial outer membrane acts as a major diffusion barrier in situ, whereas most of its barrier function is lost during isolation of mitochondria (17). This hypothesis has been supported by several experimental observations. For instance, the $K_{app}$ for the isolated permeabilized myocytes was reported to range from 150 (9) to 250 (14) μM, although the radius of a myocyte is merely 6–8 μm. Furthermore, incubating skinned fiber bundles briefly in hypoosmotic medium, which causes mitochondrial swelling and breakage of mitochondrial outer membranes, results in increased affinity of the bundles to ADP at low ADP levels (16). Finally, mild proteolytic treatment of skinned fiber bundles results in a significant decrease of $K_{app}$, ascribed to a loss of control of outer mitochondrial membrane permeability (12).

On the other hand, in skinned fiber bundle studies, the ADP concentration is determined in the incubation medium. There exist diffusion gradients between the medium and the cores of such bundles; however, the magnitude of these gradients is not known. Using a mathematical reaction-diffusion model, we designed an experiment: in bundles where large diffusion gradients exist, these gradients can be minimized by adding an ATP-consuming enzyme that produces ADP inside the bundles.

In this study, we examined the effect of yeast hexokinase (HK) as an ATP-consuming enzyme on $K_{app}$ of skinned fiber bundles from rat heart and soleus. We found that HK is able to cause a remarkable decrease of $K_{app}$, suggesting the existence of large ADP gradients in these bundles that are reduced by increased intrafiber ADP production. We thereby show that the affinity of in situ mitochondria to ADP is of the same order of magnitude as that of isolated mitochondria.

GLOSSARY

<table>
<thead>
<tr>
<th>General</th>
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<tr>
<td>AK</td>
<td>adenylate kinase</td>
</tr>
<tr>
<td>HK</td>
<td>hexokinase</td>
</tr>
<tr>
<td>NS-ATPase</td>
<td>all nonspecific background ATPases of the sample together</td>
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<tr>
<td>IMS</td>
<td>mitochondrial intermembrane space</td>
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Tight coupling of respiration and ATP synthesis is usually described as a Hill curve, characterized by Michaelis-Menten type kinetics with Michaelis constants ($K_m$) ranging from 10 to 20 μM. With permeabilized cell techniques (chemical skinning) it became possible to study the regulation of respiration on mitochondria in situ (9). Interestingly, it was found that mitochondria in saponin-skinned heart or soleus fibers possess an apparent $K_m$ ($K_{app}$) to ADP of 200–400 μM (for review, see Ref. 17). This affinity to ADP is an order of magnitude lower than that observed after isolation of mitochondria from these tissues.

The large difference between mitochondrial affinities in situ and in vitro was explained as follows: the diffusion gradients of ADP between oxygraph medium
Concentrations

ATP, ADP, AMP, P_i concentration of ATP, ADP, AMP, or P_i, respectively
Met, Met_c, Met_o concentration of a metabolite (ATP, ADP, AMP, or P_i) in the mitochondrial intermembrane space (i), cytosolic compartment (c), or oxygraph medium (o), respectively
Mg Free magnesium level

Kinetic constants

\[ V_{\text{max}} \] maximal rate
\[ K_m \] Michaelis-Menten constant
\[ K_{\text{app}} \] apparent \( K_m \) of the respiration of the sample for ADP
\[ K_{\text{mito}} \] \( K_m \) of mitochondria for ADP in the sample
\[ V_{\text{syn}}^f \] maximal mitochondrial ATP synthesis rate
\[ K_{\text{ADP}}, K_{\text{Pi}} \] \( K_m \) values of ATP synthesis for the IMS concentrations of ADP and Pi, respectively
\[ Q_{\text{P/O}} \] ADP-to-O ratio of oxidative phosphorylation
\[ V_{\text{AK}}^f, V_{\text{AK}}^b \] maximal forward and backward reaction rates of adenylate kinase, respectively
\[ K_{\text{1 to K4}}, K_{-1 to K4, K1} \] binding constants of adenylate kinase
\[ V_{\text{hyd}}^f \] \( V_{\text{max}} \) of NS-ATPases
\[ K_{\text{hyd}} \] \( K_m \) of NS-ATPases
\[ V_{\text{HK}}^f \] \( V_{\text{max}} \) of hexokinase
\[ K_{\text{HK}} \] \( K_m \) of hexokinase
\[ K_{\text{DT}}, K_{\text{DD}} \] magnesium binding constants of ATP and ADP, respectively

Diffusion

\[ R_{\text{eff}} \] effective diffusion distance for metabolites in a sample, effective radius of the bundles in a sample
\[ D_{\text{Met}} \] in vitro diffusion coefficient of the metabolite Met
\[ P_{\text{ic}} \] characteristic diffusion restriction of the outer mitochondrial membrane

