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EVIDENCE FOR IDENTITY BETWEEN THE HEXOKINASE-BINDING PROTEIN AND THE MITOCHONDRIAL PORIN IN THE OUTER MEMBRANE OF RAT LIVER MITOCHONDRIA

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Hexokinase-binding protein and mitochondrial porin were isolated from rat liver mitochondria by different procedures. It was found that the hexokinase-binding protein made lipid vesicles permeable to ADP and formed asymmetric pores in lipid bilayer membranes identical to those obtained from the mitochondrial porin. On the other hand, the mitochondrial porin confers the ability to bind hexokinase. In addition, evidence is presented that both hexokinase-binding protein and mitochondrial porin bind glycerol kinase.

Introduction

Evidence for channel structures in the outer mitochondrial membrane has been reported from electron-microscope studies [1,2] and X-ray diffraction [3,4]. Channel-forming proteins have also been incorporated into planar lipid bilayers from Triton X-100-solubilised outer membranes by Colombini and co-workers [5–7]. Meanwhile, it has been demonstrated that a polypeptide of M_r 32000 is responsible for the pore-forming activity [8–11] in the outer membrane of mung bean, rat liver and *Neurospora* mitochondria. This protein was named 'mitochondrial porin' because it has some properties in common with the bacterial porin. The outer mitochondrial membrane seems to contain only one type of porin. In reconstitution experiments with outer membrane subfractions, all membranes were impermeable to saccharides [8] and ADP [9] unless the mitochondrial porin was present. Therefore, ADP-ATP exchange with the cytosol across the outer membrane is possible only through the pore formed by the mitochondrial porin.

In view of these findings we proposed that the hexokinase present at the mitochondrial surface binds to the mitochondrial porin, because it has been reported that the bound hexokinase could directly and exclusively exchange ATP for ADP with the inner mitochondrial compartment [12]. This assumption was further supported by the description of a specific binding structure for hexokinase in the outer membrane [13] and the characterization of that as a polypeptide of M_r 30000 in SDS-polyacrylamide gel electrophoresis [14].

In the present paper we have compared the isolated mitochondrial porin and the hexokinase-binding protein for specific binding of hexokinase and glycerol kinase. In addition we have investigated the hexokinase-binding protein for pore-forming activity in planar lipid bilayers and ADP exchange activity in reconstituted vesicles. Both proteins turned out to have identical properties in all these measurements and the same molecular weight as determined by SDS-polyacrylamide gel electrophoresis.

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Abbreviation: Hepes, 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid.

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Experimental procedures

Materials

Octylglycoside was purchased from Calbiochem. Asolectin (L-α-phosphatidylcholine Type II-S from soybean) was from Sigma. 1,2-Diphytanoyl-3-phosphatidylcholine was obtained from Avanto Biochemicals, Birmingham. All other chemicals were pro analysi grade and were obtained from Boehringer, Mannheim, and Merck, Darmstadt, F.R.G.

Methods

Preparation of outer mitochondrial membrane and subfractions of it. Outer mitochondrial membrane was prepared as described by Sottocasa et al. [15] from rat liver mitochondria which had been isolated by differential centrifugation in a medium containing 0.25 M sucrose/10 mM Hepes (pH 7.4)/0.1 mM EGTA/0.1 mM phenylmethylsulfonyl fluoride.

The hexokinase-binding protein was prepared from the isolated outer membrane according to the method of Felgner et al. [14]. The pore-forming protein ('mitochondrial porin') was isolated as described recently [9].

Reconstitution and determination of permeability. The fractions containing either the hexokinase-binding protein or the mitochondrial porin were dialysed together with 5 mg asolectin against 3 × 5 l 10 mM NaH_2PO_4 buffer (pH 7.4). The dialysed samples or the outer membrane together with a portion of 50 mg asolectin were dried as a film at the bottom of a glass tube. To each tube 0.2 ml 100 mM ADP was added. Resuspension of the dried film was achieved by mixing the contents of the tube on a Vortex mixer for 10 min and incubating subsequent at room temperature for 1 h. The contents of the tubes were applied to a column (0.8 × 26 cm) of Sephadex G-50 and then eluted with 20 mM Hepes (pH 7.4) in the cold. The peak fraction of the eluted vesicles was collected and its ADP content was determined in the presence of Lubrol by an enzymatic test system.

Incorporation into planar bilayers. Aliquots of the vesicles reconstituted in the absence of ADP with isolated mitochondrial porin or hexokinase-binding protein were used for these studies. Black lipid bilayer membranes were obtained [16] from a 1–2% (w/v) solution of the different lipids in *n*-decane. The chamber used for bilayer formation was made of Teflon. The circular holes in the wall between the two aqueous phases had an area of either 1.5 mm^2 (in the case of the macroscopic measurements) or 0.2 mm^2 (for the single channel experiment). The temperature was maintained at 25°C in all experiments. The aqueous salt solutions were buffered with 5 mM Tris-HCl and were adjusted to pH 7.5. Variations of the pH between 6 and 7.5 did not affect the properties of a single channel nor those of the macroscopic conductance. The protein was added to the aqueous phase prior to membrane formation or after the membrane had turned black. The protein-containing aqueous solutions were prepared immediately before use in order to prevent protein inactivation. The protein (0.5–1 mg/ml) was dissolved by sonication for 30 s in 1% (w/v) Triton X-100 and 5 mM Tris-HCl (pH 7.5). Ag|AgCl or calomel electrodes (with salt bridges) were inserted in both aqueous compartments. The macroscopic conductance measurements were performed using a 610 C Keithley electrometer. In the single-channel experiments the membrane current was measured by using a Keithley 427 current amplifier, which was monitored by a Tektronix 5115/5A22 storage oscilloscope and recorded on a strip chart recorder. The bandwidth of the single-channel experiments was between 300 Hz and 3 kHz. The zero current potentials were measured as described elsewhere [17].

