

MACROMOLECULES INCREASE THE CHANNELING OF ADP FROM MITOCHONDRALLY ASSOCIATED HEXOKINASE TO THE MITOCHONDRIAL MATRIX

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INTRODUCTION

Several kinases are localized at the mitochondrial periphery [1]. These include creatine kinase, adenylate kinase, nucleoside diphosphate kinase and acyl CoA synthase, which are localized in the intermembrane space, and certain hexokinase isozymes and glycerol kinase, which are associated to the outer mitochondrial surface by interaction with porin, the pore protein through which metabolites can permeate the outer membrane [2]. For creatine kinase and hexokinase-I it was demonstrated that they are enriched in intermembrane contact sites.

The mitochondrial localization has been proposed as an advantage in supplying the kinases with mitochondrially formed ATP and by analogy, supplying oxidative phosphorylation with ADP generated by kinase activity [3,4]. For example, it was shown that functional relations exist between mitochondrial hexokinase and oxidative phosphorylation [3,5-9]. Binding of hexokinase to mitochondria increases the affinity for ATP under phosphorylating conditions. Both binding *per se* and intramitochondrial ATP regeneration jointly contribute to this effect [5]. However, the kinetic advantage of bound hexokinase decreases with increasing (more physiological) ATP concentrations. Therefore, it was proposed that an important task of the mitochondrial localization of hexokinase is to channel ADP into the mitochondrion.

The structural organization within the peripheral mitochondrial compartment may be important for the functional coupling between kinase activity and oxidative phosphorylation. Most studies on this functional coupling have been done *in vitro* with isolated mitochondria in isotonic media. Under these conditions, the intermembrane space is strongly enlarged as compared to the *in vivo* situation. The structural changes in mitochondria which occur upon isolation can be explained by missing colloid osmotic pressure and can be counteracted by addition of macromolecules (like dextrans and albumin) [10-13]. Besides reduction of the intermembrane space, addition of macromolecules increases the number of contact sites between outer and inner mitochondrial membranes [12].

The aim of the present study was to determine whether macromolecules influence the delivery of ADP from mitochondrially associated hexokinase to oxidative phosphorylation under *in vitro* conditions. As macromolecules we used (bovine serum) albumin, because mitochondria are embedded in a protein solution in the cell, and dextran 20, because the effect of this macromolecule on the mitochondrial structure is similar to that of proteins and has been described extensively [12,13].

METHODS

Isolation of mitochondria: Rat liver mitochondria (RLM) were isolated as described previously [14].

Enzyme preparations: Rat brain hexokinase isoenzyme I (HK-I) was purified according to [15]. A non-bindable form of HK-I was prepared by α -chymotrypsin treatment [16] which results in removal of 7-9 N-terminal amino acids while not affecting the catalytic properties as such.

Binding of HK-I to rat liver mitochondria: Freshly isolated RLM, devoid of endogenous HK, were incubated with HK-I (0.2 U HK/mg mitochondrial protein) for 30 minutes at 4 °C in 0.25 M sucrose supplemented with 3 mM MgCl₂.

Respiration measurements: Respiratory rates of RLM containing bound HK-I (0.2 U HK/mg mitochondrial protein) were measured at 25 °C in a closed vessel of an OROBOROS® Oxygraph (ANTON PAAR KG, Graz, Austria). RLM were added in a concentration of 0.79 mg mitochondrial protein/ml in a medium containing 110 mM mannitol, 30 mM sucrose, 25 mM Hepes, 10 mM succinic acid, 10 mM MgCl₂, 20 mM glucose, 1 mM disodium EDTA, 5 mM potassium phosphate, 0.5 mM PEP, 2 μ M rotenone, and, where indicated, varying amounts of pyruvate kinase, and dextran 20 or BSA (pH 7.4). To avoid inhibition of HK activity, due to glucose-6-phosphate accumulation, the medium additionally contained 0.63 mM NAD⁺ and 4.4 U/ml glucose-6-phosphate dehydrogenase. HK-stimulated respiration was initiated by the addition of 2 mM ATP. The oxygen concentration at air saturation of the medium was assumed to be 229 and 194 nmol O₂/ml in the absence and presence of 10% dextran, respectively. The specific oxygen consumption rates (nmol O₂·min⁻¹·mg⁻¹ mitochondrial protein) were calculated from the first derivative of the oxygraph trace (DATGRAF 2.2 Analysis Software, OROBOROS®). Respiratory states were as defined by Chance and Williams [17].