Volumes

\[ V_{\text{cyt}}, V_{\text{IMS}} \] fractional volumes of cytosolic (cyt) and IMS compartments with respect to the cell volume
\[ Q_{\text{c/o}} \] ratio of the sample volume to the oxygraph medium volume

METHODS

Preparation. Skinned fiber bundles were prepared according to the method described by Seppet et al. (19). Briefly, unanesthetized male or female Wistar rats weighing 300–350 g were decapitated, chests were opened, and hearts were excised while still beating and put into cooled solution A (see Solutions). Cooled hearts were cut into halves, and muscle strips (3–5 mm long and 1–1.5 mm in diameter) were cut from the endocardium of the left ventricles along the fiber orientation to avoid mechanical damage of the cells. Muscle strips (3–4 mm long, ~1 mm in diameter) were also taken from the soleus (oxidative, slow-twitch muscle). By using sharp-ended needles, fiber bundles were partly separated from each other, leaving only small areas of contact of bundles with radii of 10–50 \( \mu \)m (see DISCUSSION). Thereafter, bundles were transferred into Eppendorf test tubes (volume 2 ml) with cooled solution A (see Solutions) containing 50 \( \mu \)g/ml of saponin and were incubated in ice while being mildly shaken for 30 min for complete solubilization of the sarcolemma. Permeabilized (skinned) fiber bundles were then washed in solution B for 10 min at 4°C; this procedure of washing was repeated two times to remove metabolites and soluble enzymes.

Preincubation with HK. In most of the respiration measurements, we used the yeast type IV HK as an exogenous soluble ADP-regenerating enzyme. In this case, the three washings in solution B (see above) were done in the presence of the same activity of HK (in IU/ml) as used in the subsequent respiration measurement.

Respiration rate measurements. The skinned fiber bundles were incubated in an oxygraph in 1 ml solution B with 1 mg/ml BSA added. HK, if present, was activated by adding 25 mM glucose. Steady rates of oxygen uptake were recorded at various ADP levels during the linear phase. Determinations were carried out at 25°C using a Clark-type oxygen electrode. The medium was stirred vigorously. The medium equilibrated with air contained 215 \( \mu \)M oxygen.

Measurements of the rate of background ATPases. Nonspecific background ATPase (NS-ATPase) activity of the fiber bundles was measured using an assay in which formation of ADP was coupled to NADH oxidation. Skinned fiber bundles of rat heart or soleus were prepared as described above, except that washing solution B (see above) were done in the presence of the same activity of HK (in IU/ml) as used in the subsequent respiration measurement.

Solutions. All solutions contained 2.77 mM CaK2EGTA, 7.23 mM K2H2EGTA, 6.56 mM MgCl2, 0.5 mM dithiothreitol, and 20 mM imidazole. The calculated buffered free calcium level was 0.1 \( \mu \)M, a condition that prevents contraction of the bundles. Solution A additionally contained 50 mM potassium 2-(N-morpholino)ethanesulfonate (K-MES), 5.3 mM Na2ATP, 15 mM phosphocreatine, and 50 \( \mu \)g/ml saponin. Solution B contained 100 mM K-MES, 3 mM K2HPO4, 5 mM glutamate, and 2 mM malate. Solution C was the same as solution B but was complemented with 25 IU/ml pyruvate kinase, 25 IU/ml lactate dehydrogenase, 0.24 mM NADH, 1 mM phosphoenolpyruvate, and 3.2 \( \mu \)M antimycin to inhibit oxidative phosphorylation. The pH of all solutions was adjusted to 7.1 at 25°C. The chemicals were obtained from Sigma or Boehringer.
Statistics. Data are presented as means ± SE except for Fig. 3, where they are given as means ± SD to demonstrate the significant difference of variances (F-test) between the groups. If the variances were not significantly different, then the hypotheses about means were tested with Student’s t-test. Otherwise, a more general Welch’s t-test was used. We chose a P value of 0.05 as the determine of significance.

MATHEMATICAL MODEL

The samples used in our experiments contain bundles of various sizes per sample cross section, surrounded by oxygraph medium. Measured $K_{\text{app}}$ of a sample therefore represents a weighted average of apparent $K_m$ values of the bundles in a sample. The model represents the “average” bundle of a sample, surrounded by oxygraph medium, that has the same $K_{\text{app}}$ as the whole sample.

The model has two unknown parameters. The first of these, $K_m$, is the $K_m$ of the mitochondrial respiration to stimulation by ADP. The second, $R_{\text{eff}}$, characterizes the effective diffusion distance from the well-mixed oxygraph medium to the core of the bundle. $R_{\text{eff}}$ encompasses the diffusion paths through the permeabilized cells in the bundle and through the unstirred water layers surrounding the bundle. By fitting our model output to the experimental results, we found $K_m$ = 25 μM and $R_{\text{eff}}$ = 35 μm (see MODELING RESULTS).