Dissociation-association studies

The studies of dissociation by glucose 6-phosphate and of the binding of hexokinase were performed with asolectin vesicles, reconstituted either with hexokinase-binding protein or mitochondrial porin, as well as with outer membrane and mitochondria. This was achieved by a 15 min incubation at room temperature of the samples with the 300000 × *g* supernatant of a liver homogenate in mitochondrial isolation medium in the presence of 10 mM $MgCl_2$ and 10 mM glucose 6-phosphate as indicated.

After incubation the mitochondrial sample was diluted 20-fold with 0.25 M sucrose/10 mM Hepes (pH 7.4) and centrifuged for 10 min at 10000 × *g* in a Sorvall rotor SS-34. The other samples were

diluted in the same way and centrifuged for 60 min at 200000 × *g* in a Beckman 60 Ti rotor.

Assays. Hexokinase (EC 2.7.1.1) and glycerol kinase (EC 2.7.1.30) was determined according to Ref. 18. ADP was measured enzymatically as described in Ref. 19.

Other methods. SDS-polyacrylamide gel electrophoresis was performed as described by Lämmli [20]. Protein was determined by the method of Lowry et al. [21].

Results

Binding of hexokinase to the mitochondrial porin

The hexokinase binding protein was isolated as in Felgner et al. [14] and the mitochondrial porin as described recently [9]. The proteins were incorporated into asolectin vesicles. The vesicles were tested for ability to bind hexokinase from the particle-free liver homogenate. The data listed in Table I clearly show that the mitochondrial porin binds hexokinase with a capacity similar to that of the hexokinase-binding protein.

The binding of hexokinase to the vesicles into which the proteins were incorporated can be specifically reduced by glucose 6-phosphate, as is observed in intact mitochondria and the outer membrane fraction (Table II). With increasing purity of the hexokinase-binding protein as well as of the mitochondrial porin, this specific detaching effect of glucose 6-phosphate becomes more and more pronounced. This could suggest that non-specific binding of the hexokinase to contaminating structures is reduced by increasing the purity of both protein preparations.

Binding of other kinases to the mitochondrial porin

We have observed that glycerol kinase also binds to a comparable extent to the hexokinase-binding protein as well as to the mitochondrial porin (Table III). Glycerol kinase seems to compete with hexokinase for the same binding site because glucose 6-phosphate must be present for glycerol kinase binding to be observed. In the experiment described, glycerol kinase is bound from a supernatant of a liver homogenate when hexokinase is also present. Thus the binding of glycerol kinase becomes more prominent when the binding of hexokinase is hindered by glucose 6-phosphate.

Comparison of the molecular weights

The mitochondrial porin from rat-liver outer membrane has been shown recently to be a 32 kDa polypeptide by differential extraction of the membrane with octylglycoside and subsequent purification on a sucrose density gradient [9]. A fraction containing the high molecular weight polypeptides of the outer mitochondrial membrane (as seen in Fig. 1, trace 'hexokinase-binding protein') as a contaminant neither rendered reconstituted vesicles permeable to ADP nor produced any pores in planar bilayer experiments. The same was shown by Zalman et al. [8] for outer membrane from mung bean mitochondria and by Freitag et al. [11] for *Neurospora* mitochondria.

The hexokinase-binding protein isolated by the method of Felgner [14] migrates to the same point as the mitochondrial porin in SDS-polyacrylamide gel electrophoresis. This may be seen in Fig. 1, trace 'mitochondrial porin' and 'hexokinase-

TABLE I

BINDING OF HEXOKINASE TO MITOCHONDRIAL MEMBRANES AND TO ASOLECTIN VESICLES CONTAINING THE HEXOKINASE-BINDING PROTEIN (HKB) OR MITOCHONDRIAL PORIN (MP), RESPECTIVELY

Hexokinase was bound by incubation of the samples with the high-speed supernatant of a liver homogenate in the presence of 10 mM Mg^{2+}.

	Mitochondria	Outer membrane	Asolectin vesicles		
			+HKB	+MP	pure
Hexokinase (HK) bound (mU/mg)	0.64 ± 0.2	6.7 ± 0.72	121 ± 29	86 ± 51	0.0
Increase in binding capacity	1	10.5	189	134	—

TABLE II

SPECIFICITY OF BINDING OF HEXOKINASE TO MITOCHONDRIAL MEMBRANES AND TO ASOLECTIN VESICLES CONTAINING THE HEXOKINASE-BINDING PROTEIN (HKB) OR THE MITOCHONDRIAL PORIN (MP)

Hexokinase was bound to the different samples by incubation with a high-speed supernatant of a liver homogenate in the presence or absence of 10 mM glucose 6-phosphate (G-6*P*). Values are mU hexokinase bound/mg.

