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Effect of macromolecules on the regulation of the mitochondrial outer membrane pore and the activity of adenylate kinase in the inter-membrane space

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

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

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Macromolecules as components of the physiological mitochondrial environment were substituted by dextrans of different molecular weight. The addition of 10% dextran (molecular weights varying between 20 and 500 kDa) affected neither basic mitochondrial parameters (state 4 and state 3 respiration) nor kinetic properties of soluble kinases. A significant increase by 10% dextran was however observed of the voltage sensitivity of isolated porin when reconstituted in planar bilayers. The pores adapted the low conducting state already at a voltage of 10 mV. This effect of the macromolecules may explain the higher diffusion resistance of adenine nucleotides across the outer membrane as observed in different experiments: (i) the Michaelis constant of adenylate kinase in the inter-membrane space increased, in contrast to the soluble enzyme, from 118 ± 10 μM to 193 ± 20 μM ADP, (ii) in the presence of competing external pyruvat kinase, the mitochondrial utilization of ADP, produced by adenylate kinase in the inter-membrane space, was improved 3-fold suggesting a reduced ADP diffusion out of the outer mitochondrial compartment. The influence of the various dextrans correlated with the increase in molecular weight of the dextrans. The effect on the kinetic constants was dependent on the dextran concentration in terms of weight and not of molarity. The oncotic pressure and viscosity of dextran solutions with different molecular weight showed a comparable dependence. In general, the data indicate that the outer membrane pore responds to an increased oncotic pressure by reducing adenine nucleotide permeability. This suggests the physiological existence of a third adenine nucleotide compartment between the two envelope membranes which may be important especially at high metabolic fluxes.

Introduction

In the intact cell, mitochondria are embedded in a 30% protein solution [1, 2], but so far, isotonic media without macromolecules are used for the isolation and investigation of mitochondria. Because of the missing oncotic pressure, isolated mitochondria exhibit a large inter-membrane space in these media compared to mitochondria in tissue sections [3–5]. The artificially increased mitochondrial inter-membrane space can be reduced by adding macromolecules such as poly(vinyl pyrrolidone), ficol or albumin to isolation and incubation media [3,4,6]. Besides the effects of macromolecules on the structure of the outer mitochondrial compartment a direct influence on the conductance of the mitochondrial outer membrane pore has been described [7]. The conductivity of porin pores, reconstituted in artificial lipid membranes, was found to be voltage-dependent [8–10]. Addition of macromolecules increased the voltage sensitivity. As postulated by the authors, this was caused by osmotic stress which forced the pore to the low conductance conformation [7]. The mitochondrial outer membrane is permeable for metabolites due to the existence of porin pores. This restriction of the metabolite transport to the pores may explain the observation of adenine nucleotide compartmentation in the inter-membrane space of heart [11–19], liver [20] and brain [21] mitochondria. We demonstrated this aspect by partial reconstitution of the cellular ATP regenerating metabolisus in a system consisting of mitochondria and pyruvate kinase which, both as phosphorylating entities, compete for ADP formed by kinases of varied localizations. It was observed that the ADP produced by mitochondrial creatine kinase was preferentially metabolized by the oxidative phosphorylation and was badly liberated from the inter-membrane space [17]. Comparable results were obtained recently concerning the ADP produced (via AMP) by

Correspondence to: F.N. Gellerich, Institut für Biochemie, Medizinische Akademie Magdeburg, O-3090 Magdeburg, Germany.

adenylate kinase in the inter-membrane space of liver and heart mitochondria [20].

Depending on the turnover rate of the investigated system, the diffusion of adenine nucleotides through the outer membrane pores requires concentration gradients. These gradients were found to be 13 μM adenine nucleotides at fluxes of about 1000 nmol adenine nucleotides/min per mg [16,17]. This suggests that the exchange of charged metabolites through the outer membrane may be restricted for all compounds with intracellular levels in the same order of magnitude. In the intact muscle cell this seems to be the case for ADP (approx. 40 μM,[22]) but not for ATP (approx. 5 mM [22]).

The finding that macromolecules induced morphologically and biophysically detectable changes in the structure of the outer mitochondrial compartment [6] and the outer membrane [7] provided the basis to specifically investigate the effects of macromolecules on the outer compartment. Our efforts were mainly addressed to the question, whether the physiologically present macromolecules would influence the exchange of extra- and intramitochondrial adenine nucleotides through the pores in the mitochondrial outer membrane. We replaced intracellular proteins by dextrans and investigated their influence on the permeability of the outer membrane pore by analyzing the effects on (i) basic properties of rat liver mitochondria and soluble enzymes, (ii) the conductivity of porin pores in black membranes and (iii) the permeability of the outer membrane for adenine nucleotides in intact mitochondria.

It will be shown, that dextrans significantly reduce the permeability of the outer membrane pore without having effects on the basic properties of mitochondrial and soluble enzymes.

Materials and Methods

Chemicals and enzymes

Dextrans T20, T40, T70 and T500 were obtained from Pharmacia (Uppsala, Sweden), Dextran M70 from Serumwerke (Bernburg, Germany). Bovine serum albumin (fatty acid free) and Hepes were bought from Serva (Heidelberg, Germany). Sucrose, phosphate and $MgCl_2$ were products of Merck (Darmstadt, Germany). Oligomycin was obtained from Calbiochem (San Diego, U.S.A.), TTFB (4,5,6,7-tetrachloro-2-trifluormethylbenzimidazol) was a gift from Dr. Beechy (U.K.). All other chemicals and enzymes were purchased from Boehringer (Mannheim, Germany).

Isolation of mitochondria

Rat liver mitochondria were isolated as previously described [23] in 0.25 M sucrose adjusted to pH 7.4 with small amounts of Tris.

Porin isolation

Mitochondrial porin was isolated from rat liver mitochondria essentially as described by De Pinto et al. [24].