Figure 1 depicts a schematic representation of the model. The model consists of a cylindrical fiber bundle with radius $R_{\text{eff}}$ where the reaction-diffusion processes of metabolites are considered, surrounded by an oxy-

![Fig. 1. Schematic representation of the mathematical model. A: fiber bundle, modeled as a cylinder with radius $R_{\text{eff}}$. The bundle has a uniform distribution of mitochondrial and background ATPase activities, and it exchanges metabolites with the surrounding medium via diffusion. Inside the bundle, the background ATPase, adenylate kinase (AK), and ATP synthesis (OxPhos) reactions are modeled. B: enlarged representation of the mitochondrion and its immediate environment. The metabolites are exchanged between the cytosolic and mitochondrial intermembrane space (IMS) compartments via diffusional fluxes through the mitochondrial outer membrane. The permeability of the latter is manipulated to control $K_m$, the Michaelis-Menten constant of respiration to ADP, in the model.](http://ajpcell.physiology.org/)

graph medium compartment with a homogeneous distribution of metabolites. Within the bundle exist two compartments, the cytosolic and IMS compartments, with uniform distributions of the enzyme concentrations and of the fractional volumes of compartments over the bundle (bidomain principle). Diffusion is allowed only in the cytosolic compartment.

In the model, the following metabolites are considered: ATP, ADP, AMP, and Pi. The cytosolic and the IMS compartments are separated by a partly permeable mitochondrial outer membrane. In the IMS compartment, we consider the AK and ATP synthesis reactions. The latter takes place in the matrix but is driven by ADP and Pi concentrations in the IMS. In the cytosol, ATP hydrolysis by both NS-ATPases and HK occurs. A detailed description with equations is given in the APPENDIX.

EXPERIMENTAL RESULTS

Figure 2 demonstrates that the presence of 100 IU/ml yeast HK with 25 mM glucose as a substrate causes a remarkable decrease of $K_{\text{app}}$ without significant effect on VO2 max. To estimate the influence of incubation with HK to VO2 max, we measured VO2 at 2 mM ADP, which yielded $5.30 ± 0.15$ μmol·g wet wt$^{-1}$min$^{-1}$ for control and $5.26 ± 0.20$ μmol·g wet wt$^{-1}$min$^{-1}$ for HK-incubated fibers (means ± SE; n = 8 and 7, respectively; P > 0.05).

Figure 3 shows the dependence of $K_{\text{app}}$ on the activity of the exogenously added HK. $K_{\text{app}}$ decreased gradually (means ± SD) from 216 ± 59 μM (n = 12) for control to 50 ± 9 μM (n = 5) for 100 IU/ml HK. The reduction of $K_{\text{app}}$ was accompanied by a significant decrease of its variance.

Figure 4 demonstrates the influence of the location of the HK activity on $K_{\text{app}}$. For some bundles (denoted as groups L and N in Fig. 4), we applied the 30-min preincubation procedure. However, for groups K and M, HK was added only to the oxygraph medium immediately before the measurement, so that no time was
available for HK to equilibrate between the medium and the fiber interior. If glucose was absent (no HK activity; groups K and L), then \( K_{app} \) remained unaffected. With glucose present, \( K_{app} \) decreased. \( K_{app} \) was significantly lower for bundles preincubated with HK compared with the nonpreincubated bundles (groups M and N), demonstrating that the lowering of \( K_{app} \) is due to enzymatic kinase activity inside the bundles.

We also tested the effect of HK for skinned soleus fiber bundles. HK (100 IU/ml) reduced the \( K_{app} \) from 218 ± 46 (SD) \( \mu M \) (n = 5, control) to 49 ± 12 \( \mu M \) (n = 3; Welch’s test, \( P = 0.0015 \)).

To be able to simulate the influence of NS-ATPases on \( K_{app} \) (see Modeling Results), we measured their apparent activity in rat heart and soleus bundles (Fig. 5). Different amounts of heart bundles were used in these measurements (5.5–15.2 mg wet wt tissue); however, the results did not depend on the amount of tissue. The ATP dependence of both heart and soleus NS-ATPases is consistent with Michaelis-Menten kinetics.

**Modeling Results**

In this section, we present an analysis of the experimental results using the mathematical reaction-diffusion model of the fiber bundle.

