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# What controls the outer mitochondrial membrane permeability for ADP: facts for and against the role of oncotic pressure

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**Abstract**

In our study 10% of bovine serum albumin was added to the physiological incubation medium to mimic the oncotic pressure of the cellular cytoplasm and to test for its effect on the respiration of isolated rat heart mitochondria, saponin- or saponin plus crude collagenase (type IV)-treated heart muscle fibers and saponin-treated rat quadriceps muscle fibers. Pyruvate and malate were used as substrates. We found that albumin slightly decreased the maximal ADP-stimulated respiration rate only for saponin-treated heart muscle fibers. The apparent $K_m$ ADP of oxidative phosphorylation increased significantly, by 70–100%, for isolated heart mitochondria, saponin plus collagenase-treated heart muscle fibers and for saponin-treated quadriceps muscle fibers but remained unchanged for saponin-treated heart muscle fibers. The saponin-treated heart muscle fibers were characterized by a very high control apparent $K_m$ ADP value (234 ± 24 μM ADP) compared with other preparations (14–28 μM ADP). The results suggest that in vivo the oncotic pressure is not the relevant factor causing the low outer mitochondrial membrane permeability for ADP in cardiomyocytes, in contrast to quadriceps muscle cells. It is likely that the outer mitochondrial membrane-bound protein(s) which is supposed to remain in saponin-treated heart muscle fibers is responsible for this property of the membrane. © 2001 Elsevier Science B.V. All rights reserved.

*Keywords:* Macromolecule; Rat heart mitochondria; Skinned heart fiber; Skinned skeletal muscle fiber; Oxidative phosphorylation; Kinetic constant

## 1. Introduction

Intensive studies of permeabilized cardiac and slow- or fast-twitch skeletal muscle fibers as well as isolated hepatocytes in the past decade [1–4] have led to the conclusion that the outer mitochondrial membrane in vivo, in cardiac and liver tissues and slow-twitch skeletal muscle, in contrast to isolated mitochondria, possesses low permeability for ADP which is supposed to be regulated by some specific cytoskeleton-related protein(s) bound to porin pores. It was shown that the treatment of saponin skinned cardiac fibers with proteases caused the disappearance of an approx. 27 kDa protein [5]. This correlates with a several-fold decrease in the initially very high value of app. $K_m^{ADP}$ of oxidative phosphorylation from approx. 300 μM to 50 μM ADP, which is close to the apparent $K_m$ for ADP (app. $K_m^{ADP}$) of isolated mitochondria. In contrast, in saponin skinned fibers from fast-twitch skeletal muscles (gastrocnemius and

Abbreviations: BSA, bovine serum albumin; app. $K_m^{ADP}$, apparent $K_m$ for ADP; HMT, isolated rat heart mitochondria; HSF, saponin-treated rat heart muscle fiber(s); HSCF, saponin plus collagenase-treated rat heart muscle fiber(s); QSF, saponin-treated rat quadriceps muscle fiber(s)

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quadriceps), this value was initially very low (12–22 μM ADP) and was not decreased after treatment with trypsin [4]. It is worth mentioning that the incubation medium used for the investigations of saponin-treated cardiac and skeletal muscle fibers [4,6–8] did not contain macromolecules and therefore lacked oncotic pressure. It is well known that mitochondria, isolated and incubated in isotonic medium free of macromolecules, do not resemble the mitochondria in vivo, in contrast to those located in the saponin-treated cardiac and skeletal muscle fibers [1,4,6]: they are less intact, swollen and exhibit a greatly enlarged intermembrane space and a dramatically decreased number of contact sites [9,10]. The morphological appearance can be reversed by the addition of macromolecules, e.g. bovine serum albumin (BSA), dextran or polyvinylpyrrolidone [9–11]. Moreover, it was also demonstrated [12] that the initially high permeability of the outer mitochondrial membrane of isolated rat heart mitochondria for ADP could be decreased and, accordingly, the low initial app. $K_m^{ADP}$ value (20 μM) significantly increased by adding macromolecules of non-protein origin, i.e. 5–25% dextran of 15–20 kDa. Based on the above findings, the effects of dextran on the app. $K_m^{ADP}$ of oxidative phosphorylation were explained by the importance of the in vivo intracellular oncotic pressure in the maintenance of low outer mitochondrial membrane permeability for adenine nucleotides [12]. Thus, the opinions concerning the factors controlling the outer mitochondrial membrane permeability for ADP are different. One can assume if only one factor, either the specific cytoskeleton-associated protein(s) [4,6–8] or the intracellular oncotic pressure [12], is relevant, the effect of macromolecules on the app. $K_m^{ADP}$ of oxidative phosphorylation should be different in the case of different mitochondrial preparations, such as isolated mitochondria, mitochondria located in the saponin plus protease-treated fibers and mitochondria in the saponin-treated cardiac and quadriceps muscle fibers, due to their differences with regard to the outer mitochondrial membrane permeability for ADP [8]. Thus, to get a deeper insight into the problem, we investigated the effect of natural macromolecules, i.e. 10% of BSA, on the kinetic properties of the oxidative phosphorylation system. For this purpose, isolated rat heart mitochondria (HMT), saponin-treated rat heart muscle fibers (HSF), saponin plus collagenase-treated rat heart muscle fibers (HSCF) and saponin-treated rat quadriceps muscle fibers (QSF) were chosen. It was found that BSA significantly increased the app. $K_m^{ADP}$ of HMT, HSCF and QSF, whereas this value for HSF remained unchanged. The obtained data suggest that in vivo the intracellular oncotic pressure is not the relevant factor determining the low permeability of the outer mitochondrial membrane for ADP in heart muscle cells, in contrast to quadriceps muscle cells. The results of this work were presented in abstract form [13,14].