RESULTS AND DISCUSSION

To investigate the effects of macromolecules on the communication between mitochondrially associated HK-I and oxidative phosphorylation, the following approach was used. Rat liver mitochondria, devoid of endogenous HK, were incubated with HK-I (0.2 U HK/mg mitochondrial protein) in the presence of 3 mM MgCl₂. Under the conditions used, 86 ± 3% ($n = 6$) of the HK was bound. In oxygraph experiments, the stimulation of respiration by ADP formed by bound HK was measured after addition of a saturating amount of ATP (2 mM) in the presence of 0.5 mM PEP and increasing amounts of extramitochondrial pyruvate kinase. The ADP formed by HK can either (1) diffuse to the matrix via porin and the nucleotide translocator to be utilized by oxidative phosphorylation, or (2) remain outside the mitochondrion to be consumed by pyruvate kinase.

Fig. 1 shows the original traces of a typical experiment performed in the presence of 10% (w/v) dextran 20. In the absence of pyruvate kinase (left), bound HK stimulated the rate of respiration from 20 to 63 nmol O₂·min⁻¹·mg⁻¹), which is lower than state 3 respiration. Addition of 1 mM ADP increased respiration shortly to state 3 until the oxygen in the cell was exhausted. The respiratory control index (RCI) was about 4.7, indicating that the mitochondria were functionally intact under these experimental conditions. In the right panel of Fig. 1, the effect of excessive pyruvate kinase activity on HK-induced respiration is shown. It is obvious that also in the presence of an extramitochondrial ADP consuming enzyme, a remarkable stimulation (40 nmol O₂·min⁻¹·mg⁻¹) of the

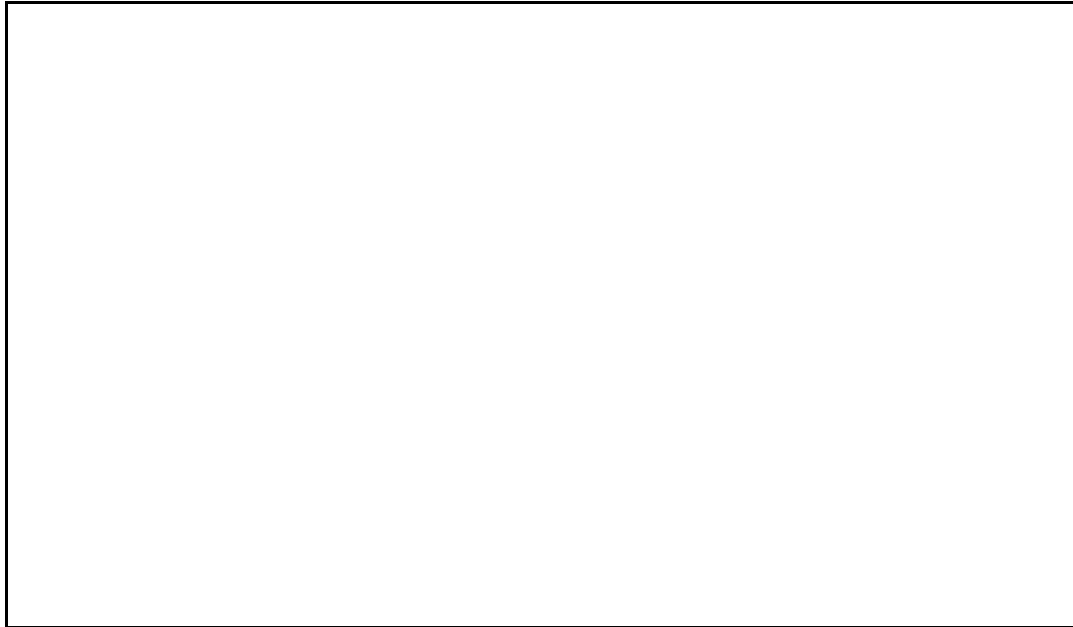


Fig. 1. Competition between RLM and pyruvate kinase for ADP regenerated by outer membrane-bound HK-I in the presence of 10% (w/v) dextran 20. Original recordings of an *Oxygraph* experiment, showing oxygen concentration [$\mu\text{M O}_2$] over time. The (negative) time derivative of the signal indicates the rate of respiration, J_{O_2} . Additions: M, 0.79 mg RLM/ml (containing 0.2 U HK-I/mg); ATP, 2 mM ATP; ADP, 1 mM ADP; AT, 50 μM atractylate. The experiment shown on the right was performed in the presence of 25 U pyruvate kinase/mg protein.

mitochondrial respiration persisted. Addition of 50 μM atractylate, an inhibitor of the nucleotide translocator, reduced the rate of respiration to $17.5 \text{ nmol O}_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$.