Figure 6 shows the simulated ADP concentration profiles along the bundle radius at a half-maximal respiration rate of the bundle (\( V_O_2 = 10 \mu mol/g dry wt^{-1} \cdot min^{-1} \)) for \( K_{mito} = 25 \mu M \). Figure 6A shows the ADP profile calculated at the parameter values (including \( R_{eff} = 35 \mu m \)) that best fit the experimental results in Fig. 3 (see below). In this case, a relatively high \( K_{app} \) results from the high ADP gradients inside the bundle. In the outer layer, the mitochondria respire above their half-maximal rate. In contrast, the core of the fiber negligibly exchanges adenine nucleotides with the outer layer or the medium and respires at a very low rate, which is determined by the activity of the NS-ATPases. The kinetics of the NS-ATPase used in the model were determined from Fig. 5. Reducing \( R_{eff} \) to a radius of a typical cardiomyocyte (Fig. 6B) results in a decrease of \( K_{app} \). Figure 6C shows the influence of the ATP-consuming activity on \( K_{app} \). In addition to the NS-ATPase activity, we assumed the presence of 100 IU/ml HK in the cytosolic compartment. The increase of the ATP-consuming activity means that smaller ADP fluxes from the oxygen graph medium are needed to keep the bundle respiration rate at the half-maximal level. Reduced ADP flux from the medium results in a reduced \( K_{app} \).

In the medium, the ADP concentration is set to be equal to \( K_{app} \), and the ATP and AMP concentrations are set to zero. Because the AMP concentration remains everywhere in the bundle below 5 \( \mu M \) in these simulations (data not shown), the local ATP concentration can be approximated by \( K_{app} = ADP \).

Figure 7 shows \( K_{app} \) as a function of \( V_{HK} \) for varying \( K_{mito} \) and \( R_{eff} \). The solid line is the optimal solution,
obtained at $K_{\text{mito}} = 25 \mu M$ and $R_{\text{eff}} = 35 \mu M$. This is an identical line to that in Fig. 3, obtained by fitting the model to the experimental data. The thick dashed line shows the simulation assuming that the mitochondrial outer membrane is the major diffusion barrier ($K_{\text{mito}} = 200 \mu M$ and $R_{\text{eff}} = 8 \mu M$). The increase of $K_{\text{mito}}$ was obtained by reducing the mitochondrial outer membrane permeability in the model while keeping the $K_{\text{ADP}}$ of oxidative phosphorylation seen from the intermembrane space at $25 \mu M$. Figure 7 also shows the sensitivity of the optimal solution (solid line) to the parameters $K_{\text{mito}}$ and $R_{\text{eff}}$ (see legend to Fig. 7 for details). Briefly, changing $K_{\text{mito}}$ results in a nearly parallel shift of the $K_{\text{app}}$ while $R_{\text{eff}}$ affects the $K_{\text{app}}$ mostly at low $V_{\text{HK}}$ values.

Simulations of the NS-ATPase measurements indicate that the $K_{\text{m}}$ values of the NS-ATPases given in Fig. 5 are overestimated by ~10−15 $\mu M$ because of small ATP gradients.

**DISCUSSION**

The present study was designed to investigate the finding that in situ rat cardiac mitochondria in saponin-skinned fiber bundles possess low affinity to ADP compared with isolated mitochondria. We manipulated the intrafiber ATP-consuming activity by incubating the skinned fiber bundles with 100 IU/ml yeast HK and observed a significant decrease of $K_{\text{app}}$ (see Fig. 3). The lowest $K_{\text{app}}$ measured (50 $\mu M$) shows an upper limit for $K_{\text{mito}}$. We observed a similar decrease of $K_{\text{app}}$ for skinned soleus fiber bundles. These results were obtained without affecting the maximal respiration rate or modifying the mitochondrial outer membrane permeability. Therefore, the mitochondrial outer membrane cannot be a major diffusion barrier in these bundles.

An important assumption in interpreting our experiments has been that the yeast HK does not affect the mitochondrial outer membrane permeability. This assumption is supported by the following observations. First, many groups have failed to show the significant binding of yeast HK by mammalian or yeast mitochondria while, in contrast, those mitochondria bind the mammalian type I HK avidly (for review, see Ref. 24). Although the yeast HK and mammalian type I HK have similar amino acid sequences, the former lacks the hydrophobic NH2-terminal segment that has been shown to be critical for binding of the type I isoenzyme (13). Furthermore, adding dextran to the medium of suspended mitochondria tends to decrease the affinity of mitochondria to ADP (5). Therefore, the change of osmolarity caused by adding a high concentration of HK would increase, rather than decrease, $K_{\text{app}}$.