## 2. Materials and methods

### 2.1. Preparations

Male Wistar rats were used in the experiments. After decapitation beating hearts were excised and rinsed in ice-cold 0.9% KCl solution. Mitochondria were isolated in medium containing 160 mM KCl, 10 mM NaCl, 20 mM Tris, 5 mM EGTA (pH 7.7 adjusted with KOH at 2°C) and suspended in 180 mM KCl, 20 mM Tris, 3 mM EGTA (pH 7.3 adjusted with KOH at 2°C). The mitochondrial protein concentration was determined by the biuret method [15]. Bundles of heart and skeletal muscle fibers, approx. 0.4 mm in diameter, were prepared using sharp-ended needles from the muscle strips cut out from the left ventricular endocardium or from quadriceps muscle, respectively, in cooled 20 mM imidazole, 20 mM taurine, 0.5 mM dithiothreitol, 7.1 mM $MgCl_2$, 50 mM MES, 5 mM ATP, 15 mM phosphocreatine, 2.62 mM $CaK_2EGTA$ and 7.38 mM $K_2EGTA$ (pH 7.0 adjusted with KOH at 2°C; solution A). The fibers were transferred to Eppendorf test tubes containing 1 ml of solution A with 50 μg/ml saponin (from Gypsophila; sapogenin content 17+%; Sigma) or, in the case of SCF, saponin plus 3 mg/ml crude collagenase (type IV; EC 3.4.24.3; Sigma), and incubated for 30 min. Then the bundles were washed for 10 min in a solution containing 20 mM imidazole, 20 mM taurine, 0.5 mM dithiothreitol, 1.61 mM $MgCl_2$, 100 mM MES, 3 mM $KH_2PO_4$, 2.95 mM $CaK_2EGTA$ and 7.05 mM $K_2EGTA$ (pH 7.1 adjusted with KOH at 37°C; solution B). This washing procedure was repeated two times only for quad-

riceps muscle fibers. All procedures were carried out under intensive shaking (120 times/min). The washed bundles of fibers were once rinsed in solution B, transferred to test tubes with the same solution and stored on ice for a period of 6–7 h.

### 2.2. Assays

The rates of oxygen uptake were recorded at 37°C by means of the Clark-type electrode system with intensive stirring in solution B (see Section 2.1), containing 5–6 mM of both pyruvate and malate as substrates. The medium was supplemented with 2 mg/ml, as control, or with 100 mg/ml BSA (fraction V; A4503, Sigma) (pH 7.1 adjusted with KOH). The solubility of oxygen was taken to be 422 ngatoms/ml in the control medium and 401 ngatoms/ml in the medium with 100 mg/ml BSA. Respiration rates were expressed as ngatoms O/min/mg mitochondrial protein or fiber dry weight. For dry weight calculations (dry weight = wet weight before respiration measurement/factor $W$), the factor '$W$' was taken to be 4.85 for heart muscle fibers [2] and 4.78 for quadriceps muscle fibers. The final mitochondrial protein concentration in the oxygraph chamber was 0.5 mg/ml. The ADP regenerative system, consisting of 1.2 IU/ml lyophilized yeast hexokinase (type V; EC 2.7.1.1; Sigma) and 24 mM glucose (Sigma), was added to the chamber before addition of isolated mitochondria or heart muscle fibers. Titration was made by different ADP concentrations in each separate probe. The concentrations covered a range from 5 to 6 μM (for HMT, HSCF and QSF) or 18 μM (for HSF) to 1000 μM (at least seven points). $\Delta V$ was expressed as the difference between the respiration rates in the presence and in the absence of added ADP. App. $K_m^{ADP}$ and $\Delta V_{max}$ were estimated from the least-squares fit to the Michaelis-Menten equation ($\Delta V$ vs. ADP concentration) by GraphPad Prism v3.0. The results are presented as means ± S.E. Statistical analysis was performed using paired Student's $t$-test and $P < 0.05$ was taken as the level of significance.