	Mitochondria	Outer membrane	Asolectin vesicles	
			+ HKB	+ MP
10 mM Mg^{2+}	0.92	7.45	142	56.0
10 mM Mg^{2+} + 10 mM G-6*P*	0.38	1.5	4.7	1.1
Ratio −/+ G-6*P*	2.4	5.0	30.2	51.0

binding protein' by comparing the polypeptide bands in the M_r 32000 region of the gel and in addition in the trace (MP + HKB) where the mitochondrial porin and the hexokinase-binding protein preparations are mixed. We therefore conclude that the hexokinase-binding protein resembles the mitochondrial porin in subunit structure.

Permeability of the reconstituted vesicles to ADP

We have recently carried out reconstitution assays with subfractions of the outer mitochondrial membrane and observed that a polypeptide of M_r 32000 was responsible for pore-forming activity in planar bilayers as well as for ADP permeability in reconstituted vesicles. Similar experiments were performed both with complete outer membrane and subfractions of the membrane, which were obtained during preparation of the hexokinase-binding protein by differential extraction with octylglycoside according to Felgner et al. [14]. As seen in Fig. 2, the active principle in ADP permeability corresponds to the hexokinase-binding protein. This can be seen since the vesicles containing this protein exhibited a similar permeability for ADP as vesicles reconstituted with complete outer membrane which contained 25-times more protein. The fractions without the binding protein did not render the vesicles permeable to ADP.

Macroscopic conductance measurement

A strong conductance increase in lipid bilayer membranes made from asolectin or diphytanoylphosphatidylcholine was observed if the isolated hexokinase-binding protein was added to the aqueous phase prior to or after membrane formation. A typical experimental result is shown in Fig. 3. A membrane from asolectin/*n*-decane was formed in an aqueous solution of 1 M KCl/5

TABLE III

BINDING OF GLYCEROL KINASE TO MITOCHONDRIAL MEMBRANES AND TO ASOLECTIN VESICLES CONTAINING THE HEXOKINASE-BINDING PROTEIN (HKB) OR MITOCHONDRIAL PORIN (MP), RESPECTIVELY

Glycerol kinase was bound by incubation of the samples with the high-speed supernatant of a liver homogenate in the presence of 10 mM glucose 6-phosphate and 10 mM $MgCl_2$.

	Mitochondria	Outer membrane	Asolectin vesicles		
			+ HKB	+ MP	pure
Glycerol kinase bound (mU/mg)	0.49 ± 0.17	7.5 ± 0.4	78.7 ± 4.0	81.5	0.0
Increase in binding capacity	1	15.3	160.6	166.3	–

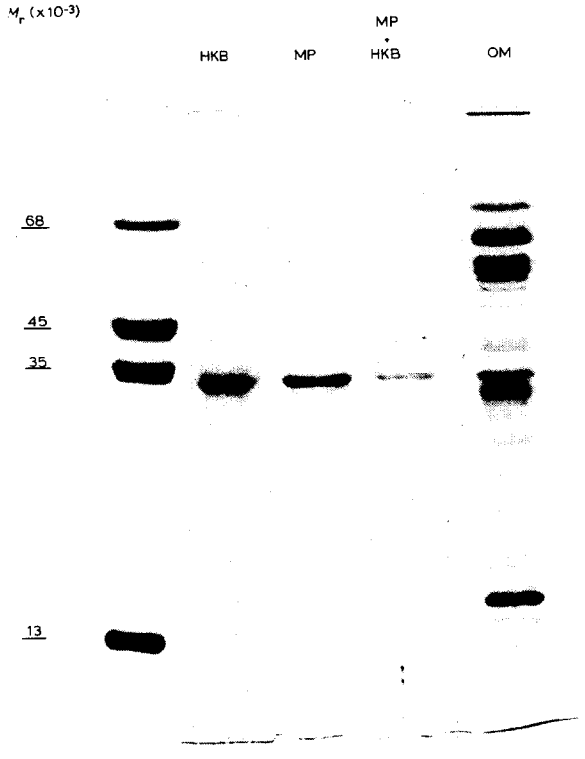

Fig. 1. SDS-polyacrylamide gel electrophoresis of outer membrane (OM), hexokinase-binding protein (HKB), mitochondrial porin (MP) and a mixture of HKB and MP. Molecular weight standards from top to bottom: bovine serum albumin, aldolase, lactase dehydrogenase and cytochrome *c*.

