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# Effect of macromolecules on the structure of the mitochondrial inter-membrane space and the regulation of hexokinase

Ulrike Wicker [a], Klaus Bücheler [a], Frank N. Gellerich [b], Michael Wagner [b], Matthias Kapischke [b] and Dieter Brdiczka [a]

[a] *Fakultät für Biologie, Universität Konstanz, Konstanz (Germany) and* [b] *Institut für Biochemie, Medizinische Akademie Magdeburg, Magdeburg (Germany)*

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Macromolecules as components of the physiological mitochondrial environment were substituted by addition of 10% dextran 70. This led to a significant reduction of the space between the two envelope and the crista membranes and to an increase of contact sites as observed by freeze-fracture analysis. The preferential binding of hexokinase in these sites was employed to further analyze the dextran effect: (i) desorption of the enzyme by digitonin treatment was found to be significantly reduced in the presence of dextran although liberation of adenylate kinase and monoamine oxidase were not affected, (ii) the affinity of isolated hexokinase isozyme I to liver mitochondria was increased by dextran. Generally the binding of hexokinase to intact mitochondria (also control mitochondria) followed a co-operative mechanism and led to an activation. Cooperativity and activation were not observed when the contact formation was suppressed by dinitrophenol or glycerol. The binding of hexokinase to the isolated outer membrane resembled that of mitochondria in the absence of contacts (i.e., no cooperativity and activation). Conversely to the observation in intact mitochondria, dextran rather reduced the affinity of hexokinase to the isolated outer membrane. Kinetic analyses of the dextran effect served to explain the function of contact site specific hexokinase binding. We observed that dextran improved the hexokinase dependent stimulation of the oxidative phosphorylation (state 3 respiration), while the activity of the enzyme with internal or external ATP remained unaffected. The results suggest three things: (i) that contact sites are probably more frequent in the intact cell than in vitro in the absence of macromolecules, (ii) that the contact preference of hexokinase serves rather the ADP supply of the translocator than the ATP transfer to the enzyme and (iii) that the total cellular hexokinase activity may be regulated by specific binding of the enzyme to the contact sites, either because of a different pore structure or because of additional components exclusively exposed in these sites.

## Introduction

Contact sites between the two mitochondrial envelope membranes have been described as specific structures in conventionally fixed and embedded [1], as well as in freeze-fractured mitochondria [2,3]. Two functions have been attributed to these structures: uptake of mitochondrial precursor proteins [4,5] and improvement of mitochondrial energy transfer [6,7]. The latter function was discussed because of the observation, by electron microscopy [8,9] and isolation of the contacts [9–11] that several kinases (e.g., hexokinase, creatine kinase, nucleoside diphosphate kinase) at the mitochondrial surface were specifically concentrated in these sites. Kinetic analyses of the kinases in intact mitochondria revealed a functional coupling to the ATP in the inner mitochondrial compartment [12–14]. Furthermore, the kinase product ADP was found to induce the contact sites as analyzed in freeze-fractured mitochondria [15].

Based on these results it was postulated that contact sites between the two envelope membranes are not pre-existing structures, but are formed as adaptations to changing metabolic conditions. To meet this function, both mitochondrial envelope membranes and the intermembrane space contain components which are capable to induce contacts. Generally these components are supposed to be proteins which, besides their specific functions (kinase, pore, transport system) have the ability to interact with other proteins or phospholipids to form functional complexes, the contact sites [7]. In support of this postulate, preferentially the octameric structure of mitochondrial creatine kinase

Correspondence to: D. Brdiczka, Fakultät für Biologie, Universität Konstanz, 7750 Konstanz, Germany.
Abbreviations: DNP, dinitrophenol; RCI, respiratory control index.

was found to be present in the contact sites of brain mitochondria [16]. It has been reported that especially the octamer of mitochondrial creatine kinase in contrast to the dimer is capable of linking together two artificial membranes [17]. Moreover, it was observed that the isolated contact sites had a higher affinity for hexokinase compared to outer the membrane fraction [10,18].

In the intact cell mitochondria are embedded in an approx. 30% protein solution [19] whereas, upon isolation applied, isotonic sucrose media are free of macromolecules. Due to the free sucrose permeability of the outer membrane, mitochondria isolated and suspended in conventional isotonic media exhibit a swollen intermembrane space and a round shape which counterpoints the elongated shape and narrow intracrista space observed in situ by applying freeze substitution [20].

The swelling of the intracrista space and the associated dilution of proteins in this compartment should, on one hand, have implications on the structure and the organization of enzymes located in this compartment and, on the other hand, might directly effect the permeability of the pore because of interruption of the capacitive coupling between the two envelope membranes in the contact sites, as postulated recently [21].

To date, little has been learned concerning these aspects. In the present investigation we attempted to adapt the mitochondrial environment closer to the physiological situation by applying polymers like dextran to substitute intracellular proteins. We observed structural changes induced by the presence of macromolecules in freeze-fractured mitochondria which resulted in an increase of contact sites. On the basis of these results we tried to obtain insight into the function of contacts in the specific organization of kinases such as hexokinase at the mitochondrial surface. We analyzed this question by studying whether the effect of macromolecules to induce contacts resulted in different binding and kinetics of hexokinase in intact mitochondria when compared to the isolated outer membrane.

## Materials and Methods

Dextran M70 was from Serumwerke (Bernburg, Germany). Dextran 70 and Hepes were bought from Serva Heidelberg, Germany. Sucrose, phosphate, $MgCl_2$ were products of Merck Darmstadt, Germany. Oligomycin was obtained from Calbiochem (San Diego, USA). All other chemicals and enzymes were purchased from Boehringer Mannheim, Germany.

*Preparation of mitochondria from rat liver and brain*

Liver mitochondria from rats (250 g body weight) were isolated by differential centrifugation in 0.25 M sucrose, 10 mM Hepes (pH 7.4). The mitochondrial sediment was washed two times using $6000 \times g$ and $3000 \times g$ (Sorvall, rotor SS-34) for sedimentation. Mitochondria from rat brain were prepared according to Rehncrona et al. [22] in a medium containing 0.25 M mannitol, 0.075 M sucrose, 1 mM EGTA, 5 mM Hepes (pH 7.4) and 0.1% fatty acid free bovine serum albumin. The mitochondria were further purified by 35 min centrifugation at 38000 rpm in a 60 Ti rotor (Beckman) on a 20% percoll gradient. Percoll was removed from the mitochondrial fraction by washing two times with the above medium.

*Isolation of the outer membrane*

The outer membrane from rat liver mitochondria was isolated according to Sottocasa [23] on a discontinues sucrose density gradient after disrupting the mitochondria by osmotic shock and mild sonification.