Incubation conditions

Mitochondria were incubated for oxygraphic and kinetic measurements in a medium containing: 110 mM sucrose, 60 mM KCl, 15 mM glucose, 10 mM KH_2PO_4, 5 mM $MgCl_2$ and 0.5 mM EDTA, pH 7.4. Further additions were as indicated in the legends of the figures. The respiratory rates and their first derivatives were measured at 25°C in a closed vessel by a custom built rate meter equipped with a CLARK-type electrode. The oxygen content of the incubation medium was assumed to be 229 nmol O_2/ml [25]. The specific oxygen consumption (nmol O_2/mg mitochondrial protein) following adenine nucleotide additions was calculated from the peaks area of the first derivative [20].

Assays

The concentrations of adenine nucleotides in the stock solution were determined by enzymatic standard procedures [26]. The protein content of the mitochondrial suspensions was determined by a modified biuret method [27].

Kinetic measurements

The influence of dextrans on kinetic properties of enzymes was investigated by the optical test. Commercially available yeast hexokinase (EC 2.7.1.1) and pyruvate kinase from muscle (EC 2.7.1.40) as well as mitochondrial creatine kinase (EC 2.7.3.2) were determined by standard procedures [26].

For measuring the mitochondrial adenylate kinase (EC 2.7.4.3) [26] the medium contained 0.7 mM NADP, 1.6 IU hexokinase/ml and 2 IU glucose-6-phosphate dehydrogenase/ml. Mitochondrial functions were inhibited by the presence of 11 μM antimycin A, 250 μg/ml oligomycin, 11 μM carboxyatractyloside. Reactions were started by addition of ADP ranging from 0 to 4 mM. The same assay was used to study the effect of dextran on released mitochondrial adenylate kinase. Released mitochondrial adenylate kinase was prepared from rat liver mitochondria which were incubated for 5 min with 500 μg digitonin/mg mitochondrial protein. After centrifugation for 10 min at $100\,000 \times g$ the supernatant was stored in liquid nitrogen until use. Soluble muscle pyruvate kinase was determined in a medium additionally containing 0.31 mM $NADH_2$, 0.83 mM PEP and 5.7 IU lactate dehydrogenase/ml. Reactions were started by addition of ADP in concentrations ranging from 0 to 0.8 mM.

The kinetic constants were calculated by means of a nonlinear regression computer programme BMDT which allows a profound statistical analysis. All data

points of 5–6 independent measurements (each with double determinations at 10 different substrate concentrations) were used together for calculation of means ± S.D. Further, the confidence range for the confidence of 0.990 was estimated.

Lipid bilayer experiments

Experiments with artificial membranes were performed as described previously [10]. Membranes were formed from a 1% (w/v) solution of diphytanoyl phosphatidylcholine (Avanti Biochemicals, Birmingham, AL, U.S.A.) in *n*-decane across circular holes (surface area about 1 mm^2) in the thin wall of a Teflon cell separating the two aqueous compartments. Porin was added from a concentrated stock solution (1 mg/ml porin, 0.1% Triton X-100) to the aqueous phase under stirring to allow immediate equilibration prior to membrane formation, or after the membranes had turned optically black in reflected light. The current through the membranes was measured with two silver electrodes switched in series with a voltage source and current amplifier. The amplified signal was monitored with storage oscilloscope and recorded on a strip recorder.

Measurement of the oncotic pressure

The oncotic pressure was determined with a Colloidosmometer MOD-1 of the Forschungsinstitut für Medizinische Labortechnik, Dresden, Germany, measuring the surface tension of a membrane with an exclusion value of about 25 kDa.

Measurement of the viscosity

Viscosities of the media were measured by means of an Ubbelohde viscosimeter at 25°C.

Results

Influence of macromolecules on functional properties of mitochondria and soluble enzymes

Before analyzing the effect of macromolecules on the exchange of adenine nucleotides across the mitochondrial outer membrane we studied the influence of macromolecules on basic properties of mitochondria and different soluble enzymes.

Effects on mitochondrial functions

As observed previously various concentrations of dextran M70 had no effect on the basic mitochondrial parameters [6]. We further compared different macromolecules at a constant concentration of 10% (Table I). The active rate of respiration increased slightly from 88.4 to 93.9, 94.4 and 91.1 nmol O_2/mg per min after addition of dextran M70, T40 and BSA respectively. The resting state remained unchanged except with BSA. As a consequence of that the respiratory control index (RCI) was slightly increased by addition of macromolecules. Moreover we studied the influence of the different macromolecules on the ADP/O ratio which was found to be slightly decreased from 2.0 to 1.8 in the case of dextrans and to 1.9 in case of albumin.

TABLE I

Influence of dextrans and bovine serum albumin on functional properties of rat liver mitochondria

Mitochondria (2–2.2 mg/ml) were incubated in a medium described in Materials and Methods additionally containing 10 mM succinate, 1 μM rotenone and 10% of macromolecules. The active rate of respiration was adjusted by addition of 500 μM ADP. The resting rate of respiration was measured after phosphorylation of that ADP. The ADP/O ratio was calculated from the oxygen consumption induced by ADP addition according to Lemasters [32] as described previously [20]. The values are means of four independent experiments each with 3–4 separate assays.

Additions	Respiratory rate (nmol O_2/mg per min)		RCI	ADP/O
	active	resting		
None	88.4 ± 4.3	16.2 ± 1.3	5.5	2.00 ± 0.20
Dextran M70	93.9 ± 2.5	16.4 ± 2.0	5.7	1.77 ± 0.04
Dextran T40	94.4 ± 4.1	16.5 ± 2.9	5.7	1.76 ± 0.01
RSA	91.1 ± 4.1	13.3 ± 2.8	6.8	1.91 ± 0.14

Obviously the basic mitochondrial properties were not influenced by addition of up to 10% of the various investigated macromolecules. Similar results were obtained with dextran T500 (not shown).

Effects of dextran on soluble enzymes

The influence of dextran M70 on the kinetic properties of soluble hexokinase from yeast, pyruvate kinase and creatine kinase from muscle tissue, as well as of extracted mitochondrial adenylate kinase was investigated by optical test. As shown in Table II, addition of 10% dextran M70 to the assay had no effect on the kinetic properties of the soluble enzymes. Neither the Michaelis constants nor the maximal velocities of the investigated enzymes changed significantly. Similar results were obtained for 10% dextran T40 (not shown).