## 3. Results and discussion

It is known that the cytosolic protein concentration is higher than 20% [16]. However, several per cent of macromolecules (2–5%) can put them into resemblance to the mitochondria in vivo [9–11]. Therefore, to simulate the oncotic conditions of the intact cell, we used 10% BSA.

The investigation of mitochondrial respiration demonstrates (see Table 1) that in case of HMT, 10% BSA decreased, by 41%, the resting state 4 respiration rate ($V_0$), only. This caused an increase in the respiratory control index by 55%. No effect was observed on the respiratory parameters of HSCF. However, BSA increased significantly, by 70–90% ($P < 0.01$), the app. $K_m^{ADP}$ in both cases (see Fig. 1), which is in good agreement with our previous data [13,14,17].

Respiration medium usually contains BSA to bind fatty acids present in the mitochondrial preparations. As 0.1–0.5% BSA are used to bind fatty acids in fatty acid rich mitochondrial preparations from brown adipose tissue and larvae of the wax moth [18,19], we supplemented the control respiration medium with 0.2% BSA. To check whether the effects of

Table 1
Effect of BSA on respiration parameters of isolated rat heart mitochondria, skinned heart and quadriceps muscle fibers

| | Isolated heart mitochondria ($n = 3$) | | Saponin+collagenase-treated heart fibers ($n = 4$) | | Saponin-treated heart fibers ($n = 6$) | | Saponin-treated quadriceps fibers ($n = 3$) | |
|---|---|---|---|---|---|---|---|---|
| | Control | +BSA | Control | +BSA | Control | +BSA | Control | +BSA |
| $V_0$ | 57 ± 5 | 34 ± 2* | 37 ± 2 | 34 ± 3 | 42 ± 2 | 34 ± 2** | 11 ± 2 | 10 ± 2 |
| $V_{ADP}$ | 341 ± 13 | 316 ± 6 | 259 ± 11 | 250 ± 8 | 147 ± 8 | 118 ± 6** | 44 ± 9 | 44 ± 3 |
| $V_{ADP}/V_0$ | 6.04 ± 0.31 | 9.36 ± 0.25* | 7.08 ± 0.20 | 7.56 ± 0.47 | 3.48 ± 0.04 | 3.43 ± 0.07 | 4.10 ± 0.23 | 4.32 ± 0.48 |

$V_0$, respiration rate with 5–6 mM of both pyruvate+malate and, in addition, in the case of isolated mitochondria and skinned heart muscle fibers, with glucose+hexokinase (24 mM+1.2 IU/ml); $V_{ADP}$, maximal respiration rate in the presence of ADP (1–1.2 mM). Every separate experiment was performed in 2–3 repeats. *$P < 0.05$, **$P < 0.01$ vs. control. Respiration rates are expressed as ngatoms O/min/mg mitochondrial protein or fiber dry weight.