*Isolation of hexokinase*

Hexokinase isozyme I was isolated from rat brain according to Wilson [24].

*Respiration measurements*

The respiratory rates were measured at 25°C in a closed vessel by a custom built rate meter equipped with a Clark-type oxygen electrode. The oxygen content of the incubation medium was assumed to be 229 nmol $O_2$/ml [25].

*Enzyme assays*

Binding of hexokinase (EC 2.7.1.1) to mitochondrial membranes was performed by incubation for 10 min at room temperature in 0.25 M sucrose, 10 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 1 mM ADP, 5 mM succinate and glucose. Outer membrane or mitochondria were separated by centrifugation. The activity of hexokinase was measured in agreement with Bücher et al. [26] in the supernatant and resuspended sediments.

Activity of hexokinase in brain mitochondria was determined in a medium containing 0.25 M mannitol, 0.075 M sucrose, 1 mM EGTA, 5 mM Hepes (pH 7.4) and 0.1% fatty acid free bovine serum albumin.

*Mitochondrial samples for freeze-fracture*

For freeze fracturing mitochondria were suspended to a concentration of about 15 mg per ml in a medium containing 113 mM sucrose, 10 mM $K_2HPO_4$, 5 mM $MgCl_2$, and 12.5 mM succinate (pH 7.4). The hypotonic medium was originally described by Hackenbrock [27,28]. We used this medium to compare our results with those of Hackenbrock and to reduce the unphysiologically large volume of the outer mitochondrial compartment, yet without effects on the frequency of contact sites [3]. To avoid anaerobiosis these samples were

gassed with carbogene (95% $O_2$, 5% $CO_2$). Under these conditions the back-diffusion of $O_2$ per ml oxygen free sample was 860 nmol $O_2$/min, while the maximal $O_2$ consumption of a mitochondrial suspension (with 15 mg/ml of protein) was 675 nmol/min per ml. The activity of the oxidative phosphorylation, the degree of coupling and the oxidation rates were measured in parallel with the oxygen electrode after 10-fold dilution of the mitochondrial suspension.

*Freeze-fracture analysis*

Mitochondria were directly subjected to rapid freezing by the sandwich technique as described by Knoll et al [29]. The time to cryofixation was approx. 15 s. The samples were broken in a Balzers BAF 301 freeze etch device at $-100$°C and $(2-7)\cdot 10^{-7}$ Torr, followed by Pt/C and C shadowing.

For electron microscopy, a Hitachi H7000 instrument at 75 kV was used. The morphological evaluations were performed using a CRP graphic board connected to an IBM compatible AT computer. The nomenclature of the exposed membranes follows that of Branton et al. [30].

As a means of quantifying the difference in fracture-plane deflections, the length of the edge where the fracture plane deflects was measured as related to the corresponding mitochondrial area. In convex fractures, the edge of the exoplasmic face of the outer membrane was measured, whereas, in concave fractures, measurements were made of the exoplasmic face of the inner membrane. The values $L$ were expressed as length ($\mu$m) per unit of mitochondrial fractured membrane area. In every population of mitochondria, there were some which were completely void of fracture plane deflections. Due to the fact that the size of this part of mitochondria changed according to the metabolic state of the whole sample, we corrected our calculation of fracture plane deflections in order to compensate for these differences. The adjustment was made by first determining on survey pictures the total area of mitochondria with no deflections, $M_s$, and those with deflections, $M_p$, and then normalizing these values with the expression $M_p/(M_p + M_s)$. Accordingly, the final value for quantification of freeze-fracture deflections, $L_p$, was calculated from the equation: $L_p\ (\mu m/\mu m^2) = L \cdot M_p/(M_s + M_p)$. The stated statistical differences were obtained by applying the $U$-test.

The quantification was made in the areas where the curvature was low to avoid large distortions of the measured edge lines. As the length $L_p$ was based on the corresponding area it was important to establish a method for standardization of the area which was used to determine $L$. The area was chosen according to the intensity of shadowing, meaning that those parts were excluded where the curvature was high and the shadowing was strong or almost not present respectively.

## Results

*Influence of dextran on functional properties of mitochondria*

Before analyzing the effect of macromolecules on the structure of mitochondria we studied the effect of different dextran 70 concentrations on basic parameters of mitochondria. The 2 ml volume of the oxygraph chamber was stirred by a strong magnetic stirrer located on a pin in the middle of the chamber in order to obtain homogenous suspensions despite of increasing viscosities due to the macromolecules. Addition of up to 15% (w/v) dextran M70 had little effect on the respiration rates with succinate as substrate (Fig. 1). The respiration rate in the presence of 0.5 mM ADP (state 3) increased slightly from 75.5 to 80 nmol $O_2$/mg as well as that of the resting (state 4) state, from 14.9 to

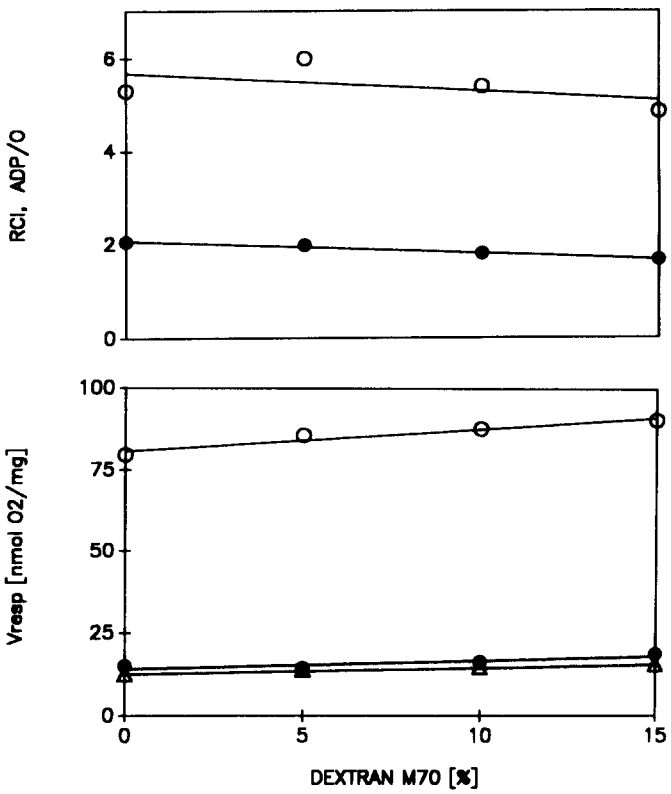


Fig. 1. Influence of dextran on functional properties of rat liver mitochondria. Incubation of rat liver mitochondria (0.83 mg/ml) in 110 mM sucrose, 60 mM KCl, 10 mM $KH_2PO_4$ (pH 7.5), 5 mM $MgCl_2$ and 10 mM succinate as well as in the presence of the indicated concentration of dextran M70. The active rate of respiration (state 3 = circles, lower panel) was induced by addition of 1062 nmol ADP. The resting rate of respiration (state 4 = filled circles, lower panel) was measured after phosphorylation of the ADP. The carboxyatractyloside inhibited state (triangles) was recorded in the presence of 1.5 $\mu$mol/mg carboxyatractyloside. The P/O ratio (ADP/O = filled circles, upper panel) was calculated from the oxygen consumption induced by the ADP addition as described by Lemasters [53]. The respiratory control index (RCI = circles, upper panel) is the quotient between state 3 and state 4 respiration rates. Data are means of four independent experiments.