Effect of dextrans on the voltage dependence of reconstituted porin

Single-channel measurements. Experiments were performed with small amounts of the isolated and purified pore protein (20 ng/ml final concentration) added after blackening to both sides of the diphytanoyl lecithin membrane. Dextrans of different molecular weights (20, 70 or 500 kDa) were present in a concentration of either 10% (w/v) or 0.5 mM in the medium bathing the membrane. The current at 10 mV membrane potential increased stepwise due to the insertions of pores into the membrane. Histograms of 40–100 single

TABLE II

Effect of dextran M70 on kinetic constants of soluble enzymes and mitochondrial compartmentalized adenylate kinase

The kinetic constants were determined as described in Materials and Methods in the absence and in the presence of 10% dextran M70. Numbers in brackets represent the confidence range.

Enzyme	Without	With dextran M70
Adenylate kinase soluble		
K_{ADP} (μM)	168±9 (145–193)	172±9 (154–191)
V_{max} (IU/mg)	0.90±0.02 (0.82–0.99)	0.90±0.02 (0.85–0.95)
Pyruvate kinase soluble		
K_{ADP} (μM)	372±20 (324–426)	378±20 (328–432)
V_{max} (IU/mg)	223±33 (191–243)	216±29 (186–233)
Hexokinase soluble		
K_{ATP} (μM)	306±15 (270–346)	312±13 (287–339)
V_{max} (IU/mg)	280±4 (260–299)	282±3 (266–310)
Creatine kinase soluble		
K_{ATP} (μM)	181±19 (161–200)	180±16 (168–203)
V_{max} (IU/mg)	1.78±0.04 (1.61–1.90)	1.77±0.03 (1.68–1.88)
Adenylate kinase compartmentalized		
K_{ADP} (μM)	118±10 (95–145)	193±20 (148–251)
V_{max} (IU/mg)	0.85±0.02 (0.79–0.91)	0.76±0.03 (0.70–0.86)

current steps at 10 mV are shown in Fig. 1. In the absence of dextran most of the incorporated pores (35%) had a conductance of about 4.8 nS and a smaller group of 20% produced a current step of 2 nS. The addition of 10% of the different dextrans caused a shift of the current steps towards a lower amplitude of 1–2 nS. When 0.5 mM of the diverse dextrans were added, the frequency of smaller current steps increased correlated to the increase of molecular weight of the dextrans.

Macroscopic conductance measurements. In order to measure the effect of different dextrans on the current voltage dependence approximately 1 μg porin/ml was added to both sides of a diphytanoyl lecithin membrane. In the course of 20 min after addition of the protein the rate of conductance increase slowed down and the conductance was almost constant thereafter. At this time a voltage varying between 10 and 50 mV was applied to the membrane. The current decreased with a time constant of several seconds. The maximal conductance (G_o) was determined measuring the instantaneous current response to the transmembrane voltage and the remaining voltage-independent current (G) after about 30 seconds when no further current change was observed. As commonly observed, the con-

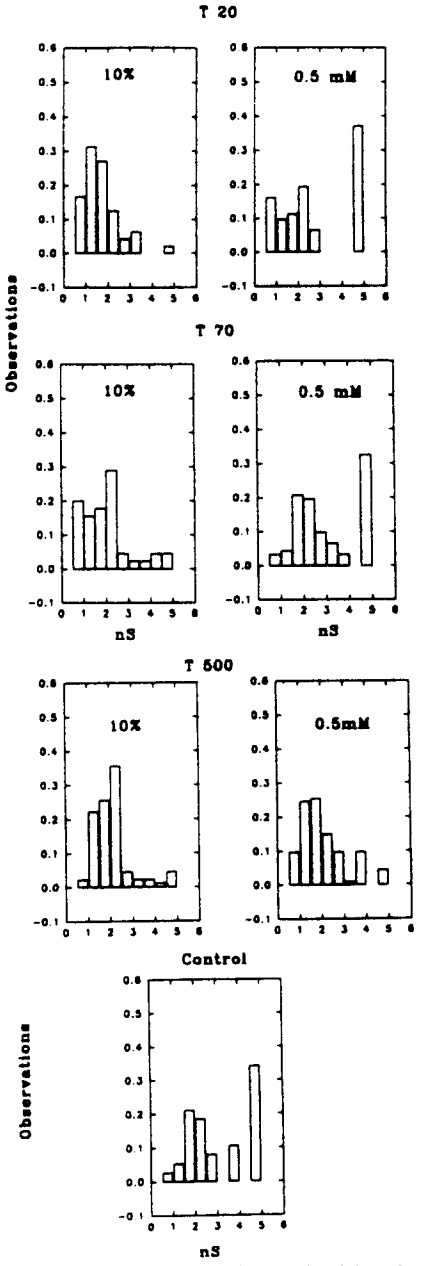

Fig. 1. Effect of different dextrans on the conductivity of porin pores in artificial membranes. Histograms of conductance fluctuations of pores in membranes formed of diphytaloylphosphatidylcholine/n-decane in 1 M KCl solution obtained as described in Materials and methods. 2 ng/ml rat liver porin were used and 10 mV were applied. The histograms result from 50–110 single insertions into the membrane. Different dextranes were present in a concentration of either 10% or 0.5 mM as indicated on both sides of the membrane. Due to the different molecular weights the concentration of 0.5 mM dextran corresponds to 1% dextran T20, 3.5% dextran M70 and 25% dextran T500.