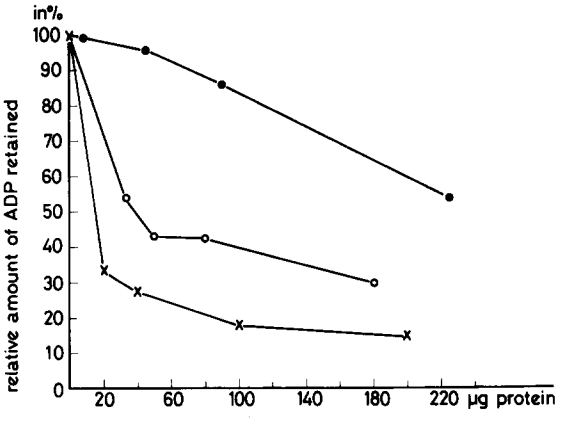

Fig. 2. Permeability of asolectin vesicles reconstituted with complete and different subfractions of outer membrane. The vesicles were loaded with ADP during reconstitution and were analysed for ADP content after they had been chromatographed on Sephadex G-50. The protein concentration used for reconstitution was multiplied by 4 in the case of the outer membrane (X) and by 100 for the hexokinase-binding protein (○) in order to fit the curves to the same protein concentration scale. Line ●——● is the pellet fraction remaining after extraction of hexokinase-binding protein from the membrane according to Felgner et al. [14]. The ADP retained in the different vesicles was referred to the ADP content of pure asolectin vesicles.

mM Tris-HCl (pH 7.5). Hexokinase-binding protein was added with stirring to the aqueous phase to a final concentration of 2.8 µg/ml 15 min after the membrane had turned completely black (arrow in Fig. 3). The membrane conductance began to increase after an intial lag of about 2–3 min, which is presumably due to the need for diffusion of the protein through the unstirred layers. After this lag period the conductance increased rapidly by about three orders of magnitude. This process slowed down 20 min after protein addition: only a slight further increase was then observed. This time-course of conductance remained the same if the protein was added to only one side or to both sides of the membrane.

In general, the time-dependence of the conductance increase was found to be very similar to that found for the pore-forming protein from outer mitochondrial membrane or for porins from outer bacterial membrane [9,22]. The conductance increase shown was not observed by adding the same Triton X-100 concentration to the membrane cell which was normally supplied together with the hexokinase-binding protein.

In order to measure the protein dependence of the conductance, use was made of the observation that the rate of conductance increase slows down 20 min after the addition of the protein. Therefore, we measured at that time the conductance of different protein concentrations in the aqueous phase. The measurements shown in Fig. 4 were performed in the presence of 1 M KCl with membranes made from asolectin. A linear relationship could be observed over a wide range between protein concentration and specific conductance. While the time-course of the conductance increase was almost the same for membranes from different lipids a considerable difference in the absolute conductance was observed. The use of oxidized cholesterol resulted in much higher conductance

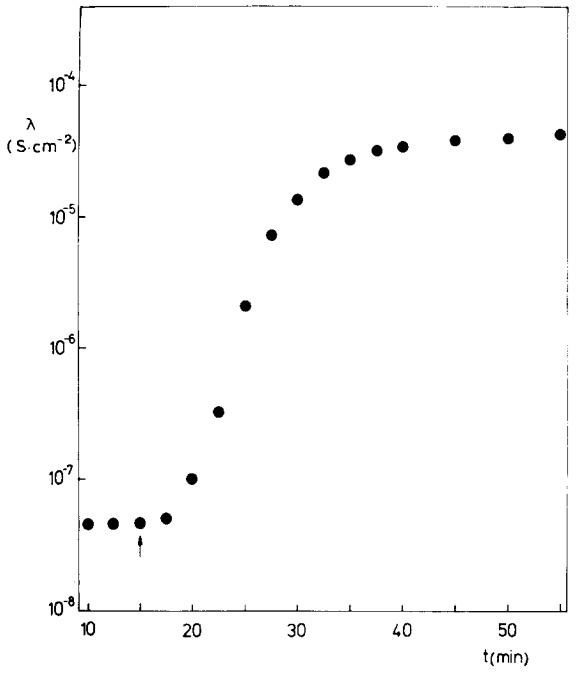

Fig. 3. Increase of the specific membrane conductance after the addition of 2.8 μg/ml hexokinase-binding protein to the aqueous phase. The protein was added 15 min after blackening of the membrane from asolectin/*n*-decane. The aqueous phase contained 1 M KCl and 2 μg/ml Triton X-100; $T = 25°C$. The applied membrane potential was 5 mV.

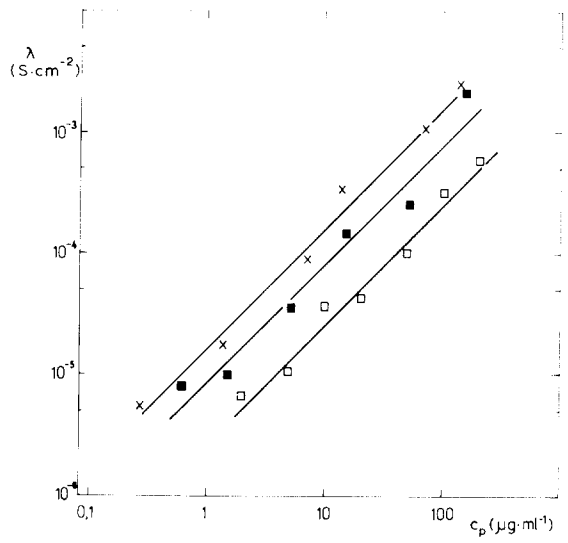

Fig. 4. Specific membrane conductance, λ, as a function of the protein concentration c_p in the aqueous phase. The membranes were formed from asolectin dissolved in *n*-decane. The aqueous phase contained 1 M KCl and less than 100 μg/ml Triton X-100; $T = 25°C$. The values were obtained 20 min after addition of the protein or after blackening of the membrane when the protein had been added prior to membrane formation; the membrane potential was 5 mV. Mean values of three experiments. ×, hexokinase-binding protein; ■, outer mitochondrial membrane; □, mitochondrial porin.

