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# The kinetic consequences of binding of hexokinase-I to the mitochondrial outer membrane

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**Abstract**

In a number of tissues, a major fraction of the hexokinase isozyme species present is bound at the mitochondrial outer surface. This study addresses the kinetic consequences of binding of hexokinase to the outer membrane of isolated, phosphorylating mitochondria. The primary aim was to separately measure the relative contributions to changes in the kinetic properties of hexokinase which (1) directly result from the binding as such, and (2) are caused by binding in close proximity to the site of mitochondrial ATP regeneration. Hexokinase isozyme I was purified from rat brain and then bound to intact rat liver mitochondria or outer membrane vesicles derived from these mitochondria. The apparent affinity ($K_{m\ app}$) for ATP and the $V_{max}$ of the bound hexokinase were determined by the spectrophotometric measurement of its activity as a function of the ATP concentration in the medium. The data obtained for the bound enzyme in the two systems were compared to the kinetic characteristics of hexokinase-I present in a non-bound form. Non-bindable hexokinase was obtained by mild protease treatment, such that bindability was completely abolished while the intrinsic catalytic properties remained unaltered. Parallel determinations of the steady-state ATP and ADP levels in mitochondrial suspensions with bound or non-bindable hexokinase present provided additional information on the consequences of binding. Binding of hexokinase to phosphorylating mitochondria decreased the $K_{m\ app}$ for ATP from 0.168 to 0.081 mM while not changing the $V_{max}$. It appeared that both binding per se ($K_{m\ app}$ for ATP decreased from 0.168 to 0.105 mM and from 0.194 to 0.103 mM upon binding to non-phosphorylating mitochondria and outer membrane vesicles, respectively) and intramitochondrial ATP regeneration (causing a further reduction in $K_{m\ app}$ for ATP from 0.105 to 0.081 mM for the system of phosphorylating mitochondria) jointly contributed to this reduction in $K_{m\ app}$ for ATP caused by binding to phosphorylating mitochondria. The kinetic effect exerted by intramitochondrial ATP regeneration persisted in the presence of an excess of extramitochondrial ATP regenerating activity. ATP and ADP measurements in hexokinase-mitochondria incubations demonstrated that: (i) up to 7 mM ATP in the medium, higher ADP concentrations were maintained for the case of non-bindable enzyme as compared to the bound enzyme; and (ii) ATP levels were not significantly different and therefore not responsible for the kinetic difference between bound and non-bound hexokinase. The present findings are compatible with previous suggestions for local channelling of adenine nucleotides between bound hexokinase and oxidative phosphorylation.

*Keywords:* Hexokinase; Mitochondrion; Oxidative phosphorylation; Outer membrane; Channeling; (Rat liver)

## 1. Introduction

Hexokinase (ATP; D-hexose 6-phosphotransferase; EC 2.7.1.1) catalyses the phosphorylation of glucose to glucose-6-phosphate, the initial step of glycolysis. In mammals, there are four different isozymes of hexokinase (labelled hexokinase-I, -II, -III and -IV) which

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; HK-I, hexokinase isozyme I; PEP, phospho*enol*pyruvate; PK, pyruvate kinase.

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differ in their kinetic properties and in their cellular and subcellular distribution (for review see Refs. [1,2]). Although glycolysis is a cytosolic process, hexokinases are partly found in a membrane-associated form. The current view (extensively reviewed in Ref. [2]) is that hexokinase-I and -II are partly bound at the mitochondrial outer surface and that type III is partly localized at the nuclear membrane [3]. By contrast, type IV is distributed throughout the cytosol in a free form. In tissues which largely rely on blood-born glucose for their energy supply, the mitochondrion-associated fraction of hexokinase is very prominent. In brain, for example, over 80% of the most abundant isoenzyme hexokinase-I is bound to mitochondria.

EM studies [4,5] and mitochondrial subfractionation studies [4,6–8] have shown that the mitochondrion-associated hexokinase species are localized at the outer surface of the outer mitochondrial membrane and suggest that they are enriched in contact sites between outer and inner membrane. A receptor protein for hexokinase has been identified in the mitochondrial outer membrane [9–11,37]. This receptor protein appeared to be identical to porin, the pore protein through which adenine nucleotides and other metabolites permeate the outer membrane.

It has previously been proposed that this outer membrane binding of hexokinase might have important functional consequences. Two aspects have been primarily considered. First, that binding changes the kinetic (Michaelis–Menten) constants of the enzyme [1]. It has for example been shown that bound hexokinase is less sensitive to product inhibition by glucose-6-phosphate than the non-bound form [12,13]. Secondly, hexokinase-mitochondrion interaction has been suggested to enable privileged delivery of mitochondrially synthesized ATP to the bound hexokinase and, by analogy, to enable privileged delivery of ADP generated by hexokinase activity to oxidative phosphorylation [14,15]. Anchoring of hexokinase at the level of the outer membrane pore protein and in intermembrane contact sites seems to uniquely facilitate this proposed functional coupling of the activities of hexokinase and oxidative phosphorylation.