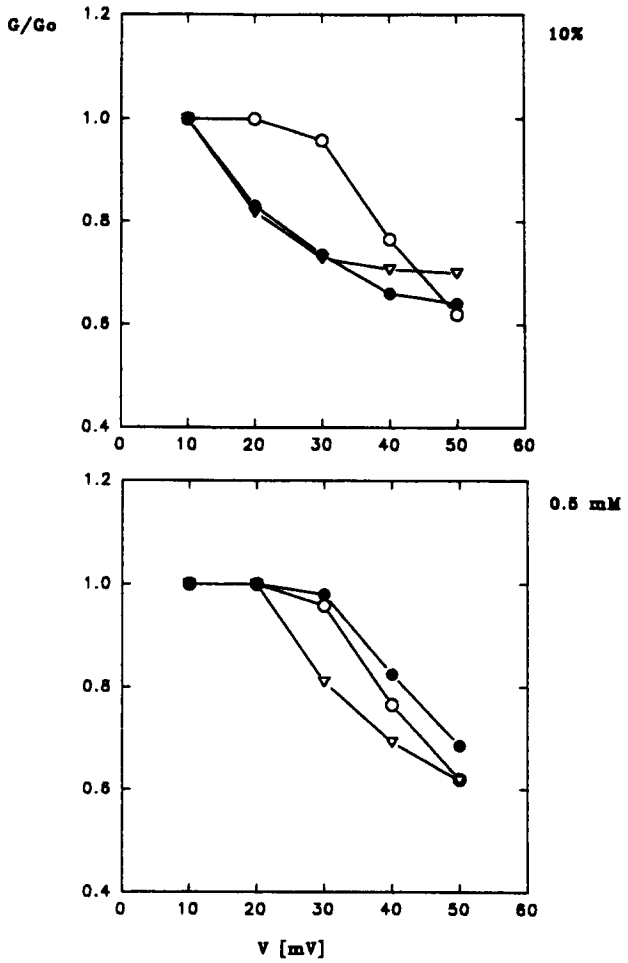

Fig. 2. Effect of different dextrans on the current voltage dependence of porin in artificial membranes. Relation between the voltage independent current and the maximal conductance of the pore in the presence of different dextrans T20 (•) and T70 (▽) in concentrations as indicated. 1 μg of pore protein was added to both sides of a membrane formed of diphytanoyl phosphatidyl choline/n-decane in 1 mM KCl solution. (○), control. The maximal conductance Go was determined measuring the instantaneous current response to the transmembrane voltage which was varied between 10 and 50 mV. G represents the voltage independent current after approx. 30 s.

ductance of the pores incorporated into the membrane decreased at a voltage above 30 mV (Fig. 2). In the presence of 10% dextrans T20 and T70, a significant increase in voltage sensitivity was observed, while this effect was less pronounced adding the two dextrans at a concentration of 0.5 mM.

The influence of dextran on the exchange of adenine nucleotides across the outer mitochondrial membrane

To investigate the influence of macromolecules on the flux of adenine nucleotides across the outer membrane of intact mitochondria, we made use of the fact that high activities of adenylate kinase as an adenine nucleotide converting enzyme are present in the intermembrane space of rat liver mitochondria. Two different experimental approaches were used: (i) the exchange of ATP and ADP between the inter-membrane and the extramitochondrial space was measured by determination of kinetic constants of adenylate kinase, (ii) the liberation of ADP produced by this enzyme from AMP and ATP was measured by following the stimulation of the oxidative phosphorylation in the presence of competing ADP-consuming external pyruvate kinase.

Effects on the exchange of ATP and ADP across the outer membrane. The exchange of ADP versus ATP was measured by recording the activity of adenylate kinase in the inter-membrane space by means of the optical test. In this system the ATP produced by adenylate kinase diffuses to the extramitochondrially added hexokinase and the ADP regenerated by the latter enzyme diffuses back to the adenylate kinase. Supposing the macromolecules increase the diffusion resistance between the intra- and extramitochondrial ADP pools one would expect a higher Michaelis constant of mitochondrial adenylate kinase for ADP in the presence of dextran. The oxidative phosphorylation was inhibited with carboxyatractyloside, antimycin A and oligomycin. In the presence of these inhibitors the dependence of adenylate kinase activity on ADP was measured. The addition of dextran had a significant effect exclusively on the compartmentalized enzyme (Fig. 3 top) compared to the soluble enzyme (Fig. 3 bottom). In this experiment the K_{ADP} of the mitochondrially organized enzyme increased from 107 to 165 μM ADP and V_{max} decreased from 0.92 to 0.82 IU/mg in the presence of dextran. The mean values of 6 independent experiments are shown in Table II. Since the confidence ranges for a probability of 99% did not overlap for the Michaelis constant of the compartmentalized enzyme, the difference seems to be highly significant. In contrast to that, the difference in the V_{max} was not significant.

The K_{ADP} of the released enzyme (128–172 μM (Fig. 3, Table II)) was some higher than for the compartmentalized enzyme. The reason for that is unclear and needs further investigations. It could be caused by effects of the microenvironment on the kinetic properties of adenylate kinase. On the other hand the difference is small and could be caused also by experimental variations since we measured in one experiment the effect of dextran on either the released or the mitochondrial enzyme but we did not compare the compartmentalized adenylate kinase with the released enzyme.

Effects on ADP export. The liberation of ADP from the intermembrane space was investigated with intact mitochondria and soluble pyruvate kinase competing for the ADP produced from ATP and AMP via mitochondrial adenylate kinase [20]. In this assay the ADP formed in the intermembrane space can react in two ways: (i) it can be transported via the adenine nu-

cleotide translocator into the matrix space to be utilized by oxidative phosphorylation and (ii) it can diffuse through the porin pores into the extramitochondrial compartment to be consumed by pyruvate kinase. The mitochondrial oxygen consumption was measured with a Clark electrode (Fig. 4, Trace A). The first derivative of this signal (Trace B) indicates that the rate of respiration and the area of the peaks correlates directly with the phosphorylation of that part of ADP which remains in the inter-membrane space. The first section of the experiments was performed without pyruvate kinase. Addition of 2.4 mM ATP increased the oxygen consumption due to the presence of ADP and AMP in the stock solution. When the oxidation rate returned to the resting state, 76 nmol AMP were added activating the active rate of respiration. The area of the AMP peak (Trace B, Fig. 4) represents the amount of oxygen which is consumed by the phosphorylation of the total ADP produced via adenylate kinase (47.7 nmol O_2/mg). In the presence of pyruvate kinase (in non-rate limiting activity of 111 IU/mg mitochondrial protein), the AMP-induced oxygen consumption decreased to 5.8 nmol O_2/mg. This indicated that 12% of the ADP formed in the inter-membrane space were transported into the matrix space by the adenine nucleotide translocator, while 88% of the ADP diffused through the outer membrane pore and became available to extramitochondrial pyruvate kinase (Fig. 4, left panel). In the presence of 10% dextran, only 74% of the ADP (produced from 76 nmol AMP) were available to pyruvate kinase (Fig. 4, right panel) and 17.1 nmol O_2/mg were consumed by the oxidative phosphorylation. Further additions of 152 and 228 nmol AMP led to a larger oxygen consumption pointing to an increased uptake of total ADP by the mitochondria. Neither in the absence nor in the presence of dextran the increase in oxygen consumption was proportional to the added amount of ADP. This may be explained by an increased efflux of ADP because of a higher gradient across the outer membrane.