levels than did the use of asolectin (which is shown in Fig. 4) or diphytanoylphosphatidylcholine. The conductance increase caused by addition of the complete outer membrane was related in Fig. 4 to the amount of the 32 kDa polypeptide supplied together with the other outer membrane polypeptides. Interestingly, the activity of the isolated hexokinase-binding protein was about twice as high as that of 32 kDa polypeptide, while the isolated mitochondrial porin showed a 3-times lower activity. These differences in the macroscopic conductance measurements are believed to reflect uncertainty in protein determinations and/or partial damage of the protein by the purification procedure.

We had already observed in preceding experiments that the mitochondrial porin upon asymmetric addition responds to membrane voltages of the opposite sign with a strong difference in conductance. Therefore, we attempted to investigate the hexokinase-binding protein for the same property. The protein was added 10 min after blackening to one side of the membrane (*cis*-side) at a concentration of 140 μg/ml. A rapid increase in the membrane conductance was observed for 20 min. Then a voltage of 30 mV and opposite polarity was applied to the membrane. If the *cis*-side was negative, the membrane current decreased with a time constant of several seconds (lower trace in Fig. 5). For positive polarity on the *cis*-side no or only a little decrease of the membrane current was observed in the same time range (upper trace in Fig. 5). We could, however, also observe a current decrease, when the *cis*-side was positive, if the voltages applied to the membrane were larger than 40 mV. However, the time constant of this decay was different by about two orders of magnitude compared to that observed when the *cis*-side was negative.

This asymmetric behaviour of the hexokinase-binding protein in lipid bilayer membranes is similar to that which has been described by us for the mitochondrial porin from rat liver and *Neurospora*

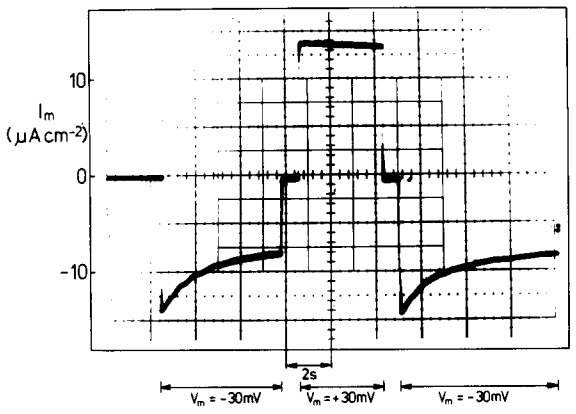

Fig. 5. Asymmetric current response to voltages of opposite sign as a result of asymmetric addition of the hexokinase-binding protein. The membrane was made from asolectin/*n*-decane. The protein concentration in the 1 M KCl solution at the *cis*-side was 140 μg/ml; $T=25°C$. A voltage of −30 mV or +30 mV (as referred to the *cis*-side) was applied to the membrane about 20 min after addition of the protein. The membrane current decreases exclusively if the *cis*-side is negative.

crassa [9,11]. It suggests an asymmetric insertion into the bilayer membrane as well as an asymmetric construction of the protein.

Single-channel measurements

Experiments with small amounts of hexokinase-binding protein using a high current resolution show that the conductance described in the previous section is caused by the formation of pores. A typical experiment of this type is shown in Fig. 6, where the hexokinase-binding protein was added 10 min after blackening to both sides of a asolectin membrane in a final concentration of 20 ng/ml. The current at 5 mV membrane poten-

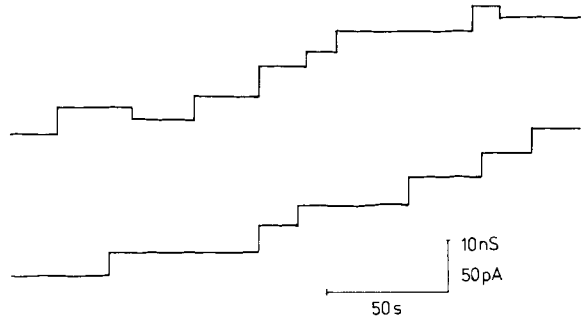

Fig. 6. Stepwise increase of the membrane current after the addition of the hexokinase-binding protein. The aqueous phase contained 1 M KCl, 20 ng/ml protein and 100 ng/ml Triton X-100; $T=25°C$. The membrane was formed from asolectin dissolved in *n*-decane. The current prior to the addition of the protein was less than 0.5 pA. The record starts at the left-hand end of the lower trace and continues in the upper trace; $V_m=5$ mV.