Saks et al. (15) claimed that the diffusion of metabolites between the oxygraph medium and the bundle interior is so rapid that considerable concentration differences between the interior of fibers and medium (tens or hundreds of micromolars) are not possible. The underlying assumption has been that the effective diffusion distance (the effective fiber bundle radius $R_{\text{eff}}$) is similar to the radius of the average cardiac myocyte ($6–8 \mu M$). The $K_{\text{app}}$ for isolated permeabilized myocytes has been reported to range from 150 (9) to 250 (14) $\mu M$. However, to our knowledge, nobody has ruled out the possible formation of small aggregates of permeabilized myocytes in the oxygraph in these measurements, nor has the $R_{\text{eff}}$ for skinned fiber bundles been
reliably measured. Visual inspection of the samples under a microscope revealed an inhomogeneous geometry of the bundles with their apparent radius ranging from 10 to 50 μm. Model fitting of the data yielded $R_{\text{eff}} = 35 \mu m$ and suggested the existence of considerable diffusion gradients (see Fig. 6).

The $K_{\text{app}}$ of the bundles varied strongly between our experiments, ranging from 150 to 400 μM. The reason might be the variance of $R_{\text{eff}}$, which depends on the mechanical separation procedure of bundles (see Methods). We found the average $K_{\text{app}}$ to be ~200 μM.

HK (4 IU/ml) has been observed earlier to have no effect on kinetic parameters (16, 19), which is in agreement with our findings (Fig. 3). Only if there were a rapid and unrestricted diffusion of metabolites between the bundle core and medium would 4 IU/ml HK provide an excess amount of total ATP-consuming activity compared with the total ATP production capacity of a sample. However, much higher HK activity is needed specifically inside the bundles.

Modification of mitochondrial or NS-ATPase activity of the samples by chemical and potentially nonspecific means was avoided by changing the overall ATP-consuming activity with known amounts of HK. This approach allows more straightforward interpretation of the results than some previously used approaches in which calcium or magnesium levels were manipulated (15). In those experiments, modifying either free calcium or free magnesium caused no significant effect on $K_{\text{app}}$. However, one cannot exclude that changes in intrafiber ADP production rate are balanced by parallel effects on mitochondria.

The model we used for our simulations is only an approximation. The effective bundle radius $R_{\text{eff}}$, found by data fitting (see Fig. 3), can differ from the real average bundle radius in our samples because un-stirred water layers may exist around the bundles. The geometry of the sample (e.g., average number of fibers in bundles) does depend on the mechanical fiber separation procedure. However, our simulations show that the choice of $R_{\text{eff}}$ affects the simulated $K_{\text{app}}$, only for low ATP-consuming activities, whereas it has a minor effect at high activities (see Fig. 7). The value of 25 μM for $K_{\text{mito}}$ found by fitting the experimental $K_{\text{app}}$ data at high ATP-consuming activities, is only slightly higher than the typical $K_m$ of isolated mitochondria (10–20 μM). However, it is an order of magnitude lower than $K_{\text{app}} = 200–400 \mu M$.

Independent support of a low $K_{\text{mito}}$ in skinned rat heart fiber bundles comes from the observations that, when respiration was activated by ADP that was produced endogenously by NS-ATPase reactions, the half-maximal respiration rate was achieved at ~25 μM ADP (19). In these experiments, the authors added different amounts of ATP to the oxygraph, allowed steady concentrations to be established, recorded the respiration rate, and measured the ADP level in the oxygraph medium by HPLC. Because there was no enzyme in the medium that could actively take up ADP, the measured ADP level was equal to the ADP level inside the bundles. Our model analysis indicates that the endogenously produced ADP level that yielded 50% of the respiration rate (25 μM ADP) reflected the real $K_{\text{mito}}$ (Fig. 8).

Mild proteolytic treatment of skinned heart and soleus fiber bundles causes a decrease of $K_{\text{app}}$ from 300–400 to 50–80 μM (12). The possibility that the decrease was caused by the increase in NS-ATPase activity was recently examined by Saks et al. (15). They measured NS-ATPase activities for five groups of skinned heart fiber bundles treated with 0 (control) to 5 μM of trypsin during 5 min at 4°C (see Fig. 7B of Ref. 15) and concluded that the NS-ATPase activity was not affected by a trypsin treatment. However, the NS-ATPase activities differed from each other by ~50% between the 0 (control) and 50 nM groups ($K_{\text{app}} = 300$ and 123 μM, respectively). A two-sided $t$-test ($n = 2$ /group) performed by us yielded $P = 0.0224$. Therefore, we conclude that trypsin treatment may increase the NS-ATPase activity. This is in accordance with observations that the mild trypsin treatment of canine cardiac microsomes, consisting largely of sarcoplasmic reticulum vesicles, activates the rate of the oxalate-facilitated calcium uptake up to 2.8-fold compared with control (8).

The increase of the intrafiber diffusion coefficient by depleting the fibers of myosin by treatment with 800 mM KCl has been observed to increase rather than decrease $K_{\text{app}}$ (16). This observation has been used to support the hypothesis of a low mitochondrial outer membrane permeability. However, the expected decrease of $K_{\text{app}}$ may be compensated by reduced NS-ATPase activity, because part of this activity is likely linked to the extracted myosin.