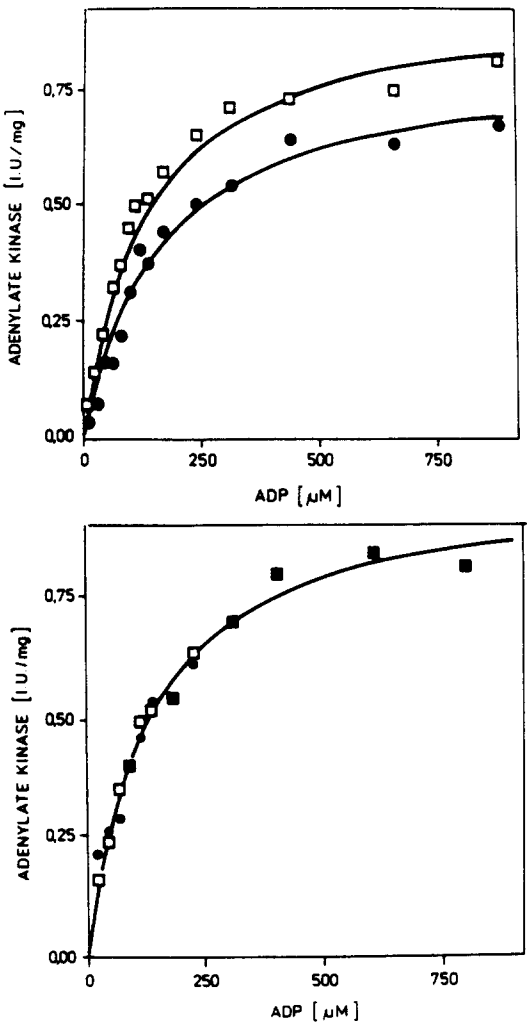

Fig. 3. Investigation of the effect of dextran M70 on kinetic properties of mitochondrial organized and soluble adenylate kinase. Enzyme activities were measured by means of the optical test as described in Materials and Methods. Top. Mitochondrial compartmentalized adenylate kinase: Liver mitochondria (1.5 μg/ml) were incubated in the presence (●) and in the absence (□) of 10% dextran M70. Data from one typical experiment. The Michaelis constant increased from 107±8 μM to 165±21 μM ADP in the presence of dextran whereas V_{max} decreased from 0.92±0.02 to 0.82±0.04 IU/mg. The mean values from six independent experiments are shown in Table II. Bottom. Soluble adenylate kinase: the experiment was performed as at the top but with soluble adenylate kinase released from rat liver mitochondria. The Michaelis constant were 128±11 μM ADP in the absence and 130±15 μM ADP in the presence of dextran. The V_{max} were 0.99±0.04 and 0.99±0.03 IU/mg mitochondrial protein in the absence and in the presence of dextran, respectively.

The same experiments were performed with bovine serum albumin instead of dextran. As shown in Table III, the AMP-induced mitochondrial oxygen consumption in the presence of exceeding pyruvate kinase increased from 17 over 25 to 34% after addition of 14 and 20% bovine serum albumin respectively. These data clearly show that albumin and dextran have comparable effects on the dynamic ADP compartmentation in the mitochondrial inter-membrane space.

In further experiments the concentrations as well as the molecular weights of the used dextrans were varied. Such as shown in Fig. 4, the AMP-induced oxygen consumption in the presence of pyruvate kinase was related to that in the absence of the enzyme. These differences (relative oxygen consumption) indicating changes in ADP compartmentation were plotted versus concentration (mM) and content (mg/ml) of the various dextrans. As shown in Fig. 5, the effect on ADP compartmentation in the inter-membrane space of the diverse dextrans correlated with the contents in mg/ml although the molecular weights were very different. Similar as in the experiment shown in Fig. 4 the amount of ADP consumed by the oxidative phosphorylation in the presence of pyruvate kinase was 10% of

TABLE III

Effect of bovine serum albumin on dynamic ADP compartmentation in the inter-membrane space of rat liver mitochondria

Incubation of rat liver mitochondria (0.47 mg/ml) similar to that described in Fig. 4 and the addition of bovin serum albumin as indicated. The mitochondrial oxygen consumption following the addition of 58 μM AMP was measured in the absence (control) and in the presence (PK) of 320 U pyruvate kinase/mg mitochondrial protein.

Addition of BSA (%)	Oxygen consumption after AMP addition (nmol O_2/mg)	
	control	PK
0	98.7	16.4 (16.6%)
13	105.7	27.3 (25.8%)
20	101.3	34.2 (33.8%)

that in the absence of the enzyme. However, correlated to gradual additions of dextran, the ADP increased which was kept in the inter-membrane space and utilized by oxidative phosphorylation. At 10% of dextran it was almost 3-times higher than in the absence of the macromolecules. By plotting the data versus the molar dextran concentration ((mM), Fig. 5), it became evident that ADP compartmentation increases with the molecular weight of the dextrans examined. In agreement with the experiments with isolated porin (Figs. 1 and 2), dextran T500 was effective already at concentrations in which the smaller dextran molecules were still inactive.

The influence of dextran on the oncotic pressure and the viscosity of incubation media. In experiments with reconstituted porin and intact mitochondria, we observed effects of dextran on transport rates through the pores and a dependence of these effects on the molecular weight of macromolecules. This restriction of transport processes my be explained in three ways: (i) the diffusion of molecules through the homogenous medium may be influenced by the viscosity of the medium, (ii) the permeability of the porin pore might be influenced by osmotic stress and (iii) the lateral diffusion within the inter-membrane space may become more difficult because of increased contacts between the two envelope membranes [6].