So far, the knowledge on the relative importance of the different functional and kinetic consequences of hexokinase binding is limited. This study was aimed at unravelling the factors involved in the modification of the kinetic properties of hexokinase induced by binding to respiring mitochondria. The apparent affinity constant ($K_{m\ app}$) of hexokinase for ATP which was measured spectrophotometrically was used as a key parameter. In particular, we focused on separating the primary consequences of binding per se from the secondary effects of binding in close proximity to the site of intramitochondrial ATP regeneration. Hexokinase isoenzyme I was purified from rat brain in a bindable form. Protease treatment of this hexokinase-I species yielded a non-bindable form, without altering its intrinsic catalytic properties [16]. The $K_{m\ app}$ for ATP of these two hexokinase-I species was measured in the absence and presence of hexokinase-free, intact rat liver mitochondria and right-side out outer membrane vesicles, prepared from these mitochondria.

## 2. Materials and methods

### 2.1. Materials

Rat brain mitochondrial hexokinase (> 50 U/ml) was purified according to Wilson [17]. The protein concentration of the hexokinase preparation was determined according to Bradford [18]. The specific activity was 35 U/mg.

Rat liver mitochondria were prepared as described previously [19]. The respiratory control index was always between 5 and 6. The final mitochondrial pellet was resuspended in 0.25 M sucrose to give a protein concentration of approx. 50–60 mg/ml.

Glucose-6-phosphate dehydrogenase (the $NAD^+$-specific species from *Leuconostoc mesenteroiden*) and pyruvate kinase were from Boehringer (Mannheim, Germany). Prior to use the enzymes were desalted in 0.25 M sucrose, 0.01 M Hepes, 1 mM monothioglycerol (pH 7.4) using a Centricon 10 microconcentrator (Amicon).

α-Chymotrypsin was from Merck. All other chemicals used were of the highest grade available and obtained from regular commercial sources.

### 2.2. Preparation of non-bindable hexokinase-I

Hexokinase-I (≈ 2 mg/ml) was incubated with 5 μg α-chymotrypsin/mg hexokinase-I for 1 h at ambient temperature in 0.1 M potassium phosphate, 0.1 M glucose, 0.5 mM EDTA, 0.01 M monothioglycerol (pH 7.0). Proteolysis was stopped by the addition of 1 mM phenylmethylsulfonylfluoride. α-Chymotrypsin was removed by gel filtration using a Sephacryl S300 column (Pharmacia) in the same buffer at 4°C. In a test run containing sufficient hexokinase-I and α-chymotrypsin to detect both proteins by $A_{280}$ and SDS-PAGE, it was demonstrated that the two proteins were fully separated on the latter column. Fractions containing hexokinase activity > 0.5 U/ml were pooled and concentrated to a final activity of ≈ 50 U/ml using an Amicon ultrafiltration cell with YM 30 membrane. Binding studies showed that protease treatment almost completely abolished the $Mg^{2+}$-dependent binding of hexokinase-I.

### 2.3. Subfractionation of rat liver mitochondria

Rat liver mitochondria were subjected to the swell-shrink-sonicate procedure followed by density gradient centrifugation as detailed in Ref. [19]. This procedure yields three fractions: an outer membrane fraction enriched for the outer membrane marker monoamine oxidase, an inner membrane fraction enriched for inner membrane markers and an inner membrane plus matrix fraction enriched for both inner membrane and matrix markers. The final fractions were resuspended in 0.25 M sucrose to give protein concentrations of approx. 15 mg/ml.

### 2.4. Binding of hexokinase-I to intact mitochondria and mitochondrial membrane fractions

Intact rat liver mitochondria or mitochondrial membrane fractions were incubated for 30 min at 4°C with hexokinase-I (0.1 U hexokinase-I/mg mitochondrial protein or 0.3–0.85 U hexokinase-I/mg membrane vesicle protein) in 0.25 M sucrose, 3 mM $MgCl_2$. The bindability of the enzyme was determined as follows. The bound and non-bound enzyme were separated by centrifugation (5 min at 14000 rpm in an Eppendorf centrifuge in the case of mitochondria, and 15 min at 4°C at 30000 rpm in a Beckman TLA 100.2 rotor for the mitochondrial membrane fractions). Pellets were washed once with 0.25 M sucrose (without resuspension) and then resuspended using glass homogenizing beads in 0.25 M sucrose supplemented with 0.5% Triton X-100. Hexokinase activity was measured in both the supernatant and pellet. The total recovery of hexokinase activity was always higher than 95%.

### 2.5. Spectrophotometric assay of hexokinase activity

Hexokinase activity was measured spectrophotometrically at 25°C according to Chou and Wilson [20] by coupling the NADH formation by glucose-6-phosphate dehydrogenase to the glucose 6-phosphate production by hexokinase. NADH formation was measured at 340 nm with a Hitachi U3200 spectrophotometer equipped with an integrating sphere accessory.