In conclusion, we found that increasing intrafiber ATP-consuming activity without affecting the permeability of the mitochondrial outer membrane causes a decrease of $K_{\text{app}}$ of skinned heart and soleus fiber bundles. These results cannot be explained by the theory that the outer mitochondrial membrane acts as a major diffusion barrier in these bundles. Our simulations suggest the existence of large ADP diffusion
gradients between mitochondria and the surrounding medium and the reduction of the gradients if intrafiber
ADP production is increased. We propose that the affinity of the in situ and probably also in vivo mito-
chondria to ADP in heart and soleus is of the same order of magnitude as that of isolated mitochondria.

**APPENDIX**

The reaction-diffusion equations of the model form a set of partial differential equations. The equations are given in polar coordinates. The reaction rates (in μM/s) are normalized to total cell volume. The parameters used in the model-
ing are given in Table 1.

The balance equations for the metabolite concentrations in the IMS are as follows

\[
\begin{align*}
\dot{V}_{\text{ATP}} &= (V_{\text{syn}} + V_{\text{AK}} - J_{\text{ATP}}) / V_{\text{IMS}} \\
\dot{V}_{\text{ADP}} &= (-V_{\text{syn}} - 2V_{\text{AK}} - J_{\text{ADP}}) / V_{\text{IMS}} \\
\dot{V}_{\text{AMP}} &= (V_{\text{AK}} - J_{\text{AMP}}) / V_{\text{IMS}} \\
\dot{P}_{\text{i}} &= (-V_{\text{syn}} - J_{\text{Pi}}) / V_{\text{IMS}}
\end{align*}
\]

where \( V_{\text{syn}} \) and \( V_{\text{AK}} \) are the ATP synthesis and the AK reaction rates, respectively, and \( J_{\text{Met}} \) are the diffusion fluxes from the IMS to the cytosolic compartment. The symbol \( V_{\text{IMS}} \) is the fractional volume of the IMS compartment with respect to the cell volume. Met denotes the partial derivative of Met with respect to time (dMet/dt).

The equations for the cytosolic compartment are as follows

\[
\begin{align*}
\dot{V}_{\text{ATP}} &= \nabla^2 V_{\text{ATP}} \times D_{\text{ATP}} \times V_{\text{cyt}} + (V_{\text{hyd}} - V_{\text{AK}} + J_{\text{ATP}}) / V_{\text{cyt}} \\
\dot{V}_{\text{ADP}} &= \nabla^2 V_{\text{ADP}} \times D_{\text{ADP}} \times V_{\text{cyt}} + (V_{\text{hyd}} + V_{\text{HK}} + J_{\text{ADP}}) / V_{\text{cyt}} \\
\dot{V}_{\text{AMP}} &= \nabla^2 V_{\text{AMP}} \times D_{\text{AMP}} \times V_{\text{cyt}} + J_{\text{AMP}} / V_{\text{cyt}} \\
\dot{P}_{\text{i}} &= \nabla^2 P_{\text{i}} \times D_{\text{i}} \times V_{\text{cyt}} + (V_{\text{hyd}} + J_{\text{Pi}}) / V_{\text{cyt}}
\end{align*}
\]

where \( V_{\text{hyd}} \) and \( V_{\text{HK}} \) denote the reaction rates of NS-ATPase and HK. \( V_{\text{cyt}} \) is the fractional volume of the IMS compartment, \( D_{\text{Met}} \) are the diffusion constants of metabolites Met in solution, and the products \( D_{\text{Met}} \times V_{\text{cyt}} \) represent the diffusion constants adjusted for the reduction of free diffusion space. The symbol \( \nabla^2 \) denotes the Laplace operator in polar coordinates if radial symmetry is held, i.e.

\[
\nabla^2 \text{Met} = \frac{\partial^2 \text{Met}}{\partial r^2} + \frac{1}{r} \frac{\partial \text{Met}}{\partial r}
\]

where \( r \) is the radial coordinate in the bundle.