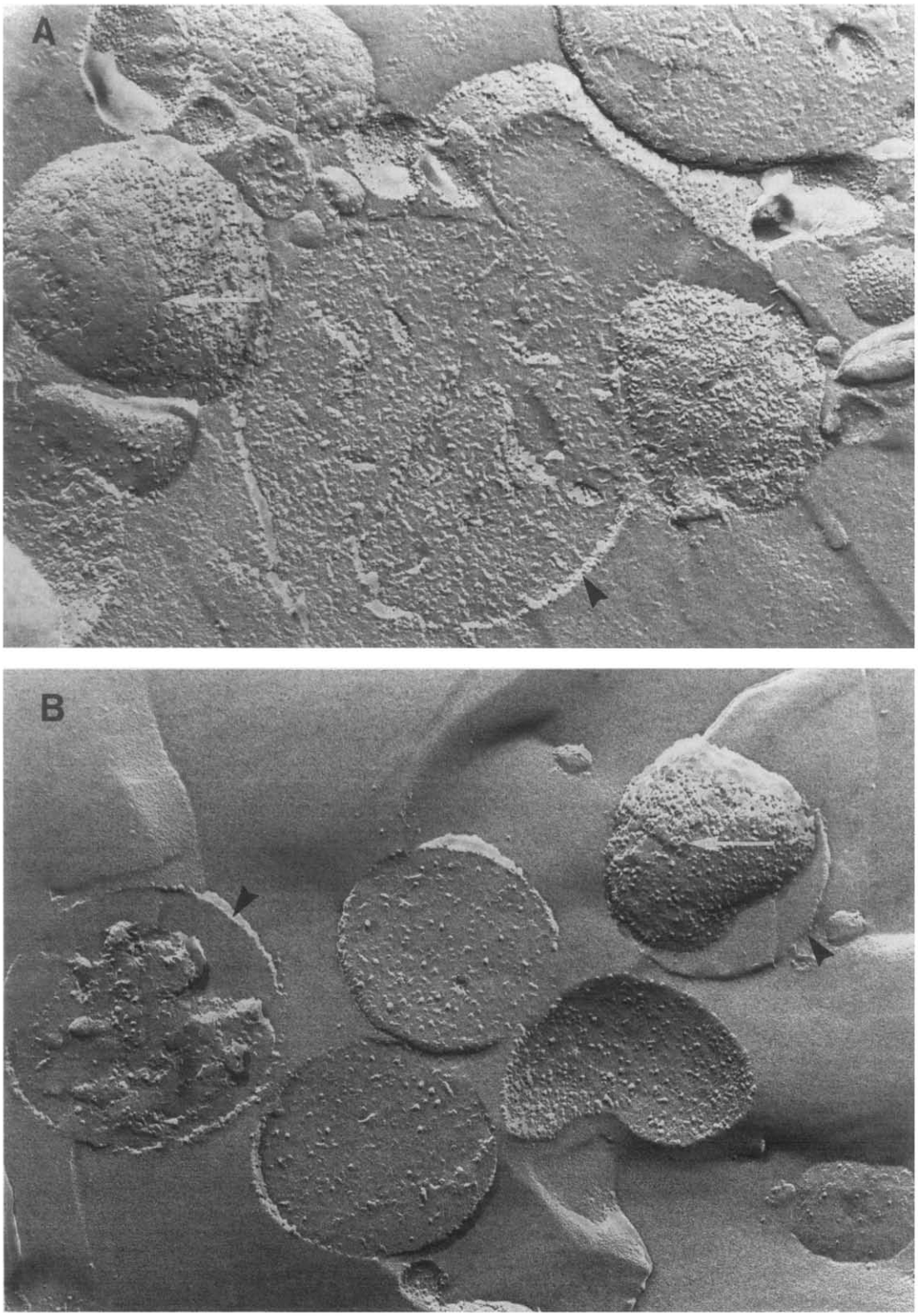


Fig. 2. Isolated rat liver mitochondria were incubated in a medium containing 113 mM sucrose, 10 mM $K_2HPO_4$, 5 mM $MgCl_2$ and 12.5 mM succinate (pH 7.4). The oxidation rate was stimulated by addition of 10 mM ADP (state 3). Convex fracture of (A) mitochondria in the presence of 10% dextran M70 (original magnification 75000) and (B) control mitochondria (original magnification 80000). Patches of the exoplasmic face of the outer membrane are covering the protoplasmic face of the inner membrane (white arrows). The arrowsheads point to the space between the two envelope membranes which upon addition of dextran appears to be almost absent.

19 nmol $O_2$/mg. As a consequence of that the respiratory control index (RCI) was slightly reduced from 5.5 to 5.0. There was almost no effect of dextran on the carboxyatractyloside inhibited state of respiration. Furthermore we studied the influence of dextran M70 on the ADP/O ratio which was found to decrease from 2.0 to 1.95 (10% dextran) and 1.8 (15% dextran).

*Structural changes of the outer mitochondrial compartment and the inter-membrane space*

For freeze-fracture analysis the mitochondria were suspended in a hypotonic medium which was originally described by Hackenbrock [27,28]. We used this medium to compare our results with those of Hackenbrock and to reduce the unphysiologically large volume of the outer mitochondrial compartment. As was observed earlier hyper- and hypotonic media did not have significant effects on the frequency of contact sites [3]. Furthermore the frequency of contacts changed also in intact cells depending on the metabolic state. This was found in tumour cells [31] and hepatocytes following different hormonal treatment [32]. In spite of the application of a hypotonic medium we found effects of dextran on the structure of the outer mitochondrial compartment. The incubation of isolated liver mitochondria in the presence of 10% dextran T70 led to a significant reduction of the space between the two envelope and the crista membranes (Fig. 2). The cristae visible in cross fractures of control mitochondria were swollen and exhibited the same, pears like, shape as in thin sections, whereas they had a long, thin structure in dextran treated mitochondria. The close attachment of the envelope membranes under dextran rose the question whether this would also affect contact formation.