The following components were added together in a final volume of 1060 μl:

(I) 1000 μl assay medium containing 200 mM mannitol, 30 mM sucrose, 25 mM Hepes, 10 mM succinic acid, 10 mM $MgCl_2$, 20 mM glucose, 1 mM disodium EDTA, 2 mM potassium phosphate and 2 μM rotenone (pH 7.4 with KOH);

(II) 10 μl $NAD^+$ (42 mg/ml in 0.1 M sodium phosphate, pH 7.0);

(III) 10 μl glucose-6-phosphate dehydrogenase (100 U/ml in 0.02 M Tris-HCl, 0.2% (w/v) bovine serum albumin, pH 7.5);

(IV) 40 μl hexokinase-I solution (0.02 U diluted in 0.25 M sucrose, 3 mM $MgCl_2$).

The reaction was started by the addition of ATP (pH 7.0 with NaOH) with final concentrations ranging from 0.05 to 7 mM and followed for at least 3.5 min.

### 2.6. Assay of hexokinase activity in the presence of mitochondria or mitochondrial membranes

In the presence of mitochondria, the activity of hexokinase-I was measured as above except that in point IV, 40 μl of hexokinase-mitochondria incubation mixtures (0.1 U hexokinase-I/mg mitochondrial protein; 0.02 U total activity) were added. In the presence of outer membrane vesicles, the activity of hexokinase-I was measured similarly, except that potassium phosphate was omitted from the assay medium and that 10 μl of the hexokinase-outer membrane vesicle mixtures (0.85 U hexokinase-I/mg outer membrane protein; 0.02 U total activity) were used.

It was routinely checked whether hexokinase-I bound initially, remained associated with the outer membrane throughout the experiment: over 95% and 70% of the bound hexokinase was recovered in the mitochondrial pellet and outer membrane vesicle pellet, respectively, after centrifugation of an aliquot taken from the cuvet.

### 2.7. Extramitochondrial ATP regeneration

In the case of extramitochondrial ATP regeneration, the above assay system was supplemented with 6 μl of 200 mM phospho*enol*pyruvate (PEP) and 6 μl of 2000 U/ml pyruvate kinase (PK). PEP and PK were added prior to starting the assay with ATP.

### 2.8. Inhibition of mitochondrial ATP conversion

In several studies, mitochondrial ATP conversion and exchange were blocked by the simultaneous addition of 5 mM KCN (to inhibit electron transport), 10 μg oligomycin/ml (to inhibit the $H^+$-ATPase), 0.5 mM diadenosine pentaphosphate (to inhibit adenylate kinase) and 5 μM carboxyatractyloside (to inhibit the ATP/ADP carrier) to the assay medium.

### 2.9. Data analysis

Hexokinase activities are expressed in units (i.e., μmol of product formed/min). Two alternative procedures were used to determine the $V_{max}$ and the apparent $K_m$ for ATP of hexokinase under the different experimental conditions. In both procedures hexokinase activity in steady-state and the initial ATP concentration added in the assay were used for calculations, unless indicated otherwise. First, the data were

fitted according to Michaelis–Menten kinetics with proportional weighting using the Enz fitter 1.05 program (Biosoft, Cambridge, UK). Secondly, linear regression of the inverse of the enzyme activity versus the inverse of the ATP concentration was performed according to Lineweaver and Burk as described in [21]. Both approaches yielded essentially identical results; only the quantitative results of the first method are presented. For clarity, the graphical representation of the data is done according to Lineweaver–Burk.

For statistical evaluation of the data, unpaired *t*-tests (two-tailed) were performed. *P*-values less than 0.05 were considered significant.

### *2.10. ATP and ADP determinations*

To determine the steady-state ATP and ADP levels in incubation mixtures, all reactions were terminated by dilution into an organic solvent mixture [22] in the following way. At 3.5 min after starting the reaction by the addition of ATP, a 675 μl sample was taken and transferred to a precooled (4°C) glass tube containing 1.5 ml phenol/chloroform/isoamylalcohol (38:24:1 v/v/v) and 225 μl 0.1 M EDTA (pH 7.4), followed by 90 s of vigorous mixing. Next the tubes were put on ice and the phases were allowed to separate. Then a 800 μl sample was taken from the water phase and centrifuged in a cooled Eppendorf centrifuge (12000 × *g*, 90 s). The supernatant was collected, frozen and stored in liquid nitrogen until ATP and ADP determinations. ATP and ADP levels were measured fluorimetrically, using standard enzymatic assays [23]. For ADP determinations, the amount of added pyruvate kinase was increased sixfold to compensate for inactivation of the enzyme by the residual organic solvents. Calibration curves of ATP or ADP, diluted in identical media as the samples, were used to calculate the unknown amount of ATP or ADP in the samples.