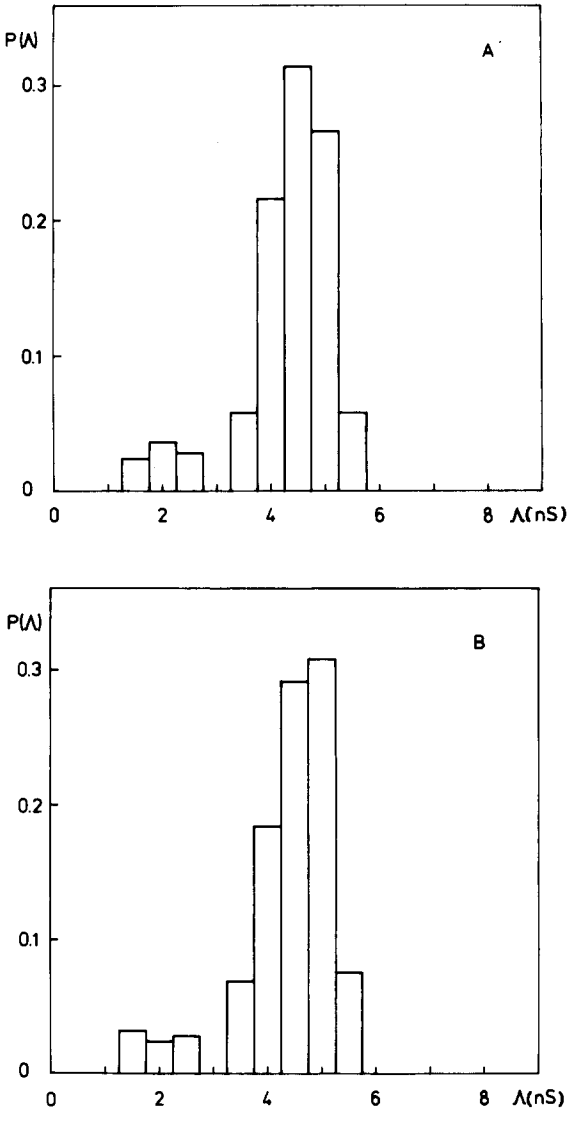

Fig. 7. Histogram of the conductance fluctuations observed with membranes from asolectin/*n*-decane in the presence of: (A) hexokinase-binding protein ($n=435$; $\bar{\Lambda}=4.3$ nS); (B) mitochondrial porin ($n=381$; $\bar{\Lambda}=4.3$ nS [9]). The aqueous phase contained 1 M KCl, $T=25°C$. The applied voltage was 5 mV.

tial increased stepwise. Current fluctuations like that are typical for the presence of the protein and are never observed by addition of Triton X-100 alone. Most of the current fluctuations are directed upward at this membrane potential. Terminating steps are only rarely observed and have in most cases half the amplitude of the upward-directed current fluctuations. A histogram of all current steps at 5 mV membrane potential is given in Fig. 7A. Fig. 7B shows for comparison the histogram derived earlier for the mitochondrial porin [9] under identical conditions. Both histograms show a strong analogy.

At 5 mV the terminating steps represent a minor fraction, whilst at higher voltages they become more and more prominent. Simultaneously, the amplitudes of the upward-directed steps decrease. Thus, at 10 mV the average conductance increment of all fluctuations is much lower than at 5 mV. This finding indicates that the pore is not completely switched off at high voltages but accepts many different conductance states depending on the magnitude of the membrane potential. This becomes evident from the data in Table IV, which summarizes the results obained from single-channel measurements with the hexokinase-binding protein in membranes made fom asolectin and diphytanoylphosphatidylcholine. The average conductance increment $\overline{\Lambda}_{on}$ and $\overline{\Lambda}_{off}$ were recorded after asymmetric addition of the protein (to the *cis*-side) and with the membrane potential negative at the *cis*-side.

Zero-current membrane potentials

Further information about the structure of the pore formed by the hexokinase-binding protein was obtained from zero-current membrane-potential measurements. In these experiments 14 μg/ml hexokinase-binding protein was added to a 10 mM aqueous solution of NaCl or KCl bathing a membrane prepared from asolectin/*n*-decane. In order to assure the proper insertion of the pores, a membrane potential was applied. When the incorporation of the pores reached an equilibration the voltage was switched to zero and the salt concentration on one side of the membrane was raised 10-fold. In most experiments described here the more dilute side became negative by about -2 to -8 mV, indicating a small anion selectively of the pore. In Table V the zero-current potential mesurements obained with the hexokinase-binding protein are compared to the results published earlier for the mitochondrial porin [9]. In addition, Table V shows the ratio of the anion permeability, P_a, versus the cation permeability, P_c, calculated according to the Goldman-Hodgkin-Katz equation [17]. The hexokinase-binding protein resembles the mitochondrial porin in having a small preference

TABLE IV

AVERAGE CONDUCTANCE INCREMENTS $\overline{\Lambda}_{on}$ AND $\overline{\Lambda}_{off}$ FOR THE ISOLATED HEXOKINASE BINDING PROTEIN AS A FUNCTION OF THE APPLIED MEMBRANE POTENTIAL

The potential was negative on the side at which the protein was added. The membranes were formed from two different lipids dissolved in *n*-decane; $T = 25°C$. The aqueous phase contained 1 M KCl. n_{on} and n_{off} are the numbers of events from which $\overline{\Lambda}_{on}$ and $\overline{\Lambda}_{off}$ are calculated.