*Frequency of boundary membrane contact sites*

Liver mitochondria were subjected to rapid freezing in different functional states (state 4 and state 3). The fracture plane in freeze fractured mitochondria was jumping between the two adjacent envelope membranes: patches of the exoplasmic face of the outer membrane were remaining on the inner membrane (Fig. 2, white arrows). As described previously [3] phosphorylating mitochondria in state 3 exhibited a higher frequency of fracture plane deflections indicating an increase of contact sites. In the presence of 10% dextran T70, mitochondria in state 4 had approximately the same $L_p$ value (representing frequency of fracture plane deflections) as control mitochondria in state 3 (Table I). When in the presence of dextran mitochondrial respiration was fully activated (state 3), a further increase of the $L_p$ value was observed, suggesting additional formation of contact sites. On the whole, electron microscopy showed that dextran reduced the space between boundary and crista membranes and induced contact site formation.

*Effect of dextran on the desorption of enzymes from liver mitochondria by digitonin*

In order to prove the increase of contact sites also by biochemical methods, liver mitochondria were treated with increasing digitonin concentrations in the presence of 10% dextran 70 (Fig. 3). Consistent with the composition of isolated contacts those enzymes remained bound to the mitochondrial membranes during digitonin treatment which were enriched in the contact sites. The addition of dextran to untreated mitochondria already resulted in more hexokinase activity, as compared to the control, which stayed bound

TABLE I

*Effect of dextran M70 on the frequency of contact sites*

Mitochondria (16.8 mg/ml) were incubated in sucrose medium in the presence of 12.5 mM succinate (state 4). The oxidation rate was stimulated by addition of 10 mM ADP (state 3). In a second sample 10% dextran 70 was present (+dextran). The oxidation rate was determined in the same assays but with a 10-times lower concentration of mitochondria. $M_p$ is the percentage of mitochondria with fracture plane deflections in the whole sample; $L$ represents the mean length in single mitochondria of the edge where the fracture plane deflects. $L_p$ gives the $L$ value corrected for the whole mitochondrial population as described in Materials and Methods. The significance of the difference between each group was tested by the U-test with $P < 0.01$.

| | State of oxidative phosphorylation | | | |
|---|---|---|---|---|
| | state 4 | state 3 | state 4 + dextran | state 3 + dextran |
| Oxidation rate nmol $O_2$/min per mg | 13.5 | 74.0 | 18.9 | 60.2 |
| $M_p$ (%) | 33.9 | 52.2 | 51.2 | 74.1 |
| $L$ ($\mu m^{-1}$) | 5.00 ± 2.01 | 8.27 ± 4.8<br>$P \leq 0.05$ | 9.29 ± 4.05<br>$P \leq 0.01$<br>(n.s.) | 10.18 ± 4.00<br>$P \leq 0.01$<br>$P \leq 0.1$<br>(n.s.) |
| $L_p$ ($\mu m^{-1}$) | 1.69 ± 0.68 | 4.33 ± 2.60<br>$P \leq 0.01$ | 4.75 ± 2.0<br>$P \leq 0.01$<br>(n.s.) | 7.54 ± 2.9<br>$P \leq 0.01$<br>$P \leq 0.01$<br>$P \leq 0.01$ |

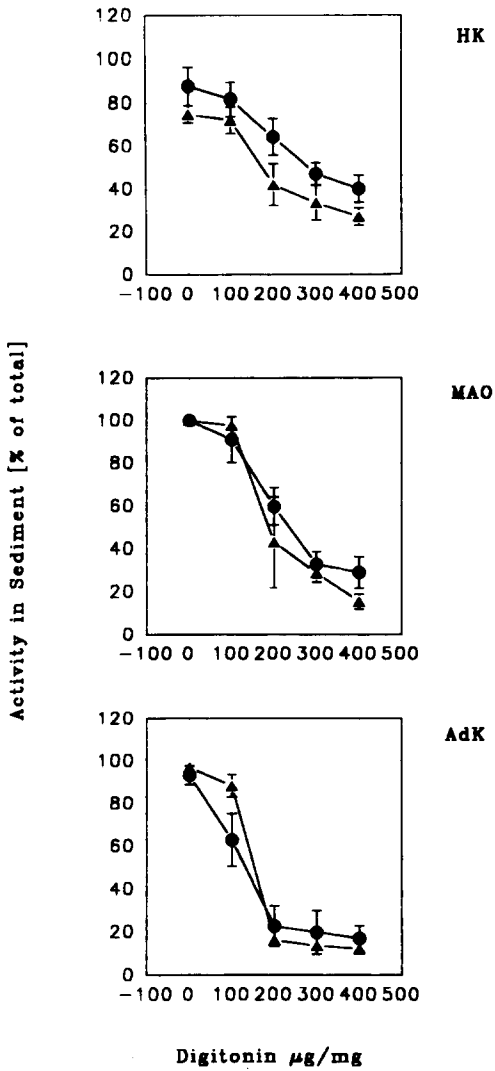


Fig. 3. Desorption of hexokinase and outer membrane by digitonin in liver mitochondria. Liver mitochondria were incubated with increasing digitonin concentrations for 30 s at room temperature and subsequently sedimented in a table top centrifuge. The activity of hexokinase (HK), monoamine oxidase (MAO) and adenylate kinase (AdK) were determined in supernate and sediment. To calculate the total activity. Circles = in the presence of 10% dextran 70, triangles = control.

during the first washing step in the absence of digitonin. This surplus of hexokinase activity in the presence of dextran was not detached during the following treatment with increasing digitonin concentrations (Fig. 3A) whereas adenylate kinase was liberated to the same extent as in the controls (Fig. 3C). Similarly the desorption of monoamine oxidase activity, representing integrated outer membrane enzymes, was not different in the presence of dextran when compared to the control (Fig. 3B). Supposing that hexokinase represents a marker enzyme for contact sites [8,9], the results would agree with the electron microscopic observation that the frequency of contacts increases in the presence of dextran. The data would furthermore propose a higher affinity of hexokinase to the contacts.

*Binding of hexokinase to mitochondria in the presence of dextran*

It was observed that hexokinase has a higher affinity to contact sites than to outer membrane [10,18], suggesting that the binding of this enzyme could be used to study the increase of these sites in the presence of dextran.