The equations for the oxygen graph medium compartment are as follows

\[
\begin{align*}
\dot{V}_{\text{ATP}} &= F_{\text{ATP}} \times Q_{\text{oxy}} \\
\dot{V}_{\text{ADP}} &= F_{\text{ADP}} \times Q_{\text{oxy}} \\
\dot{V}_{\text{AMP}} &= F_{\text{AMP}} \times Q_{\text{oxy}} \\
\dot{P}_{\text{i}} &= F_{\text{Pi}} \times Q_{\text{oxy}}
\end{align*}
\]

where \( Q_{\text{oxy}} \) is the ratio of the sample volume and the oxygen graph volume. Here

\[
F_{\text{Met}} = -\frac{2D_{\text{Met}} V_{\text{cyt}}}{R_{\text{eff}}} \times \left. \frac{\partial \text{Met}}{\partial r} \right|_{r=r_{\text{eff}}}
\]

**Table 1. Parameters of the model**

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</tr>
<tr>
<td>( K_{\text{ATP}} ), μM</td>
<td>56.0</td>
<td>6</td>
</tr>
<tr>
<td>( K_{\text{P}} ), μM</td>
<td>16.8</td>
<td>6</td>
</tr>
<tr>
<td>ATP synthesis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V_{\text{ATP}} ), μM/s</td>
<td>406</td>
<td>+^b</td>
</tr>
<tr>
<td>( K_{\text{ATP}} ), μM</td>
<td>360</td>
<td>21</td>
</tr>
<tr>
<td>( Q_{\text{P/O}} )</td>
<td>25</td>
<td>+^c</td>
</tr>
<tr>
<td>ATP hydrolysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V_{\text{ATP}} ), μM/s</td>
<td>108</td>
<td>+</td>
</tr>
<tr>
<td>( K_{\text{ATP}} ), μM</td>
<td>770</td>
<td>+</td>
</tr>
<tr>
<td>Hexokinase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V_{\text{ATP}} ), μM/s</td>
<td>0–770</td>
<td>+^d</td>
</tr>
<tr>
<td>( K_{\text{ATP}} ), μM</td>
<td>150</td>
<td>22</td>
</tr>
<tr>
<td>Magnesium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V_{\text{ATP}} ), μM/s</td>
<td>4,000</td>
<td>+</td>
</tr>
<tr>
<td>( K_{\text{ATP}} ), μM</td>
<td>24</td>
<td>2</td>
</tr>
<tr>
<td>( K_{\text{Pi}} ), μM</td>
<td>347</td>
<td>2</td>
</tr>
<tr>
<td>Diffusion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( D_{\text{ATP}} ), μm^2/s</td>
<td>397</td>
<td>3^e</td>
</tr>
<tr>
<td>( D_{\text{ADP}} ), μm^2/s</td>
<td>386</td>
<td>3</td>
</tr>
<tr>
<td>( D_{\text{AMP}} ), μm^2/s</td>
<td>374</td>
<td>3</td>
</tr>
<tr>
<td>( D_{\text{Pi}} ), μm^2/s</td>
<td>700</td>
<td>3</td>
</tr>
<tr>
<td>( P_{\text{O}_{2}} ), μm^2</td>
<td>0.005 or 93</td>
<td>+^f</td>
</tr>
<tr>
<td>Volumes, geometry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V_{\text{ATP}} ), μM/s</td>
<td>12</td>
<td>7, 11^g</td>
</tr>
<tr>
<td>( V_{\text{ATP}} ), μM/s</td>
<td>124</td>
<td>20^h</td>
</tr>
<tr>
<td>( Q_{\text{oxy}} )</td>
<td>0.006 or 0</td>
<td>+</td>
</tr>
<tr>
<td>( R_{\text{eff}} ), μM</td>
<td>35</td>
<td>+</td>
</tr>
</tbody>
</table>

Parameters marked with “+” are estimated from the measurements performed in this work. The activities of the enzymes (in μM/s) were normalized to a cell volume assuming the tissue wet weight-to-dry weight ratio = 5.5 and tissue mass-to-volume ratio = 1.06 g wet wt/ml. *Equivalent to the AK rate of 216 μmol·g dry wt^{-1}·min^{-1} toward ADP production. The rates were estimated from the total tissue AK activity toward ADP production (23), from dissociation constants and the equilibrium between the forward and the reverse reactions (6), and from the fraction of AK activity in the mitochondrial intermembrane space (18). *Equivalent to the maximal ADP-stimulated respiration rate of the bundles \( V_{\text{O}_{2,0}} = 22.5 \) μmol·g dry wt^{-1}·min^{-1}. *Estimated by fitting the model output to the data in Fig. 3. *Equivalent to the range of HK activities 0–100 IU/ml in the cytosolic compartment. Obtained by interpolating the in vitro diffusion coefficients measured at 20°C and 37°C to 25°C using formulas from Ref. 1. *These values yield \( K_{\text{max}} = K_{\text{ADP}} + 0.01 \) μM and \( K_{\text{max}} = K_{\text{ADP}} + 175 \) μM, respectively. *Estimated from the ratio of the intracellular diffusion constants for ATP and PCr in their in vitro values. *\( V_{\text{ATP}} = V_{\text{ATP}} \text{/Vcyt} \). *\( Q_{\text{oxy}} = 0 \) was used to simulate the initial rate measurements. See text for definitions.

is the diffusional flux of the metabolite Met, from the bundle to the oxygen graph medium per unit of bundle volume. The concentrations Met, are also the boundary conditions for the cytosolic concentrations, i.e.
for all metabolites in the model. At the other boundary, \( r = 0 \), the no-flux condition applies.