In order to analyze the two first possibilities we measured the effect of different dextrans on the oncotic pressure and the viscosity of the incubation medium. The dependence of the oncotic pressure on the molar concentration (mM) and the content (mg/ml) of different dextrans are shown in Fig. 6. Plotting the data versus the molar concentration, we observed differences depending on the molecular weight which were very similar to the effect on the ADP compartmentation (Fig. 5). Obviously the oncotic pressure was, under these conditions, not a colligative property, but dependent on the molecular weight of the dextran.

Finally we determined the effect of different macromolecules on the viscosity of the incubation media by means of an Ubbelohde viscosimeter. The results were shown in Fig. 7. The plot versus the dextran concentration (mM) revealed similar differences as observed for adenine nucleotide compartmentation and oncotic pressure. However, by plotting viscosity versus dextran

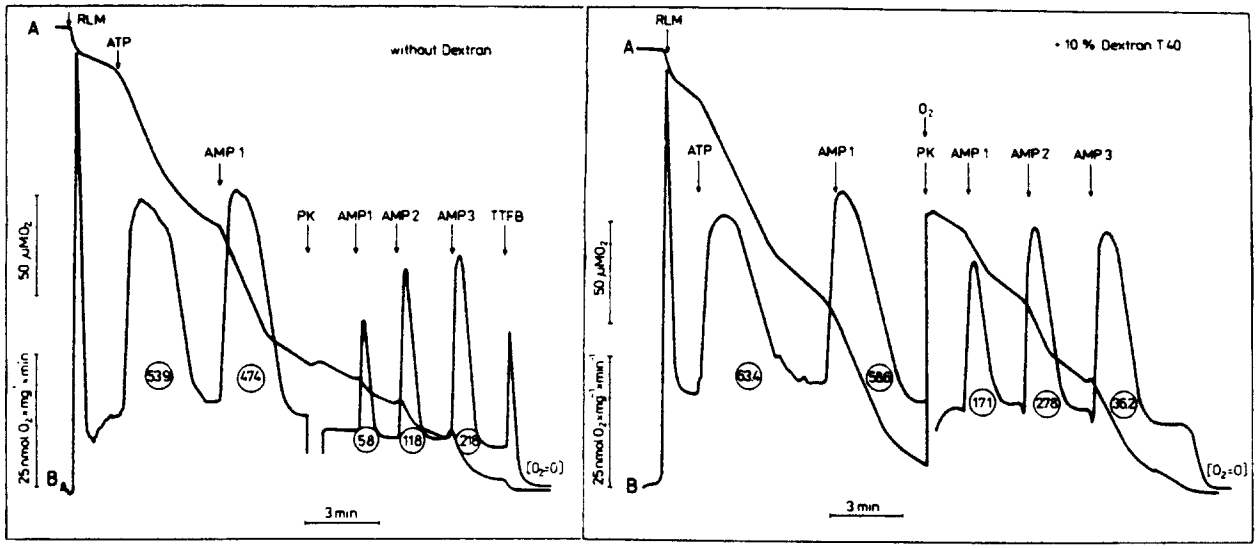

Fig. 4. Effect of 10% dextran on the ADP compartmentation in the intermembrane space of rat liver mitochondria. Mitochondria (1.15 mg/ml) were incubated in the a sucrose medium containing 2 mM PEP, 10 mM succinate and 1 μM rotenone, 10% dextran T40 as indicated. Subsequent additions: ATP, 2.4 mM ATP contaminated by 0.3 mM ADP and 0.12 mM AMP; AMP1, AMP2, AMP3 = 76, 152 and 228 nmol AMP; PK, 97 IU pyruvate kinase/mg; TTFB, 4,5,6,7,-tetrachloro-2-trifluormethylbenzimidazol = 48 μM. In the first section of the experiment mitochondria phosphorylate the ADP externally added, or formed via adenylate kinase from AMP. Addition of pyruvate kinase initiates the competition for ADP between this enzyme and the oxidative phosphorylation. The AMP-induced oxygen consumption in the absence and presence of pyruvate kinase in the same assay was taken as magnitude of the ADP compartmentation in the inter-membrane space [20].

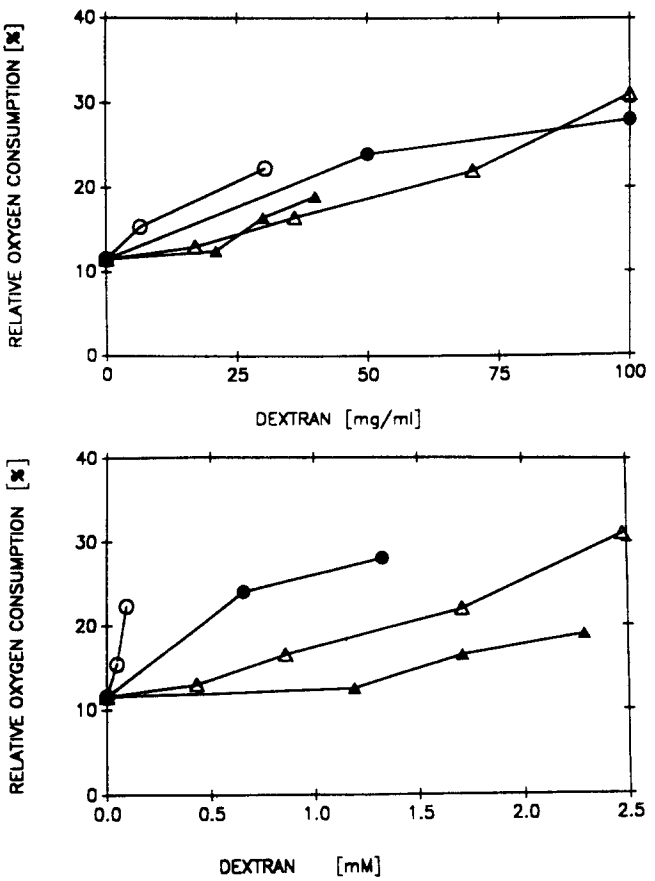

Fig. 5. Effect of different dextrans on the ADP-compartmentation in the intermembrane space of rat liver mitochondria. Mitochondria (0.9–1.2 mg/ml) were incubated as in Fig. 3 but with different concentrations of different dextrans (▲) T20; (△) T40; (●) M70; (○) T500. The relative AMP-induced oxygen consumption was calculated as described in Fig. 3 and plotted versus the dextran concentration (mM) and versus the dextran content (mg/ml).

content (mg/ml), we found an extremely different behavior of dextran T500 compared to the smaller dextran molecules.