Liver mitochondria (1–1.2 mg) were incubated for 10 min at room temperature with increasing amounts of isolated hexokinase, isozyme I, in isotonic medium in the presence of 1 mM ADP, 5 mM glucose and 5

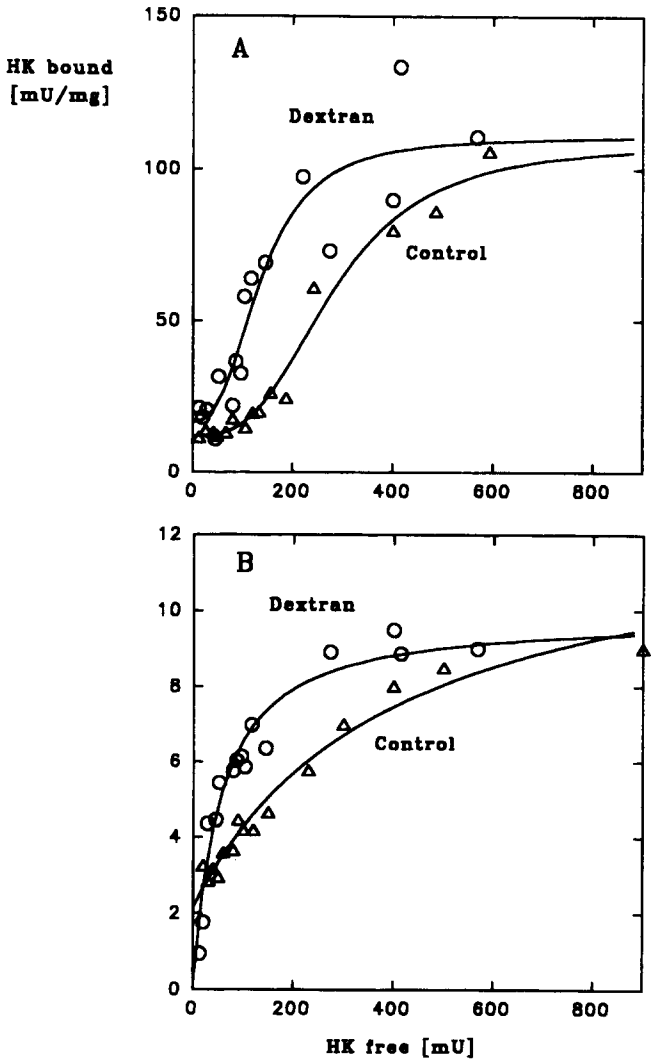


Fig. 4. Binding of hexokinase to the mitochondrial surface. The figures show single representative experiments of three which are summarized in Table II. A: 1–1.2 mg of intact liver mitochondria were incubated with increasing concentrations of isolated hexokinase isozyme I. The incubation was performed for 10 min at room temperature in 10 mM Hepes, 0.25 M sucrose medium in the presence of 2 mM glucose, 5 mM $MgCl_2$ and 10% dextran 70 as indicated. After 2 min centrifugation in a table-top centrifuge the free hexokinase activity was determined in the supernate and the bound activity in the resuspended sediment. B: same experiment as in A, but the activity in the sediment was determined in the presence of 0.5% Triton X-100.

mM $MgCl_2$. After centrifugation, the sediments were resuspended in isolation medium and the activity was determined under hypotonic conditions (0.1 M triethanolamine pH 7.4) in the supernatant and sediment. The binding appeared to be a co-operative process, the mean value of the calculated Hill coefficients was 3 (Fig. 4A and Table II). The half-maximal saturation constant was significantly lower in the presence of 10% dextran 70 than compared to the control. When hexokinase, bound to the mitochondrial sediments, was determined in the presence of 0.5% Triton X-100, we observed an approx. 10-fold decrease of activity and the cooperativity disappeared (Fig. 4B and Table II). Moreover, when the contacts were suppressed by addition of 20% glycerol or 50 $\mu$M dinitrophenol we observed almost the same binding as after destruction of the membranes by Triton (Fig. 5A). The results indicated two aspects: (i) that the contacts may have multiplied in the presence of dextran and (ii) that the induction of contacts had two effects on hexokinase: it increased the binding affinity to the membrane surface and the enzyme activity.

*Binding of hexokinase to the isolated outer membrane in the presence of dextran*

In order to prove the suggestion that the induction of contacts was responsible for the specific effects on hexokinase binding and activation we studied the binding of the enzyme to the isolated outer membrane (Fig. 5B). The experiments were performed in the presence of 10% dextran 20 because it was difficult to sediment outer membrane vesicles (0.2 mg/ml) in dextran 70. In contrast to the investigation of intact mitochondria described above the binding to the outer membrane did not show cooperativity. The binding efficiency ($U_{max}/K_s$, Table II) of 2.14 to the outer membrane was better than to mitochondria in the absence (0.33) or presence of dextran (0.6). This resulted from a higher

TABLE III

*Kinetic analysis of hexokinase in isolated mitochondria from rat brain*

The activity of originally bound hexokinase was analyzed in isotonic sucrose Hepes medium in the presence 2 mM glucose and 10% dextran 70 as indicated. The velocity of the activity was determined from the formation of glucose 6-phosphate by optical test in experiments 1 and 2, while the stimulation of state 3 respiration was measured by the oxygen electrode in experiment 3. In experiment 2 ATP was formed via activity of oxidative phosphorylation in the presence of 5 mM succinate and 5 mM phosphate and 2 mM diadenosine 5′-pentaphosphate. In experiment 3 the ADP to stimulate the oxidative phosphorylation was formed by the activity of hexokinase in the presence of 5 mM succinate and 5 mM phosphate. Hexokinase activity was calculated from the ADP/O ratio and the oxidation rate per min and ml mitochondrial suspension. The mean of three experiments is given.

| Sample | $V_{max}$ (mU/ml) | $K_m$ ($\mu$M) | $V_{max}/K_m$ | Hill $K$ |
|---|---|---|---|---|
| External ATP | | | | |
| Dextran | | | | |
| Mean ± S.D. | 1671 ± 802 | 173 ± 30.5 | 9.6 ± 3.9 | 1.0 ± 0.05 |
| Control | | | | |
| Mean ± S.D. | 1942 ± 920 | 166 ± 33.4 | 12.3 ± 6.5 | 1.2 ± 0.2 |
| ATP via ox. phos. | | | | |
| Dextran | | | | |
| Mean ± S.D. | 610 ± 186 | 39 ± 21 | 16.1 ± 1.3 | 1.2 ± 0.6 |
| Control | | | | |
| Mean ± S.D. | 919 ± 238 | 66 ± 13.1 | 14.0 ± 1.2 | 1.5 ± 0.1 |
| Ox. phos. via HK (nmol ADP/min per ml) | | | | |
| Dextran | | | | |
| Mean ± S.D. | 1902 ± 163 | 66 ± 2.4 | 30.6 ± 1.5 | 1.6 ± 0.4 |
| Control | | | | |
| Mean ± S.D. | 1983 ± 5.3 | 144 ± 46.3 | 14.8 ± 0.8 | 0.80 ± 0.1 |