## 3. Results

### *3.1. Hexokinase-I binding to phosphorylating mitochondria increases its affinity for ATP*

First, the apparent affinity ($K_{m\ app}$) for ATP and $V_{max}$ of hexokinase-I when free in solution or when bound to isolated rat liver mitochondria were determined. The experimental setup was as follows. Purified rat brain hexokinase-I was incubated with freshly isolated rat liver mitochondria, which are devoid of endogenous hexokinase, in the presence of $MgCl_2$ to promote hexokinase binding. Under our conditions, 81 ± 5% ($n = 20$) of the enzyme was bound. This incubation mixture or an equivalent amount of hexokinase-I in solution (without mitochondria) was used to determine steady-state hexokinase activities in the presence of varying amounts of ATP in a medium in which mitochondrial oxidative phosphorylation is operative. The steady-state hexokinase rates and the initial ATP concentrations were used to calculate $V_{max}$ and $K_{m\ app}$ for ATP. Fig. 1 and Table 1 show that binding of hexokinase to phosphorylating mitochondria significantly decreased the $K_{m\ app}$ for ATP from 0.168 mM to 0.081 mM. Binding had no effects on the maximal activity of the enzyme.

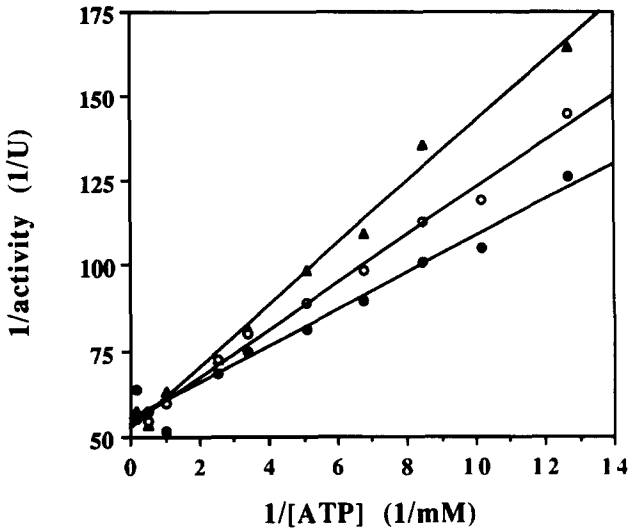


Fig. 1. Lineweaver–Burk plots of hexokinase-I activity as a function of initial ATP concentration under the following conditions: in absence of mitochondria (▲); bound to rat liver mitochondria in which ATP conversion and exchange were inhibited (○); bound to phosphorylating rat liver mitochondria (●). Rat brain hexokinase-I was incubated with rat liver mitochondria (0.1 U hexokinase-I/mg mitochondrial protein) in the presence of 3 mM $MgCl_2$. Under the used conditions 81% of the hexokinase was bound. The incubation mixture or an equivalent amount of hexokinase-I in absence of mitochondria were used to determine the steady-state hexokinase rates at various ATP concentrations in a medium with or without inhibitors of ATP conversion and exchange (for details see Section 2). Single representative experiments are depicted. Average values of apparent affinity for ATP ± standard deviation as calculated according to Michaelis–Menten kinetics from a series of identical experiments are presented in Table 1.

### *3.2. Hexokinase-I binding to non-phosphorylating mitochondria and to isolated outer membrane vesicles results in a similar increase of its affinity for ATP*

Two different factors may contribute to the increase in $K_{m\ app}$ for ATP induced by binding of hexokinase-I to phosphorylating mitochondria: (1) binding might directly change the (apparent) catalytic properties of the enzyme; (2) the $K_{m\ app}$ might be indirectly affected by binding in close proximity to the site of mitochondrial ATP regeneration. To assess the contribution of the first factor, the $K_{m\ app}$ for ATP and the $V_{max}$ were determined for hexokinase-I bound to: (1) mito-

Table 1
Apparent affinity for ATP of native and α-chymotrypsin-treated hexokinase-I under three basic conditions: in absence and in presence of rat liver mitochondria in which ATP conversion and exchange were either operative or inhibited

| Condition | $K_{m\,app}$ for ATP (mM) | | |
|---|---|---|---|
| | HK-I | non-bindable HK-I | HK-I + PEP/PK |
| No mitochondria | 0.168 ± 0.022 ($n = 8$) | 0.165 ± 0.01 ($n = 6$) | 0.121 ± 0.010 ($n = 5$) |
| 'Inhibited' mitochondria | 0.105 ± 0.012 ($n = 8$) | 0.187 ± 0.017 ($n = 6$) | 0.085 ± 0.007 ($n = 5$) |
| Phosphorylating mitochondria | 0.081 ± 0.014 ($n = 6$) | 0.174 ± 0.024 ($n = 4$) | 0.055 ± 0.014 ($n = 3$) |

Purified rat brain hexokinase-I or α-chymotrypsin-treated hexokinase-I were incubated with rat liver mitochondria in the presence of 3 mM $MgCl_2$ (0.1 U hexokinase-I/mg mitochondrial protein), resulting in binding percentages of 81 ± 5% ($n = 20$) and 11 ± 2% ($n = 11$), respectively. These incubation mixtures or equivalent amounts of hexokinase-I in the absence of mitochondria were used to measure hexokinase activities at various ATP concentrations with a coupled enzyme assay in a medium in which mitochondrial ATP conversion and exchange were either operative or blocked. The apparent affinity was calculated by fitting the steady-state hexokinase rate as a function of initial ATP concentration according to Michaelis–Menten kinetics. Column 3 shows experiments carried out in the presence of extramitochondrial ATP regenerating capacity by addition of PEP and PK. The values represent averages ± standard deviations of independent experiments performed with different mitochondrial and hexokinase preparations.

chondria, in which ATP conversion and exchange were inhibited; and (2) purified right-side out outer membrane vesicles.