Discussion

A compartmentation of adenine nucleotides in the inter-membrane space has been observed by several authors [11–21], but so far the phenomenon has remained unexplained. Saks and Jacobus postulated a functional coupling between the adenine nucleotide translocator and the mitochondrial creatine kinase [12,19]. These authors considered the role of the outer membrane as unimportant [13] which was in contrast to Bessman et al. who, by employing radioisotopes, concluded that the outer membrane is a barrier, since compartmentation effects disappeared when the outer membrane was removed [11]. Based on kinetic analysis of free and bound creatine kinase, Brooks et al. came to the same conclusion [18]. By means of stationary flux measurements we have observed that the diffusion limitations caused by the outer membrane are dynamic because the concentration gradients, of up to 13 μM ADP were dependent on the rate of adenine nucleotide fluxes [15–17]. The restriction of diffusion across the outer membrane may have several reasons. One limitation may result from the small number of pores in the outer membrane of mammalian mitochondria. Supposing the existence of 10^{10}–10^{11} pores per cm^2 [17,29] with a diameter of 2 nm, it can be calculated that only between 0.16% and 1.6% of the outer membrane surface permits free permeation of polar molecules. Besides the diffusion limitation through the outer membrane pores at high adenine nucleotide fluxes, we recently found that the binding of creatine kinase to the inner membrane became important at low fluxes [30].

In view of these results we defined two types of adenine nucleotide compartmentation which might be important in metabolic regulation: (i) intermolecular compartmentation within complexes between kinases and the inner and outer membrane and (ii) dynamic

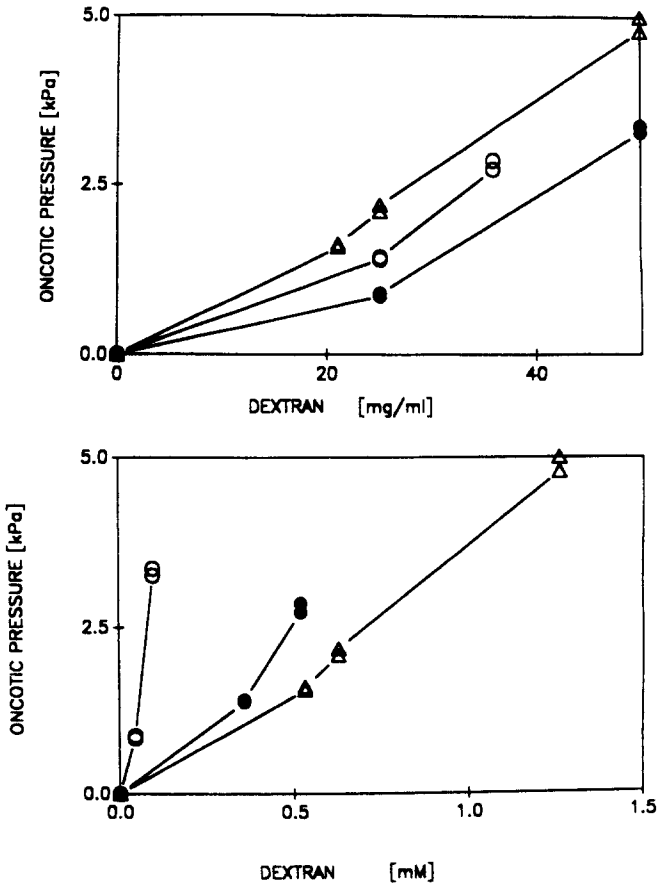

Fig. 6. Effect of different dextrans on the oncotic pressure of the medium The oncotic pressure of the different dextrans were measured as described in Materials and Methods. Dextrans were dissolved in the incubation medium in concentrations as indicated (△) T40; (●) M70; (○) T 500. The results were plotted versus the dextran concentration (mM) and the dextran content (mg/ml).

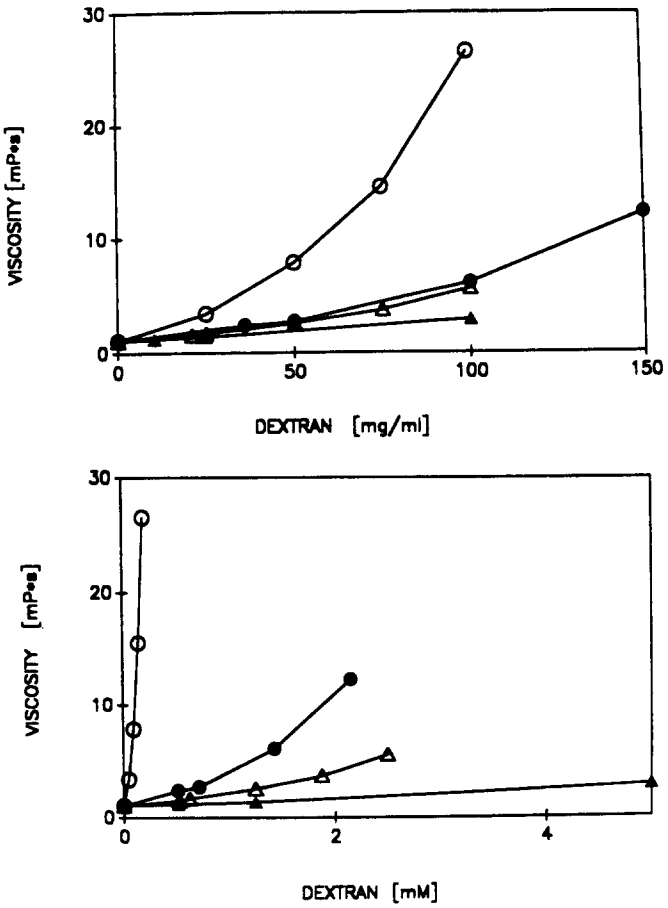

Fig. 7. Effect of different dextrans on the viscosity of the medium The viscosity of the incubation medium was measured with an Ubbelohde viscosimeter in the presence of dextrans varying in molecular size at concentrations as indicated (▲) T20; (△) T40; (●) M70; (○) T500. The results were plotted versus the dextran concentration (mM) and the dextran content (mg/ml).

compartmentation in the inter-membrane space via diffusion limitations, e.g., through the outer membrane pore.