lipid	V_m (mV)	$\overline{\Lambda}_{on}$ (nS)	n_{on}	$\overline{\Lambda}_{off}$ (nS)	n_{off}
Asolectin	5	4.3	431	4.0	81
	10	3.0	142	2.5	119
	20	2.4	157	2.2	121
	50	0.75	221	0.72	185
	100	0.45	35	0.42	30
Diphytanoylphosphatidylcholine	5	4.3	118	3.9	52
	10	2.9	97	2.6	71
	20	2.1	65	2.0	59
	50	0.87	78	0.84	70
	100	0.53	45	0.50	35

TABLE V

ZERO CURRENT POTENTIALS, V_m, OBSERVED WITH INCORPORATION OF THE HEXOKINASE-BINDING PROTEIN (HKB) AND THE MITOCHONDRIAL PORIN (MP)

The data were obtained in the presence of a 10-fold salt gradient of NaCl or KCl. The membranes were formed from two different lipids dissolved in *n*-decane. $T = 25°C$. V_m is the potential of the dilute side (0.01 M) minus the potential of the concentrated side (0.1 M). For further explanations see text. The results were obtained from at least three membranes.

Salt	V_m (mV)		P_a/P_c	
	MP	HKB	MP	HKB
Asolectin membranes				
NaCl	-7 ± 2	-8 ± 2	1.4 ± 0.16	1.5 ± 0.16
KCl	-2 ± 2	-3 ± 2	1.1 ± 0.12	1.2 ± 0.12
Diphytanoylphosphatidylcholine membranes				
NaCl	-4 ± 3	-5 ± 3	1.2 ± 0.20	1.3 ± 0.19
KCl	0 ± 2	-1 ± 2	1.0 ± 0.15	1.1 ± 0.15

for anions. This may be explained by the presence of positively charged groups in or near the pore mouth and by the higher mobility of the chloride versus the sodium ion in the aqueous phase.

Discussion

It has been described by several investigators that a polypeptide of M_r 32000 is responsible for the permeability of the mitochondrial outer membrane from rat liver for poly(ethylene glycol) up to M_r 3000 [7], dextrans up to M_r 8000 [8] and ADP [9]. No other polypeptide in the different subfractions of the outer membrane was detected which could either form channels in the lipid bilayer membranes or render the reconstituted vesicle membranes permeable to ADP. We have also observed that this channel-forming protein confers the ability to bind hexokinase with a capacity comparable to that of the isolated hexokinase-binding protein. It has been described by Rose et al. [23] that the hexokinase can be specifically desorbed from the outer membrane by glucose 6-phosphate. On the other hand, Mg^{2+} displaces the equilibrium between bound and free hexokinase towards complex formation but does not prevent the glucose 6-phosphate action. The same effects of these agents were also seen on vesicles reconstituted with isolated mitochondrial porin where glucose 6-phosphate in the presence of 10 mM Mg^{2+} desorbs most of the hexokinase. It is worth noting that this effect on desorption of hexokinase from the purified mitochondrial porin is much more pronounced when compared to either the complete outer membrane or the mitochondria. We suggest that Mg^{2+} also favours the formation of unspecific complexes with other membrane structures, which is impossible when the specific binding structure is present in the pure form. From these experiments we conclude that the mitochondrial porin is also responsible for the selective binding of hexokinase to the outer membrane. To substantitate this thesis further we investigated whether or not the hexokinase-binding protein has channel-forming properties. It was observed that the hexokinase-binding protein, when incorporated into lipid vesicles, facilitated their permeability to ADP. About 25-times less of the purified protein was necessary to restore the same rate of permeability as observed upon incorporation of all outer membrane proteins. On the other hand, a fraction which contains the high molecular weight polypeptides (pellet fraction) of the outer membrane could not establish permeability of the vesicles, although it was present at a 100-fold higher concentration. Therefore the hexokinase-binding protein seems exclusively to render lipid membranes permeable to ADP, as has recently been described for the mitochondrial porin [9]. It seems most unlikely that any other contaminating protein in the mitochondrial porin preparation or in the isolated hexokinase-binding protein fraction can confer the ability to bind hexokiase and/or to

establish permeability to ADP. As judged from the SDS-polyacrylamide gel electrophoresis, both protein preparations lead to the purification of a polypeptide of M_r 32000 and are almost free from contaminating proteins. A mixture of both preparations did not result in two different polypeptide bands.

Noteworthy is the fact that the mitochondrial porin, besides binding hexokinase, can also bind glycerol kinase. It seems probable that both kinases bind to the same binding site, because we observed an increase in bound glycerol kinase activity concomitant with a decrease in bound hexokinase upon incubation with glucose 6-phosphate.

We have further attempted to measure an influence of the bound kinases upon the permeability of the pore for ADP. Experiments with the reconstituted system were not successful but we hope to show this in the near future.