TABLE II

*Binding of hexokinase to the mitochondrial surface*

Binding of hexokinase type I to mitochondrial membranes was performed by incubation 1–1.2 mg of mitochondrial protein for 10 min at room temperature with increasing concentrations of hexokinase in 0.25 M sucrose, 10 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 5 mM glucose and 1 mM ADP. As indicated the following additions were made: 10% dextran M70, 20% glycerol, 0.5% Triton X-100. Outer membrane was isolated from osmotically shocked mitochondria by density gradient centrifugation. 0.15–0.2 mg protein from outer membrane were incubated as above with hexokinase. As indicated 10% dextran 20 was present. The amount of hexokinase bound to the outer membrane of intact mitochondria was calculated by assuming that outer membrane protein amounts to 1% of the total mitochondrial protein. All values are means of three experiments.

| Sample | Dextran | | | | Control | | | |
|---|---|---|---|---|---|---|---|---|
| | $U_{max}$ (mU/mg) | $K_s$ 0.5 mU | $U_{max}/K_s$ | Hill $K$ | $U_{max}$ (mU/mg) | $K_s$ 0.5 mU | $U_{max}/K_s$ | Hill $K$ |
| Mitochondria liver | 81.1 ± 14.4 | 140.0 ± 35.8 | 0.60 ± 0.13 | 3.1 ± 0.76 | 79.4 ± 27.1 | 239.1 ± 75.2 | 0.33 ± 0.01 | 3.3 ± 1.0 |
| + Glycerol | – | – | – | – | 4.36 ± 0.66 | 125 ± 25 | 0.04 ± 0.00 | 1.5 ± 0.5 |
| + Triton | 14.0 ± 4.3 | 126.6 ± 73.4 | 0.14 ± 0.05 | 1.1 ± 0.03 | 6.76 ± 3.9 | 274.2 ± 124.2 | 0.02 ± 0.00 | 1.1 ± 0.03 |
| Outer membrane in mitochondria | 8110.0 ± 1440 | 140.0 ± 35.8 | 58.0 | | 7940 ± 2710 | 239.1 ± 75.2 | 33.2 | |
| Isolated outer membrane | 277.2 ± 132.6 | 614.6 ± 319.6 | 0.5 ± 0.28 | 0.7 ± 0.3 | 198.6 ± 19.6 | 112.2 ± 42.2 | 2.14 ± 0.98 | 0.9 ± 0.2 |

capacity to bind hexokinase per mg of outer membrane protein compared to intact mitochondria (Table II). However, taking into account that the outer membrane represents approx. 1% of the total mitochondrial protein the data in Table II show that the binding capacity of the outer membrane in intact mitochondria is between 15- and 100-times higher.

*Functional coupling of hexokinase in brain mitochondria*

The induction of contact sites by dextran and the influence on activation of hexokinase led us to study the effect of dextran on the kinetics of the originally bound enzyme in brain mitochondria (Fig. 6, Table III). When the saturation of the bound hexokinase activity for external ATP in the presence of oligomycin was determined we observed almost no difference in the kinetic constants in the absence ($166 \pm 33$ $\mu$M ATP, $1.9 \pm 0.9$ U/ml) or presence ($173 \pm 31$ $\mu$mol ATP, $1.7 \pm 0.8$ U/ml) of 10% dextran 70 (Table III). In further experiments the ATP was provided by the

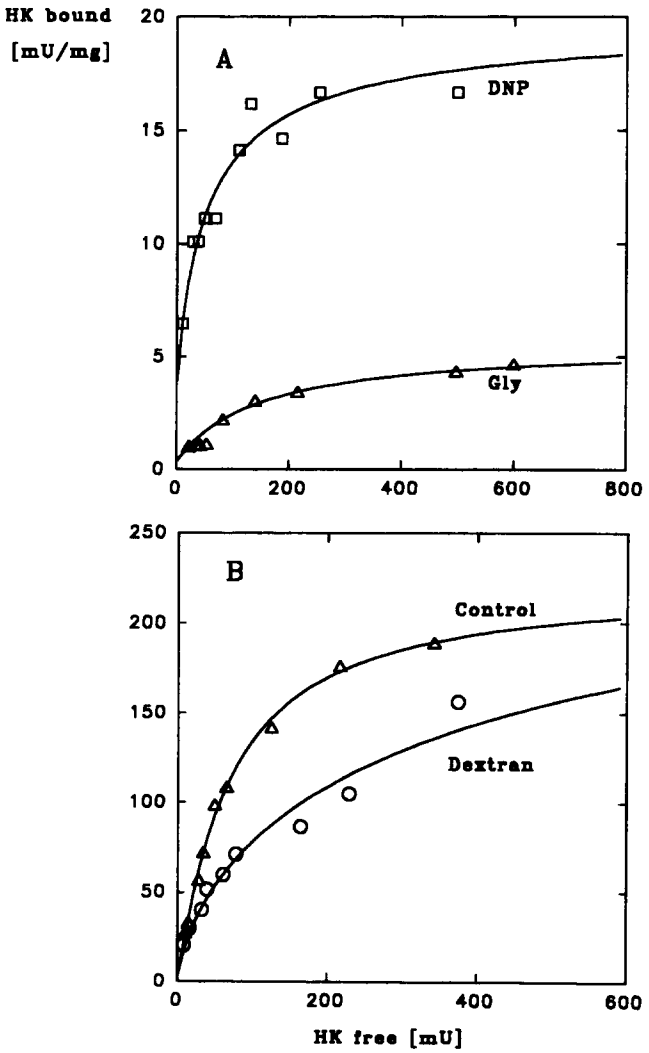


Fig. 5. Binding of hexokinase in the absence of contact sites. A: the same experiment as in Fig. 4 was performed under conditions where contacts between the envelope membranes were suppressed [3] by the presence of 20 $\mu$M dinitrophenol (DNP) or 20% glycerol (Gly). B: Outer membrane was isolated from liver mitochondria by osmotic shock and density gradient centrifugation. 0.2 mg of the outer membrane fraction were incubated with hexokinase I in the presence of 10% dextran 20 as described in Fig. 4. After incubation the membrane was sedimented by 30 min centrifugation at 200000 × $g$ in a Beckman TL 100 centrifuge.