Table 1 shows that the $K_{m\,app}$ for ATP of hexokinase-I bound to 'inhibited' mitochondria was 0.105 mM. This number was significantly lower than that for hexokinase-I in the absence of mitochondria, suggesting that binding directly changed the catalytic properties (see also Fig. 1). Importantly however, this effect cannot totally explain the increase in apparent affinity induced by binding of hexokinase to phosphorylating mitochondria: the $K_{m\,app}$ for ATP of hexokinase bound to 'inhibited' mitochondria is significantly higher than that for hexokinase bound to phosphorylating mitochondria.

A more direct approach to study changes in catalytic properties related to binding is to utilize purified outer membrane vesicles rather than whole mitochondria. Fig. 2 demonstrates that the binding of hexokinase to mitochondrial outer membrane vesicles is $Mg^{2+}$-dependent, analogous to the binding to intact mitochondria. Addition of 3 mM $MgCl_2$ increased the binding degree from 11% to 88%. In agreement with the anticipated specificity among the two mitochondrial boundary membranes, high capacity, $Mg^{2+}$-dependent binding of hexokinase was unique for the outer membrane: inner membrane vesicle preparations

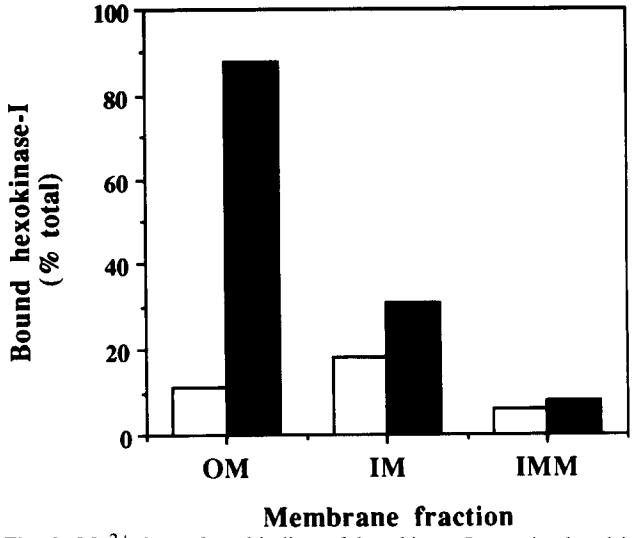


Fig. 2. $Mg^{2+}$-dependent binding of hexokinase-I to mitochondrial membrane fractions. The outer membrane (OM), inner membrane (IM) or inner membrane plus matrix (IMM) fractions, derived from rat liver mitochondria, were incubated with hexokinase-I (0.3 U hexokinase-I/mg OM, IM or IMM protein) in the absence (open bars) or presence (closed bars) of 3 mM $MgCl_2$ (for details, see text). Hexokinase binding was determined by measuring its activity in the membrane pellets and their respective supernatants after centrifugation.

showed minor binding which was largely $Mg^{2+}$-independent (Fig. 2).

Incubation mixtures containing hexokinase-I bound to outer membrane vesicles, or equivalent amounts of the enzyme in the absence of outer membrane vesicles, were used to determine the direct effects of vesicle binding on the catalytic properties of hexokinase. Binding decreased the $K_{m\,app}$ for ATP significantly from

Table 2
Apparent affinity for ATP of native and α-chymotrypsin-treated hexokinase-I in absence and in presence of purified outer membrane (OM) vesicles

| Condition | $K_{m\,app}$ for ATP (mM) | |
|---|---|---|
| | HK-I ($n = 3$) | non-bindable HK-I ($n = 1$) |
| − OM vesicles | 0.194 ± 0.018 | 0.181 |
| + OM vesicles | 0.103 ± 0.025 | 0.185 |

Purified rat brain hexokinase-I or α-chymotrypsin-treated hexokinase-I were incubated with mitochondrial outer membrane vesicles in presence of 3 mM $MgCl_2$ (0.85 U hexokinase-I/mg outer membrane protein). Binding amounted to 86 ± 1% ($n = 3$) and 5%, respectively. These incubation mixtures or equivalent amounts of hexokinase in absence of outer membrane vesicles were used to determine hexokinase activity at various ATP concentrations with a coupled enzyme assay. The apparent $K_m$ for ATP was calculated by fitting the steady-state hexokinase activities as a function of the initial ATP concentration according to Michaelis–Menten kinetics. Values for native hexokinase-I represent averages ± standard deviation of independent experiments performed with three different batches of outer membrane vesicles. Further details in Section 2.

0.194 to 0.103 mM while having no effect on the $V_{max}$ (Table 2).