For the AK reaction, we derived the full kinetic equations, because in some simulations the AK works far from equilibrium. We calculated the magnesium-bound and magnesium-free forms, denoted by prefixes \( m \) or \( f \), respectively, of ATP, ADP, and AMP as follows

\[
\begin{align*}
\text{fATP}_i &= \frac{\text{ATP}_i}{1 + \text{Mg}/K_{DF}} \\
\text{mATP}_i &= \text{ATP}_i - \text{fATP}_i \\
\text{fADP}_i &= \frac{\text{ADP}_i}{1 + \text{Mg}/K_{DD}} \\
\text{mADP}_i &= \text{ADP}_i - \text{fADP}_i
\end{align*}
\]

where \( \text{Mg} \) is the free magnesium concentration. AMP, was assumed to be mostly in the magnesium-free form (2). The AK reaction rate \( V_{\text{AK}} \) took into account the inhibitory effects of ADP and AMP via formation of the nonproductive complexes ADP\( \cdot \text{ATPi} \), ADP\( \cdot \text{AMPi} \), and ADP|AMP|Pi as follows

\[
\begin{align*}
\text{J}_{\text{ATP}} &= (D_{\text{ATP}}/P_o) \times (\text{ATPi} - \text{ATP}_i) \\
\text{J}_{\text{ADP}} &= (D_{\text{ADP}}/P_o) \times (\text{ADPi} - \text{ADP}_i) \\
\text{J}_{\text{AMP}} &= (D_{\text{AMP}}/P_o) \times (\text{AMPi} - \text{AMP}_i) \\
\text{J}_{P_i} &= (D_{P_i}/P_o) \times (P_i - P_i^*)
\end{align*}
\]

where \( P_o \) reflects the permeability of the outer mitochondrial membrane and affects the \( K_{\text{mfor}} = \text{ADP}_i - \text{ADP} + K_{\text{ADP}} \) calculated at the half-maximal ATP synthesis rate. Positive direction of the flux is from the IMS to the cytosol.

The rate equations of the model form a system [Eqs. 1–8 and Eqs. 10–13] that was solved simultaneously. The partial differential equations were transformed using a finite differences approximation scheme with a uniform grid along the bundle radius. The resulting system of ordinary differential equations was solved by a backward differentiation formula that is able to treat stiff equations, using the DVODE package. The precision of a solution was tested by comparing it with the solution for a 2-fold denser grid and for a 10-fold smaller relative tolerance of the integrator. Convergence was obtained at a grid step \( \Delta r \leq 1 \mu \text{m} \) and relative tolerance \( 10^{-7} \).

To fit to the experimental data, the model parameters were optimized by a modified Levenberg-Marquardt algorithm, using the LMDIF least-squares solver.

To improve the moity conservation in the model, the spatial derivatives of the cytosolic metabolite concentrations at the bundle surface were estimated from the quadratic approximations to the concentrations’ profiles at the boundary

\[
\frac{\partial \text{Met}_i}{\partial r} \bigg|_{r = R_{\text{eff}}} = \left( \frac{3}{2} \text{Met}_{i,n} - 2 \text{Met}_{i,n-1} + \frac{1}{2} \text{Met}_{i,n-2} \right)/\Delta r
\]

where \( \text{Met}_{i,n}, \text{Met}_{i,n-1}, \text{and Met}_{i,n-2} \) are the approximations to \( \text{Met}_i \) at \( n \)-th, \( (n - 1) \)-th, and \( (n - 2) \)-th grid point, respectively, with the \( n \)-th grid point residing at the boundary.

The implicit parameter \( K_{\text{app}} \) was calculated by optimizing \( \text{ADP}_o \) to yield the half-maximal respiration rate. At each optimization step, the levels of the metabolites in the oxygen-graph were kept constant (\( Q_{\text{so}} = 0 \)) as follows: \( \text{ATP}_o = 0 \), \( \text{AMP}_o = 0 \), \( P_i = 3 \text{ mM} \). \( \text{ADP}_o \) was modified (Levenberg-Marquardt), and the resulting respiration rate was obtained after the transients vanished (\( t = 60 \text{ s} \)). The parameter \( K_{\text{mfor}} \) was manipulated by changing \( P_{o} \) in Eqs. 25–28, and it was estimated as \( K_{\text{app}} \) for the bundle with a very small radius (\( R_{\text{eff}} = 0.1 \mu \text{m} \)) where the diffusional gradients were negligible.

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