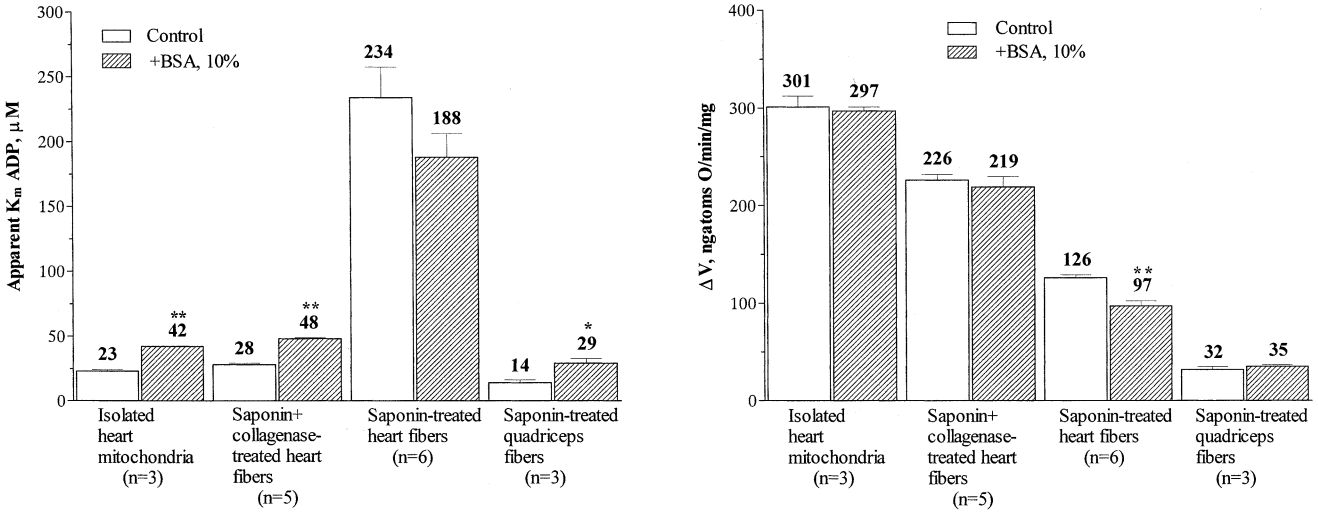


Fig. 1. Effect of BSA on the app. $K_m^{ADP}$ and $\Delta V_{max}$ of isolated rat heart mitochondria, skinned heart and quadriceps muscle fibers. $^*P < 0.05$, $^{**}P < 0.01$ vs. control.

10% BSA, i.e. the decrease of $V_0$ in HMT and the increase in the app. $K_m^{ADP}$ in HMT and HSCF, are due to the oncotic pressure and not caused by the fatty acid binding capability of BSA, we performed additional tests with HMT. For that purpose, we used four media: the first (control) supplemented with 0.2% BSA, the second supplemented with 0.2% ovalbumin, which has no capability to bind fatty acids [19,20], the third supplemented with 0.2% BSA+9.8% ovalbumin, and the fourth supplemented with 0.2% BSA+9.8% dextran T-70 (Amersham Pharmacia Biotech), which is a neutral water-soluble macromolecule. Our results showed that, indeed, ovalbumin did not bind fatty acids, because in the respiration medium supplemented with 0.2% BSA $V_0$ was smaller than in medium supplemented with 0.2% ovalbumin ($35 \pm 1$ and $49 \pm 1$ ngAt O/min/mg protein, respectively; $n = 10$). In the medium supplemented with 9.8% ovalbumin (+0.2% BSA), $V_0$ was similar to that in the medium with 10% BSA ($26 \pm 1$ and $28 \pm 1$ ngAt O/min/mg protein, respectively; $n = 9$), indicating that the decrease of $V_0$ is not due to the fatty acid binding property of BSA. However, 9.8% ovalbumin (+0.2% BSA) 3 times decreased the HMT respiration rate in state 3. Therefore, we could not use it to determine the app. $K_m^{ADP}$, and for this kind of experiments we have chosen medium supplemented with 9.8% dextran (+0.2% BSA). 9.8% dextran had no effect on the HMT respiration rate in state 4 and state 3, but increased the app. $K_m^{ADP}$ from $37 \pm 0.3$ μM ADP (control medium with 0.2% BSA, $n = 3$) to $94 \pm 6$ μM ADP (medium with 9.8% dextran +0.2% BSA, $n = 3$), i.e. by 150%. This finding is in good agreement with the effects of 10% dextran observed on isolated rat heart and liver mitochondria [21,22] and with the effect of 10% BSA (see Fig. 1). Thus, our results demonstrate that the increase in the app. $K_m^{ADP}$ by 10% of BSA is due to the oncotic pressure, but not to the fatty acid binding property of BSA.

Our results support the hypothesis that in the presence of macromolecules the outer mitochondrial membrane creates a diffusion barrier for adenine nucleotides [12]. The mechanism of regulation of the outer mitochondrial membrane permeability by macromolecules is still unclear. It was proposed [21] that dextran macromolecules might reduce the permeability of the porin pores by decreasing their diameter. However, recent investigations [12] did not confirm such an assumption. The viscosity of the incubation medium, varied by changing the molecular size of the dextrans, was also excluded from the possible factors influencing the diffusion of adenine nucleotides across the outer mitochondrial membrane [12]. Finally, it was proposed that macromolecules could create unstirred layers in the outer mitochondrial compartment which act as a diffusion barrier for adenine nucleotides [12].