### 3.3. Mitochondrial ATP regeneration increases the affinity for ATP of bound hexokinase but not of non-bindable hexokinase

As stated above, the increase in the $K_{m\ app}$ for ATP induced by binding of hexokinase-I to phosphorylating mitochondria can partially be explained by direct effects of binding. However, under phosphorylating conditions yet another factor, presumably mitochondrial ATP regeneration, indirectly also contributes to this increase. To measure the importance of outer membrane binding for the kinetic consequences of this mitochondrial ATP regeneration, a non-bindable form of hexokinase-I was used. The ability of hexokinase-I to bind to mitochondria was eliminated by α-chymotrypsin-treatment [16]. Protease treatment results in removal of the 7–9 N-terminal amino acids while not affecting the catalytic properties, as described below.

#### 3.3.1. Characterization of non-bindable hexokinase-I

Several properties of the protease-treated enzyme were determined prior to its use in functional studies. SDS-PAGE demonstrated that no detectable changes in the apparent molecular weight of the enzyme had occurred, indicating that only a small fragment was removed. Binding experiments were performed to compare the capacity of native and protease-treated hexokinase-I to bind to rat liver mitochondria. Protease treatment reduced the binding to $11 \pm 2\%$ ($n = 11$).

To exclude that α-chymotrypsin treatment had modified the kinetic parameters of hexokinase-I, steady-state hexokinase activities were measured at various ATP concentrations to determine $V_{max}$ and apparent affinity for ATP of protease-treated and native hexokinase-I. Table 1 shows that the affinity for ATP of hexokinase-I and α-chymotrypsin-treated hexokinase-I was 0.168 mM and 0.165 mM, respectively. In summary, α-chymotrypsin treatment of hexokinase-I had no significant effects on its apparent affinity for ATP and its maximal activity while almost completely abolishing the $Mg^{2+}$-dependent binding.

#### 3.3.2. Effects of mitochondrial ATP regeneration on $K_{m\,app}$ for ATP of non-bindable hexokinase-I

The experimental set up was as follows: non-bindable hexokinase-I was incubated with rat liver mitochondria in the presence of $MgCl_2$. Under those conditions, binding was marginal (i.e., $11 \pm 2\%$ ($n = 11$)). The incubation mixture or an equivalent amount of non-bindable hexokinase-I without mitochondria was used to determine $K_{m\ app}$ for ATP and $V_{max}$ in a medium supporting mitochondrial oxidative phosphorylation or supplemented with inhibitors of ATP conversion and exchange. The results are presented in Table 1. The $K_{m\ app}$ for ATP of non-bindable hexokinase-I appeared essentially identical when measured in the absence of mitochondria and in the presence of phosphorylating or non-phosphorylating mitochondria. The presence of mitochondria had also no significant effects on the $V_{max}$ of the soluble, non-bindable enzyme. These data imply that the observed decrease of $K_{m\ app}$ for ATP of bound hexokinase-I measured under phosphorylating conditions is dependent on its localization at the outer membrane surface.

Similar observations were made for non-bindable hexokinase-I in the presence of isolated outer membrane vesicles (Table 2), confirming that the increase in apparent affinity for ATP of hexokinase-I after binding to outer membrane vesicles was a typical property of the bound form and not dependent on the presence of vesicles as such.

### 3.4. Intramitochondrial versus extramitochondrial ATP regeneration

The above experiments demonstrated that binding of hexokinase to the outer mitochondrial membrane increased the apparent affinity for ATP and that this was partly due to mitochondrial ATP regeneration. We next investigated whether it makes a difference from which source the ATP regenerating capacity originates, i.e., the intra- or the extramitochondrial compartment. To that aim, the kinetic parameters of hexokinase-I in absence of mitochondria and hexokinase-I associated with mitochondria were also determined in the presence of an extramitochondrial ATP regenerating system, i.e., PEP (1.1 mM) and PK (12 units). Based on literature data of the $K_m$ for ADP of PK [24] and of oxidative phosphorylation [25], it was estimated that the ratio of the rate of PK- and oxidative phosphorylation-dependent ATP regeneration minimally amounted to 15 under the present conditions. Except for the addition of PEP and PK, the experimental setup was identical to the experiments described above.

Table 1 shows that in all three cases tested, the $K_{m\ app}$ for ATP was reduced by the addition of PEP and PK. The $K_{m\ app}$ for ATP of hexokinase-I bound to non-phosphorylating mitochondria amounted to 0.085 mM. This was significantly lower than the $K_{m\ app}$ for ATP of hexokinase-I in solution which was 0.121 mM. The apparent affinity of hexokinase bound to phosphorylating mitochondria was 0.055 mM which was significantly lower than that for hexokinase-I bound to 'inhibited' mitochondria. Interestingly, these data show that intramitochondrial ATP regeneration exerts a considerable effect on the kinetic properties of bound

hexokinase, even in the presence of an excess capacity of extramitochondrial ATP generation.

External ATP regeneration had no effect on the $V_{max}$ of hexokinase-I in solution or hexokinase-I bound to inhibited rat liver mitochondria (not shown). By contrast, the $V_{max}$ of hexokinase-I bound to phosphorylating mitochondria was significantly decreased upon addition of PEP and PK. The reason for that is not clear as yet.