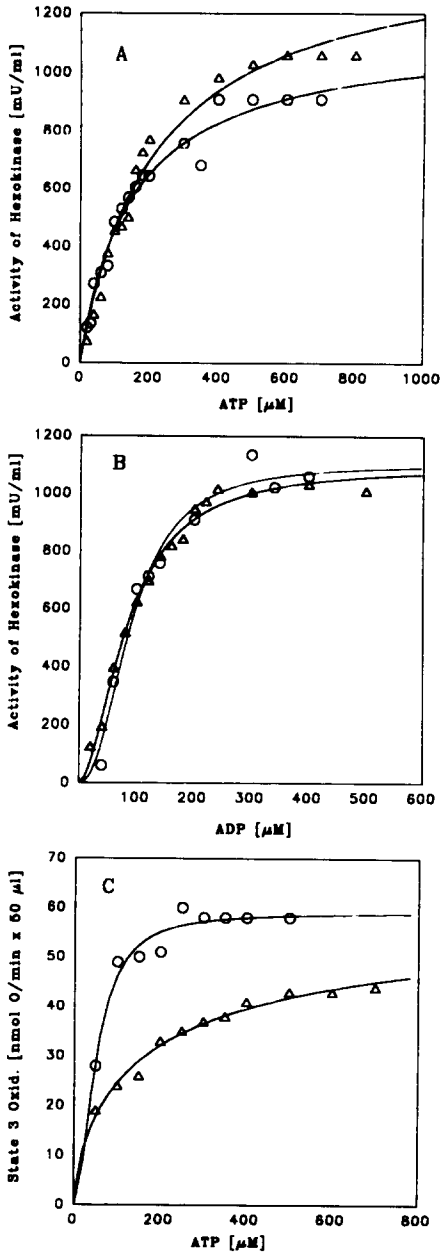


Fig. 6. Effect of dextran on the kinetic parameters of originally bound hexokinase in brain mitochondria. The figures show representative, single experiments of in total three of each group, which are summarized in Table III. The effect of 10% dextran 70 on originally bound hexokinase was analyzed in panel A and B by recording the glucose 6-phosphate production with an optical test system and in panel C by following the stimulation of state 3 respiration with the oxygen electrode. 2 mM diadenosine 5′-pentaphosphate was present to inhibit the adenylate kinase activity. Circles = in the presence of 10% dextran 70, triangles = control.

oxidative phosphorylation in the presence of adenylate kinase inhibitor (Fig. 6B, Table III). As compared to external ATP, the $V_{max}$ was approx. 30 to 50% lower and the Michaelis constants decreased remarkable to 40 $\mu$M AdN in the presence and 66 $\mu$M AdN in the absence of dextran. On the whole the catalytic efficiency ($V_{max}/K_m$) was higher when the ATP was supplied by the mitochondria. A significant effect of dextran was observed when the oxidative phosphorylation was stimulated by ATP and glucose via the ADP production of originally bound hexokinase. In the presence of dextran the $K_m$ for ATP was lower ($66 \pm 2$ $\mu$M AdN) and the catalytic efficiency was 31 compared to 15 in the control (Table III and Fig. 6C).

## Discussion

In this study, we have examined two questions: (i) whether the substitution of physiologically present macromolecules by dextran changes the structure of mitochondria and (ii) whether these structural differences have a regulatory meaning on peripheral kinases such as hexokinase.

*Effect of dextran on basic mitochondrial functions*

The effect of dextran on the basic mitochondrial parameters like oxidation rate, RCI and ADP/O ratio was investigated. In general the presence of 10% dextran resulted in minor changes only (Fig. 1). Thus the dextran effects discussed below concern mainly the regulation and not a direct affect on the mitochondrial functions.

*Effect of volume changes of mitochondrial compartments*

The investigation of freeze-fractured mitochondria was performed in a hypotonic medium which was first described by Hackenbrock [27,28] and, for comparison, routinely used by us in this type of experiments. In earlier experiments we showed that volume changes of the inner mitochondrial compartment, caused by hyper- or hypotonic sucrose media, did not affect structures of the outer mitochondrial compartment such as contact sites [3]. Thus the osmotic effect of different sucrose media appears not to interfere with the osmotic influence of macromolecules on the structure of the outer compartment. Moreover in intact liver and tumour cells, with physiological oncotic pressure on both compartments, contact sites are present and subjected to regulation [31,32].

*Induction of contact sites by dextran*

*Freeze-fracture analysis.* Earlier studies in liver mitochondria provided the methodology to test the frequency of contact sites by electron microscopy [3,15,33]. It was assumed that the points of intimate attachment between the mitochondrial envelope membranes explain the atypical behaviour of the fracture plane in freeze-fractured mitochondria, which is characterized by frequent jumping of the fracture between the adjacent membranes [2,3]. Since these observations were made in mitochondria after pure physical fixation, chemical preparation artefacts could not have interfered with the structure of attachment points.

As described above, we supposed, that contact sites were responsible for fracture plane deflections in freeze-fractured samples and that the latter would only occur in these sites. This suggested that the frequency of these deflections would correlate with the frequency of contacts. Based on this proposal we studied the deflections in freeze-fractured samples of mitochondria in different metabolic states and observed a significantly higher frequency of contacts (fracture plane deflections) in maximal phosphorylating mitochondria (state 3) compared to mitochondria in the energized state (state 4) and freshly isolated mitochondria (state 1) [3,15,33]. The same difference between mitochondria in state 4 and state 3 was observed in the present investigation. However in the presence of dextran the frequency of fracture plane deflections, representing contact sites, was higher in both states of the mitochondria compared to the control (Table I).

*Desorption of hexokinase.* The important point in terms of the present study is that besides freeze-fracture analysis, hexokinase binding can be employed to determine changes in contact sites because contacts are the preferred binding sites for this enzyme. This has been shown by a number of experiments: (i) hexokinase was located in the contacts by electron microscopy in liver and brain mitochondria [8,9], (ii) it was concentrated in the isolated contact fraction in liver, kidney and brain mitochondria [10,11], (iii) the isolated contact fraction had about 4-times higher affinity for hexokinase than the outer membrane fraction [10,18], (iv) the activity bound to the mitochondrial fraction correlated with the frequency of contacts [32,33]. Based on these results we considered the binding of hexokinase a good means to analyze the increase of contact sites by dextran.