Similar incubations were performed with a range of pyruvate kinase activities in the presence and absence of 10% (w/v) dextran 20 (Fig. 2). Increasing amounts of pyruvate kinase reduced the HK-induced respiratory rate, demonstrating that pyruvate kinase can compete with oxidative phosphorylation for ADP formed by mitochondrial HK. However, it was impossible to reduce the HK-stimulated respiration to resting state (state 4) levels, indicating that part of the ADP formed by bound HK was not accessible for pyruvate kinase and transported into the mitochondrial matrix. From the maximal respiratory rate adjusted by bound HK-I, the state 4 respiration and corresponding rates in the presence of excess pyruvate kinase activity, it could be calculated that in the absence of macromolecules, 19% of the totally produced ADP was inaccessible to extramitochondrial pyruvate kinase. In the presence of 10% (w/v) dextran 20 this value increased to 31%. Similar results were obtained for albumin.

Our results suggest that there is channeling of ADP from outer membrane-anchored HK to the mitochondrial matrix as proposed earlier by Bessman [3], since the ADP produced by HK-I did not completely mix with the extramitochondrial pool of ADP. Under more *in vivo*-like conditions, in the presence of macromolecules, this channeling was more efficient. Other studies [12] which showed that dextrans increased the apparent affinity for ATP of HK-induced respiration

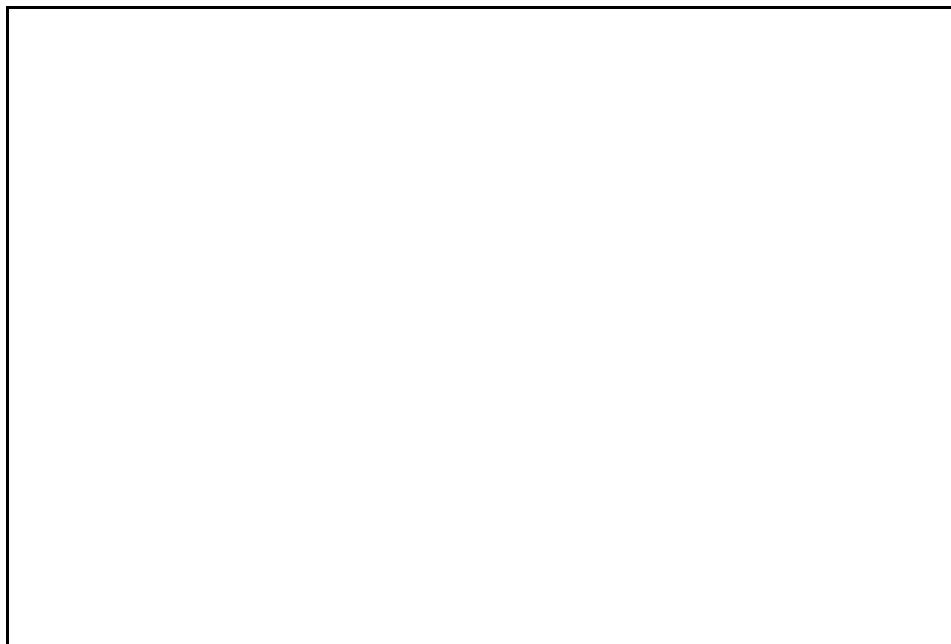


Fig. 2. Effect of 10% (w/v) dextran 20 on the competition between oxidative phosphorylation and extramitochondrial pyruvate kinase for ADP produced by mitochondrially associated hexokinase. The respiratory rates of rat liver mitochondria containing bound rat brain HK-I were measured as described in Methods and Fig. 1. HK-induced respiration was initiated by addition of 2 mM ATP (circles). Resting respiration was adjusted by addition of 50 μ M atractylate (squares). Triangles indicate state 3 respiration (1 mM ADP). Incubations in the presence of 10% dextran 20 are marked by closed symbols.

also suggested an improvement of channeling of ADP in the presence of macromolecules. Macromolecules up to 10% had no significant effect on the basic properties of rat liver mitochondria, like state 3 respiration, and the kinetic parameters of HK-I. The reduced intermembrane space, the increased number of intermembrane contacts and the enlarged unstirred layer could be involved in the tighter coupling between bound HK-I and oxidative phosphorylation in the presence of macromolecules.

The relatively high percentage of ADP which was supplied to oxidative phosphorylation was a typical property of the bound form of HK. In the presence of 10% (w/v) dextran 20, only 11% of ADP produced by a non-bindable form of HK was delivered to oxidative phosphorylation. Further information on the ADP delivery from outer membrane-anchored or non-bound HK-I to oxidative phosphorylation in the presence of macromolecules was obtained by measuring bulk phase ATP and ADP levels. At equal rates of oxygen consumption, when the intramitochondrial ADP levels and ATP/ADP ratios can be considered to be equal, the bulk phase ADP levels were lower and the bulk phase ATP/ADP ratios higher when HK-I was bound to mitochondria. In agreement with earlier studies [4], this confirms that ADP is more effectively channeled into the mitochondrion when it is produced by the bound form of HK.

In conclusion, these data show that macromolecules increase the channeling of ADP from mitochondrially associated hexokinase to oxidative phosphorylation.

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