Control experiments confirmed that the above results were exclusively due to external ATP regeneration and not to PEP or PK separately (data not shown).

### 3.5. Steady-state ATP and ADP concentrations

In the above experiments, the kinetic parameters of hexokinase were determined by measuring hexokinase activity under steady-state conditions as a function of the initial ATP concentration added to the medium. In order to assess whether the steady-state ATP concentrations differed significantly from the initial ATP concentrations, ATP concentrations were determined upon quenching of the incubation mixtures. In view of the importance of ADP in stimulating oxidative phosphorylation, ADP levels were also measured.

The experiments were done as follows. Incubation mixtures of hexokinase-I bound to phosphorylating rat liver mitochondria or α-chymotrypsin-treated hexokinase-I in the presence of phosphorylating rat liver mitochondria were used for the spectrophotometric measurement of hexokinase activity as a function of the ATP concentration in the medium. When the system was in a steady-state, a sample was taken and diluted into an organic solvent mixture to instantaneously quench all the reactions and extract the metabolites. After that, ATP and ADP levels were determined using fluorimetric methods.

The results of these experiments are listed in Table 3. The ATP levels in the mitochondrial incubation mixtures containing the bound or the non-bound enzyme were not significantly different. The apparent affinity for ATP in these experiments as calculated from the *initial* ATP concentrations were 0.081 ± 0.020 mM and 0.194 ± 0.012 mM for bound hexokinase and non-bindable hexokinase-I, respectively. The $K_{m\ app}$ values as calculated from the *actual* ATP concentrations in steady-state were 0.081 ± 0.018 mM and 0.192 ± 0.018 mM, respectively, and thus not significantly different from the values calculated from the initial ATP concentrations.

Interestingly, the ADP concentrations (Table 3) appeared lower for the case of bound than for non-bindable hexokinase-I. Consequently, higher ATP/ADP ratios were maintained when hexokinase-I was associated to mitochondria (Table 3). It was remarkable that also at saturating ATP concentrations where the hexokinase activities of the bound and non-bound form were similar, the ATP/ADP ratios remained different.

The above mentioned ATP and ADP concentrations represent values in total incubation mixtures, including contributions from both intra- and extramitochondrial nucleotides. However, direct measurements of intramitochondrial ATP and ADP showed that under our experimental conditions their contributions to the total ATP and ADP concentrations were negligible (approximately 2 μM and 0.5 μM, respectively) (Laterveer, F.D., unpublished observations).

## 4. Discussion

In this study intact rat liver mitochondria, isolated outer membrane vesicles and purified rat brain hexokinase-I were used to investigate the effects of outer membrane binding on the kinetic parameters of hexokinase, in particular the $K_{m\ app}$ for ATP. This ap-

Table 3
Effects of binding of hexokinase-I to the mitochondrial outer membrane on extramitochondrial ATP and ADP levels

| Steady state ATP (mM) | | Steady state ADP (μM) | | ATP/ADP | |
|---|---|---|---|---|---|
| Bindable HK-I | Non-bindable HK-I | Bindable HK-I | Non-bindable HK-I | Bindable HK-I | Non-bindable HK-I |
| 6.80 ± 0.34 | 6.55 [a] | 18.5 ± 1.6 | 23.8 [a] | 367 | 276 |
| 4.73 [a] | 4.67 [a] | 17.9 [a] | 21.6 [a] | 264 | 216 |
| 1.93 ± 0.06 | 2.00 ± 0.07 | 13.1 ± 1.1 | 18.1 ± 0.4 | 147 | 110 |
| 0.32 ± 0.03 | 0.33 ± 0.02 | 8.8 ± 0.5 | 10.9 ± 0.2 | 37 | 30 |
| 0.21 ± 0.02 | 0.21 ± 0.02 | 8.4 ± 0.5 | 10.8 ± 0.2 | 25 | 20 |
| 0.16 ± 0.01 | 0.16 ± 0.01 | 7.7 ± 1.0 | 8.8 ± 0.1 | 21 | 18 |
| 0.10 ± 0.00 | 0.10 ± 0.00 | 6.8 ± 0.9 | 6.7 ± 0.3 | 15 | 15 |

Native or α-chymotrypsin-treated hexokinase-I were incubated with rat liver mitochondria (0.1 U hexokinase-I/mg mitochondrial protein) in the presence of 3 mM $MgCl_2$. Binding amounted to 79 ± 2% ($n = 2$) and 12 ± 1% ($n = 2$), respectively, of the added hexokinase. The incubation mixtures were used to determine steady-state hexokinase activities as a function of ATP concentration under phosphorylating conditions. During steady-state hexokinase activities, samples were quenched with organic solvent mixtures as described in Section 2. ATP and ADP concentrations were determined by standard fluorimetric assays. Unless otherwise indicated, the values represent average values ± standard deviation of two independent experiments with different mitochondrial preparations measured in duplicate.