Hackenbrock [34] showed that, in liver mitochondria, digitonin did not remove the entire outer membrane but that remnant vesicles remained attached to the inner membrane. This suggested the existence of attachment sites between the two envelope membranes, resistant to digitonin treatment, as well as the occurrence of distinct domains in the outer membrane [35]. In support of this view, between 40–50% of total hexokinase activity remained bound to brain and liver mitochondria at digitonin concentrations (200 $\mu$g/mg) which liberated almost all (85%) of the adenylate kinase and significant amounts of outer membrane markers as was described in a number of

publications [9,10,36]. In the presence of 10% dextran the amount of hexokinase activity which was not desorbed by washing was 15–20% higher than in the control and more than 60% of the activity were resistant to digitonin (200 $\mu$g/mg) treatment (Fig. 3). The liberation of adenylate kinase and monoamine oxidase by digitonin was, however, not reduced in the presence of dextran. This correlated with the observed increase of contact sites by electron microscopy and showed in addition that monoamine oxidase may be mainly integrated in those parts of the outer membrane which were not in the contacts.

*Binding of hexokinase.* Having observed that more hexokinase remained bound to the mitochondrial membranes in the presence of dextran led us to study the effect on the enzyme binding.

As characterized by Felgner et al. [37] a specific protein in the outer membrane of rat liver mitochondria is responsible for hexokinase binding. This was later proved to be identical with the outer membrane pore protein [38,39]. The pore protein was found to be randomly distributed in the outer membrane [10,11]. To explain the preferred binding of hexokinase in the contact sites, it was postulated that the inner membrane potential effects the structure of the pore protein in these sites because of its voltage sensitivity [21]. It is known that the pore protein is voltage sensitive [40,41]: above 30 mV it reduces its conductance [40] and changes from anion to cation selectivity [21]. Furthermore it was observed that polymers such as polyethyleneglycol and dextran reduce the volume of the pore and thus effect the structure [42]. Therefore, besides the aspect to use hexokinase binding as a tool to determine contact site induction, it was interesting to study the effect of dextran on the binding of the enzyme to the pore in the isolated outer membrane (Fig. 5B). As shown earlier [10] 87% of the outer membrane vesicles were right side out, when prepared by the swelling shrinking method. Therefore the pore protein is accessible for hexokinase mainly from the same side as in intact mitochondria. We expected that this investigation would give insight into the question whether the formation of a complex between the two envelope membranes or simply the change of the pore structure is responsible for the increased affinity of hexokinase to the contact sites.

In intact mitochondria, where induction of contacts was possible, dextran increased the affinity for hexokinase. On the contrary, dextran had a negative effect on the hexokinase binding to the isolated outer membrane (Figs. 4 and 5). Considering that the outer membrane protein amounts about 1% of the total mitochondrial protein the binding efficiency ($U_{max}/K_s$) in intact mitochondria, of 58 or 33 respectively, compared to 2.14 of isolated outer membrane is significantly higher (Table II). The present data suggested that the induction of contact sites had a specific effect on hexokinase binding. This was supported by the observation that suppression of contact formation in intact mitochondria by either glycerol or dinitrophenol, as observed previously [3], led to a reduction of both the binding affinity and the activation of hexokinase (Fig. 5A). Taken together, these findings suggested two things: (i) that dextran induced contacts and thus supported the above observations by electron microscopy and digitonin treatment and (ii) and that the formation of contacts exerts regulatory effects on hexokinase namely activation and increase of the binding affinity. The direct effect of dextran on the pore structure according to Zimmerberg [42], which was presumably existent in the outer membrane, had no or a negative consequence on the affinity of the enzyme (Fig. 5B). We calculated from the data in Table II that four hexokinases bind to one porin (assuming that 7% of the outer membrane protein is porin and a specific hexokinase activity of 10 U/mg). This agrees with recent experiments of Xie and Wilson [43] who found, by employing cross-linking, that hexokinase I forms tetramers when bound to the membrane of liver mitochondria. The tetramer formation might provide a basis to explain the co-operative binding to the contacts and the activation.

*Regulatory meaning of hexokinase binding in the contact sites*

The results of the present study show that the binding of hexokinase to the contact sites may have two regulatory functions: (i) the regulation of the total cellular activity of glucose phosphorylation via the activation by binding and (ii) the saturation of the adenine nucleotide translocator in the presence of high extramitochondrial ATP/ADP quotients because of microcompartmentation of the ADP between hexokinase and the adenylate translocator.

*Activation by binding.* The activation of hexokinase, isozymes I and II, by binding to the mitochondria [8,44,45] and to liposomes [46] has been described earlier. It may be important in tissues which do not use glucose as the main substrate such as liver and muscle. In these tissues the uptake of glucose is regulated according to the availability and not dependent on the energy requirement. It thus responds to metabolite levels in the blood and to hormones. It has been observed that free fatty acids [33,47] and glucagon [32] decrease mitochondrial contact sites and hexokinase binding in hepatocytes, while insulin increased hexokinase binding to the mitochondria in fat cells [48]. These results emphasize a possible function of contacts in the regulation of cellular hexokinase activity.

*Functional coupling to the AdN translocator.* Bessman and co-workers have produced evidence that the mitochondrial hexokinase [12,49] preferentially utilizes intramitochondrially generated ATP. However, they

have also shown that this preference decreased with increasing total ATP concentrations. The same was observed in reticulocyte [50] and brain [51,52] mitochondria. These experiments were performed in the absence of dextran. Addition of dextran to brain mitochondria, however, resulted in changes of the kinetic properties of the originally bound enzyme, which point to a functional coupling with the AdN-translocator. In agreement with Bessman and co-workers [12,49] we noted a significantly lower $K_m$ for ADP (ATP) when the enzyme was utilizing internal ATP as compared to external ATP (Table III). As observed by others [51,52] the $V_{max}$ with internal ATP was about halve of that with external ATP. The addition of dextran had no effect on these parameters, suggesting that contact formation did not improve the supply of hexokinase with internal ATP. On the other hand, when we studied the ADP supply of the oxidative phosphorylation by the activity of originally bound hexokinase, we noticed a significant decrease in $K_m$ and a duplication of the catalytic efficiency in the presence of dextran. This points to the importance of contacts in optimizing the AdN exchange. Externally added ADP (to produce ATP for hexokinase) led to saturation of the AdN translocator and unimpaired uptake, while the saturation of the translocator with ADP produced by hexokinase was difficult in the presence of increasing concentrations of external ATP. In this case, induction of contacts by dextran significantly improved the saturation of the translocator because of facilitated communication with hexokinase. In other words the saturation of the AdN translocator by ADP was much more sensitive to the ATP/ADP ratio than the saturation of hexokinase with ATP.

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