To simulate the action of cellular cytosolic proteins we added 10% (w/v) dextran M70 to the incubation of mitochondria. It would been more obvious to simulate that action by using proteins. Indeed we used for some experiments bovine serum albumin too (Tables I and III) and found similar effects as with dextrans. However the use of albumin has serveral disadvantages. The most important problem arises from the high adsorption of 10% albumin containing solutions at 340 nm disturbing the optical measurements. An other serious problem is the difficulty to quench the albumin containing solutions with phenol or even with perchloric acid (Gellerich, F.N., unpublished results).

It was observed that the substitution of intracellular macromolecules by dextran affected both types of compartmentation. Dextran induced the formation of contact sites [6] which may be considered as complexes between kinases (such as creatine kinase and hexokinase) and components of the two envelope membranes (such as porin and AdN-translocator). Furthermore, dextran affected the conductance of the outer membrane pore [7] which may physiologically result in a reduced AdN exchange. The aim of the present investigation was to study the effect of oncotic pressure on the permeability of the outer membrane pore. This was performed in two ways: (i) the pore was reconstituted in artificial membranes and (ii) the dynamic compartmentation was studied by analyzing the effects of macromolecules on the kinetic parameters of adenylate kinase. In the liver this enzyme is located in the intermembrane space beyond the contact sites [31,32] and thus depends exclusively on the permeation of substrates through the pore.

Effect of dextrans on reconstituted porin

It was shown by Zimmerberg and Parsegian [7] in artificial membranes that the addition of macromolecules to reconstituted porin caused a change of the pore structure to a low conductance state. Our results completely agreed with these observations. In addition we observed (Figs. 1 and 2) that these effects did not correlate with the concentration but were dependent on the molecular weight of the added macromolecules.

Influence of macromolecules on the adenine nucleotide exchange

Since there is information about negative effects of macromolecules on metabolic properties of mitochondria [4,5], we carefully studied the influence of dextrans and bovine serum albumin (BSA) on rat liver mitochondria. We found only negligible alterations of the respiratory rates with succinate [6] (Table I) and glutamate/malate (not shown). The slightly reduced resting state respiration after addition of BSA probably results from the binding of uncoupling free fatty acids. A small but significant decrease of the ADP/O ratios by dextrans was observed. The reason for that is unclear. Some charges of dextrans caused a larger effect on the ADP/O ratio suggesting contaminations by uncouplers. These dextrans were not used in the experiments.

It was not possible to measure metabolic fluxes through reconstituted porin pores. We therefore tried to study these fluxes across the outer membrane by following the metabolite exchange between adenylate kinase in the inter-membrane space and extramitochondrial (not binding) hexokinase from yeast. Since hexokinase was used in non-rate limiting activity, the rate of the indicator reaction depends on the activity of the adenylate kinase and the AdN diffusion between both pools. The addition of dextran did not influence the kinetic properties of soluble adenylate kinase and hexokinase (Table II), while the increase of K_{ADP} of

the compartmentalized enzyme indicated an increased diffusion resistance. In the system where pyruvate kinase was used to utilize the liberated ADP, we observed that the release of ADP, formed in the intermembrane space by adenylate kinase, was diminished by the presence of macromolecules. This again pointed to an increased diffusion resistance. Such as observed with reconstituted porin, the effect of dextrans on ADP exchange was dependent on the concentration and the molecular weight of macromolecules. To rule out the possibility that the dextran effects are caused by anionic impurities of the dextrans we performed additional experiments as well as with dialysed dextrans as with dextran sulfate and König's polyanion. We observed with the dialysed dextrans the same results as with the normal one. The results obtained with negatively charged macromolecules are completely different from those obtained with dextrans: they reduce the active and the uncoupled rate of respiration drastically and reduce the dynamic ADP compartmention in the mitochondrial inter-membrane space (Gellerich, F.N., unpublished results).

These findings together with the albumine effect on the dynamic compartmentation (Table III) support our conclusion that the observed results are caused by macromolecules independently on their chemical structure.

Several aspects must be considered to explain the observed reduction of the metabolite exchange between the inter-membrane space and the extramitochondrial compartment: (i) the resistance of lateral diffusion within the inter-membrane space could have been reinforced by increasing contacts between the two envelope membranes, (ii) the permeability of the outer membrane pore might have been influenced by oncotic stress [7], (iii) the diffusion of metabolites through the homogenous medium to the pores could have been influenced by macromolecules.

Under the conditions used in our experiments the first possibility appears to be of minor importance since the macromolecule effects were also observed with pores reconstituted in artificial membranes. To distinguish between the other two possibilities we compared the oncotic pressure and the viscosity of different dextran solutions. The oncotic pressure correlated with the concentration and the molecular weight of different dextrans in a similar way as the observed biological effects. On the other hand the viscosity of the different dextran solutions was likewise dependent on the concentration and molecular weight of the dextrans. Since viscosity affects the diffusion coefficient, an influence of macromolecules on the metabolite diffusion can not be ruled out and needs further investigation.

On the whole the present results indicate that the observed effects of macromolecules on the reconstituted outer membrane pore may have physiological relevance because we observed the same response of the AdN exchange in intact mitochondria to the varied dextran concentrations and molecular weight. It appears that the permeability of the outer membrane pore may be overestimated and unphysiologically high in mitochondria isolated in the frequently used isolation media which lack macromolecules.

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