Further experiments were performed on the most native preparations, i.e. on mitochondria located in

Table 2
Different effect of crude collagenase on the kinetic properties of the oxidative phosphorylation system of rat heart and quadriceps muscle fibers

| Fibers | Treatment | Apparent $K_m$ ADP (μM) | $\Delta V_{max}$ (ngatoms O/min/mg dry wt) |
|---|---|---|---|
| Heart muscle | saponin ($n=6$) | 234 ± 24 | 126 ± 3 |
| | saponin+collagenase ($n=5$) | 28 ± 1 | 226 ± 6 |
| Quadriceps muscle | saponin ($n=2$) | 16 ± 2 | 30 ± 0.2 |
| | saponin+collagenase ($n=2$) | 20 ± 1 | 33 ± 5 |

HSF. In this case 10% BSA slightly (by about 20%) reduced the state 4 ($V_0$) and state 3 ($V_{ADP}$) respiratory rate as well as $\Delta V_{max}$ (see Table 1 and Fig. 1). It is noteworthy that, in contrast to HMT and HSCF, we did not notice any increase in the app. $K_m^{ADP}$ of HSF in the medium supplemented with 100 mg/ml BSA. This finding was also confirmed at lower temperature (20°C): control, 265 ± 13 μM and with BSA, 267 ± 13 μM ADP ($n=6$).

The effect of oncotic pressure was also investigated on QSF, fibers with a smaller amount of mitochondria and a lower respiratory activity than those of heart muscle. It is noteworthy that, in contrast with HSF, the QSF were characterized by a very low app. $K_m^{ADP}$ (22 μM ADP), i.e. the same as that of isolated muscle mitochondria [4]. This finding was also confirmed in our experiments. It appeared that in the case of QSF 10% BSA did not change the respiratory parameters ($V_0$, $V_{ADP}$, $V_{ADP}/V_0$), and significantly, by 104% ($P<0.05$), increased the initially low app. $K_m^{ADP}$ (see Table 1 and Fig. 1).

The low app. $K_m^{ADP}$ in QSF in the control medium denies the possibility that the high app. $K_m^{ADP}$ in HSF is due to the limiting diffusion of hexokinase or ADP into the preparation. This is in agreement with conclusions made by other authors [4] investigating slow-twitch and fast-twitch muscle fibers.

Thus, the isolated mitochondria and mitochondria in HSCF and QSF on the one hand, and those located in HSF on the other hand, respond differently to the same factor: oncotic pressure. BSA increased the app. $K_m^{ADP}$ of the first and did not change that of the second. According to the different effect of BSA on the app. $K_m^{ADP}$ it could be assumed that HSF possess, whereas QSF and HSCF do not contain, the factor – probably cytoskeleton-associated protein(s) – that controls the permeability of the mitochondrial porin pores for ADP (maintains it low in HSF). It was demonstrated that this factor is lost during the isolation of mitochondria or proteolytic treatment of muscle fibers [5,7,8,23]. In accordance with these diverse properties, the quadriceps muscle fibers respond differently to the treatment with crude collagenase (see Table 2), confirming other authors' data obtained with trypsin on gastrocnemius muscle fibers [4]: no protease sensitive protein(s), no effect of protease on the app. $K_m^{ADP}$ and, thus, on the outer mitochondrial membrane permeability for ADP, and vice versa.

It is also possible that the crude collagenase (type IV) itself or its contaminating proteases used in our experiments can injure, at least to some extent, the outer mitochondrial membrane and increase its permeability for ADP. However, in our experiments the cardiac and quadriceps muscle fibers, treated and non-treated with collagenase, were of good quality, i.e. the outer mitochondrial membrane was not damaged – addition of exogenous cytochrome *c* did not stimulate the state 3 respiration rate.

In conclusion, the data obtained in this work suggest that in vivo the intracellular oncotic pressure/cytosolic macromolecules cannot be considered a relevant factor determining the low outer mitochondrial membrane permeability for ADP in the intact cardiomyocytes of adult animals. However, it may be hypothesized that this mechanism of respiration regulation is important in skeletal muscle like quadriceps and other fast-twitch skeletal muscle cells, in which the outer mitochondrial membrane possesses high permeability for ADP i.e., a low app. $K_m^{ADP}$ value [4,5,7,8,24].

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