Our finding that kinases cover the outer surface of the pore may explain Parson's failure to detect pore structures in the outer membrane of rat liver mitochondria [2], although the pores have properties (a calculated diameter of 2 nm [9]) comparable to the channels in *Neurospora* [11] and plant [1] mitochondria. In the outer membranes of the latter two species, pore structures have been visualized [24,1,2]. Further, one must consider that in *Neurospora* outer membranes the mitochondrial porin amounts to about 30% of the outer membrane protein, whereas in rat liver we calculated a value for about 7% of the total outer membrane protein. Therefore the pore structures may be more scarce in rat liver mitochondria. We have attempted to calculate the number of pores in the rat liver outer membrane. By assuming 1% pore protein (per total mitochondrial protein), an M_r of 100000 for the pore [10], a mitochondrial diameter of 1 μm and the dry weight of a mitochondrion as $1 \cdot 10^{-13}$ g [25] we calculated 53 pore molecules per μm^2 of the mitochondrial surface. This is about one pore present per $(100\ nm)^2$. This is a relatively low number compared to the four pores per $(10\ nm)^2$ calculated by Freitag et al. [11], employing antibody titration, for *Neurospora* outer membrane.

The mitochondrial porin from rat liver has been widely characterized in planar bilayers by Colombini [5–7] and has been shown to form voltage-dependent anion-selective channels. We have recently described the inclination of the protein for asymmetric incorporation into planar bilayers [9].

When the hexokinase-binding protein is inserted into a planar bilayer, we observe the same properties of the channels as were found upon insertion of the mitochondrial porin. The rate of channel incorporation is proportional to the amount of the protein added to the aqueous phase. The isolated protein is more active in pore formation than is the same protein in the complete outer membrane. This does not seem to be caused by any difference in the pore structure or in the pore kinetics, but shows, as already discussed above, that the channel-forming structure has been purified in the preparation of the hexokinase-binding protein.

If the hexokinase-binding protein is added to one side (*cis*-side) of the membrane, the conductance is significantly higher when the *cis*-side is positive, whereas the pore partly switches off when the *cis*-side is negative. This finding and the observation of a slight anion selectively as well as the voltage-dependent change in conductance described by Colombini [6] in our eyes point to an asymmetric construction of the pore. The existence of a binding site for kinases exclusively on the outer surface of the pore would also require an asymmetric structure. However, no attempt has yet been made to exclude binding of kinases to the inner surface of the pore, which, however, was never observed physiologically [13,23].

An asymmetric structure of soluble as well as membrane-integrated proteins is probably of wide biological occurrence. Generally each different conformation of such proteins will have a different dipole moment because, for example, the distribution of charged groups depends on conformation. Each state of the protein has presumably a different value of dipole moment. Therefore, the energy associated with each state is differently affected by membrane voltage. A voltage-dependent change in conformation of the channel protein is indeed observed. It becomes manifest in planar bilayer experiments as a voltage-dependent change in conductance. In our experiments the pore is not completely closed at high voltages but seems to switch to a smaller cross-sectional diameter.

What function the voltage-dependent conformation change of the pore might serve is a matter of speculation at this time. On first consideration one naturally discusses the presence of a membrane potential across the outer membrane. In spite of the lower amount of pores in rat liver mitochondria supposed above, that number and the diameter of 2 nm is still too large to support any belief that an ion gradient can be established across the outer membrane. It seems more plausible to assume an intrinsic membrane potential in the outer membrane caused by asymmetry in surface charges. This has indeed been described by Hackenbrock [26] from electron-microscope investigations. He observed by binding of cationic ferritin that the inner surface of the outer membrane has more negative charges than the outer. In principle, the small fluctuations of the transmembrane potential needed for regulation of the channels could be caused by changes in metabolite concentrations and may therefore be influenced by the different metabolic states of the mitochondria. However, the observation of contacts between the outer membrane and the inner boundary membrane [27] may also be of relevance, because the number of contacts between both mmebranes is suggested to be regulated by the energetic state of the mitochondria [28]. In view of these results, it seems probable that the transmembrane potential is also influenced by the number of contacts between the two boundary membranes in correlation with the metabolic states of the mitochondria.

Two consequences of a conformational change of the pore protein must be considered with regard to the two functions of the protein. First, the binding of kinases to the outer surface of the protein might be influenced and, second, the permeability for large molecules would decrease when the pore switches to a smaller cross-sectional diameter.

The binding of hexokinase to mitochondria is probably a factor in the regulation of carbohydrate metabolism. This is suggested from the finding that insulin increases the binding of the enzyme to mitochondria [29,30] and that the bound enzyme has different kinetic properties [23,12,30,31]. The K_m of ATP is lower for the bound form and it was observed that the associated hexokinase preferentially utilizes internally generated ATP to form glucose 6-phosphate. In experiments employing radioactive isotopes, the hexokinase upon binding seemed to create a microcompartment facilitating direct exchange of ATP and ADP between the enzyme and the point of oxidative phosphorylation [12]. It was postulated by Bessman [32] that other kinases might also bind to the mitochondrial surface and be supplied with ATP in a manner similar to hexokinase. This proposal is strongly supported by the finding that the hexokinase-binding protein alternatively binds glycerol kinase with a comparable capacity.

The association of kinases to the binding protein would enable these enzymes to utilize newly synthesized ATP exclusively because the high-energy phosphate does not mix with the exogeneous pool of ATP before being used for phosphorylation [12]. It has been pointed out above that the two boundary membranes can have intimate contact which may serve to perform a compartmentation on the molecular level between the pore and binding protein in the outer membrane and the ATP/ADP translocator in the inner membrane [33]. This would allow direct exchange of ATP and ADP between the surface-bound kinase and the site of ATP generation at the inner mitochondrial membrane.

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