[a] Result of one experiment.

proach enabled us to separately quantify the primary effects of binding to the outer mitochondrial membrane per se and the secondary effects of intra- and extramitochondrial ATP regeneration.

In agreement with previous findings (for review see Ref. [1]) [38], binding of hexokinase-I to phosphorylating mitochondria was found to increase the apparent affinity for ATP. In addition, it was observed that binding had no effect on the maximal activity of the enzyme. The last observation is in agreement with previous reports [33–36] but at variance with findings by Brdizcka and coworkers [26,27] who reported a several fold increase in $V_{max}$ upon binding. Our data offer no explanation for the latter deviating findings.

The increased affinity for ATP was shown to result from two distinct contributions, i.e., outer membrane binding by itself and the fact that binding occurs in close proximity to the site of mitochondrial ATP regeneration. These factors will be discussed separately below.

The effects of binding per se on the catalytic properties of the enzyme were inferred from experiments in which hexokinase-I was bound to either right-side out outer membrane vesicles (Table 2) or mitochondria in which ATP conversion and exchange had been inhibited (Table 1). The apparent affinity for ATP was similar in these two cases and considerably higher than for the non-bound enzyme. Interestingly, this finding suggests that the effects on the $K_{m\ app}$ of hexokinase for ATP are similar for binding to the pore protein in non-contact site domains of the outer membrane (as obviously occurs in the outer membrane vesicle system) and in contact site domains of this membrane (assuming preferential binding of hexokinase in contact sites in the incubations with intact mitochondria [27–31]). It remains to be established whether, apart from the $K_{m\ app}$ for ATP and the $V_{max}$, this also applies to other kinetic parameters.

The origin of the direct kinetic effects of binding remains to be elucidated. Two major possibilities should be considered. First, that binding modifies the catalytic properties of the enzyme, and secondly that the local substrate concentrations as experienced by the catalytic center at the membrane interface are different from those in the bulk phase. On the basis of glucose-6-phosphate inhibition studies, Wilson [1] has proposed that binding of hexokinase to the outer mitochondrial membrane is accompanied by a conformational change, implying the first possibility while not excluding the second.

An additional contribution to the decrease in $K_{m\ app}$ for ATP measured for hexokinase upon association with phosphorylating mitochondria seems to result from binding in close proximity to the site of mitochondrial ATP (re)generation. This was concluded from comparative studies using non-bindable and bindable hexokinase in the presence of phosphorylating mitochondria (Table 1). Determinations of the bulk phase levels of ATP as maintained under steady-state conditions (Table 3) showed that these were not significantly different from the initially added ATP concentrations, and therefore cannot explain the observed increase in $K_{m\ app}$ for ATP. Furthermore, the bulk phase ADP levels invariably were far below the enzyme's $K_i$ for ADP which is approx. 1 mM [28]. Consequently, it seems highly unlikely that the bound enzyme's increased $K_{m\ app}$ for ATP seen under phosphorylating conditions was caused by diminished inhibition by ADP. On the basis of these considerations, it rather seems that the bound enzyme experiences higher local ATP concentrations, thereby supporting higher activity. Surprisingly, the kinetic effect exerted by intramitochondrial ATP regeneration was maintained in the presence of an excess of extramitochondrial ATP regenerating capacity (Table 1). This suggests that ATP delivery to bound hexokinase proceeds more efficient from the intramitochondrial side than from the extramitochondrial side. It seems plausible that, in contrast to the direct kinetic consequences of binding, the effect of intramitochondrial ATP (re)generation on $K_{m\ app}$ for ATP is different (i.e., stronger) for hexokinase bound in contact site regions of the outer membrane.

As described in the Introduction, the possibility for coupling between hexokinase activity and oxidative phosphorylation has been implied as an important functional consequence of hexokinase binding [14,15]. The functional coupling might provide a mechanism for coordination of glycolytic and oxidative phases of glucose metabolism [2]. On the basis of a variety of studies [14,15,29–32], it has been proposed that the coupling proceeds in a putative micro-compartment in which channeling of nucleotides between bound hexokinase and the mitochondrial matrix takes place. Channelling implies that transfer of adenine nucleotides between bound hexokinase and the mitochondrion occurs without (complete) mixing with the extramitochondrial nucleotide pools. The present data also lend some support to the channelling model. First, the persistence of the kinetic effect on $K_{m\ app}$ for ATP exerted by intramitochondrial ATP regeneration in the presence of the PEP/PK system (Table 1) suggests that there is an intimate communication between bound hexokinase and mitochondrial matrix. Secondly, our data suggest that the steady-state bulk phase ADP concentrations were lower and the bulk phase ATP/ADP concentration ratios higher for the bound enzyme than for the non-bindable enzyme (Table 3). Although not excluding alternative explanations, this finding is compatible with local cycling of adenine nucleotides between bound hexokinase and the matrix compartment. Interestingly, the difference in adenine nucleotide pattern seemed to persist across the range of ATP concentra-

tions tested, i.e., well above the $K_{m\ app}$ for ATP of bound hexokinase. We are currently investigating these channelling aspects in more detail.

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