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*Minireview*

# The Cation-Selective Substate of the Mitochondrial Outer Membrane Pore: Single-Channel Conductance and Influence on Intermembrane and Peripheral Kinases

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The polyanion-induced substate of the outer mitochondrial membrane was studied *in vivo* and *in vitro*. Study of the substate in artificial bilayers showed that it is highly cation selective. The induction of the substate in intact mitochondria leads to a complete inhibition of the intermembrane kinases, such as creatine kinase and adenylate kinase, which were excluded from the external ATP pool. Peripheral kinases, such as hexokinase, were blocked when they utilized internal ATP. The results with intact mitochondria suggested the existence of two regions of the outer membrane containing channels of different states, which may be involved in the regulation of intermembrane and peripheral kinases.

**KEY WORDS:** Mitochondrial porin; mitochondrial outer membrane; mitochondrial kinase; nucleotide transport; reconstitution; lipid bilayer membrane.

## INTRODUCTION

The specific transport systems for mitochondrial metabolites are localized in the inner mitochondrial membrane. Therefore, it has been assumed that the outer membrane is freely permeable to these compounds and does not represent a permeability barrier for small hydrophilic solutes. Their permeation through the outer membrane occurs through a slightly anion-selective general diffusion pore (Colombini, 1979; Benz, 1985), which at a voltage above 20 to 30 mV switches in a substate, characterized by low conductance (Colombini, 1979; Roos *et al.*, 1982; Ludwig *et al.*, 1988). The latter state of the pore excludes ADP and ATP permeation through the outer membrane in intact mitochondria (Benz *et al.*, 1988, 1990). The exclusion of nucleotides is of considerable interest since mitochondrial porin has been identified as a specific binding protein for glycerol kinase (Fiek *et al.*, 1982) and hexokinase (Fiek *et al.*, 1982; Lindén *et al.*, 1982).

Binding of enzymes to the pore suggests that mitochondrial ATP may be channeled to the peripheral bound kinases (Gots and Bessman, 1974; Inui and Ishibashi, 1979, Brdiczka *et al.*, 1986). Further insight into the structural organization of channel and peripheral enzymes has been obtained by electron microscopy since it has been found that hexokinase is preferentially localized in the contact sites between the two mitochondrial boundary membranes (Weiler *et al.*, 1985; Ohlendiek *et al.*, 1986; Kottke *et al.*, 1988). Furthermore, contact sites obtained from rat brain mitochondria contained high activity of the mitochondrial creatine kinase (Kottke *et al.*, 1988). Obviously, the contact sites play an important role in the organization of kinases although the pore protein has been found to be randomly distributed all over the outer membrane. This result suggested that voltage-dependent regulatin of the pore conductivity (Colombini, 1979; Benz, 1985) may play an important role in the contact sites, especially if it is assumed that the

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inner membrane potential might be transduced to the outer membrane in the contact sites.

It has been described in a large number of previous publications that the mitochondrial pore switches to substates when the transmembrane potential exceeds 20 to 30 mV (Colombini, 1979; Roos *et al.*, 1982; Benz, 1985; De Pinto *et al.*, 1987; Ludwig *et al.*, 1988). The substate of *Paramecium* porin (and most likely also of other mitochondrial porins) is cation selective (Ludwig *et al.*, 1988). This could mean, for example, that neutral and cationic solutes are still permeable through the closed channel, while anionic solutes are impermeable. A partial closure of the channel would lead to a dynamic compartmentation (i.e., a compartmentation dependent on the energetic state of mitochondria) of adenine nucleotides at the mitochondrial surface (Brdiczka *et al.*, 1986; Gellerich *et al.*, 1987). As a consequence, the study of the cation-selective closed state or substate of the mitochondrial outer membrane channel is of considerable physiological interest. The properties of the substate may be investigated at high transmembrane potential. Another possibility is to study the closed state in reconstitution experiment in the presence of a synthetic polyanion, a copolymer of methacrylate maleate and styrene in a 1:2:3 proportion (König *et al.*, 1977, 1982; Colombini *et al.*, 1987; Benz *et al.*, 1988, 1990). The addition of polyanion results in a strong inhibition of the adenine nucleotide translocator in rat liver mitochondria (König *et al.*, 1977). Furthermore, the polyanion inhibited also, but with a different sensitivity, other inner membrane transport systems such as the oxoglutarate, dicarboxylate, and tricarboxylate carriers (König *et al.*, 1982). These data have been explained on the basis of a direct binding of polyanion to the carriers and an inhibition of their function.

The existence of the outer membrane pore in intact mitochondria with a defined exclusion limit around 2,500 Da (Benz, 1985) makes a direct interaction between the carriers and the polyanion of 10 kDa unlikely. Moreover, there is good evidence that the polyanion interacts with the outer membrane channel. Its primary action is presumably based on the shift of the voltage dependence of the mitochondrial porin (Colombini *et al.*, 1987; Benz *et al.*, 1988, 1990). The symthetic polyanion was also used for the study of peripheral and intermembrane kinases localized outside the inner membrane at the mitochondrial periphery. Of these kinases two groups can be defined with respect to location and to function: (i) energy-consuming kinases which bind to the outer membrane pore protein at the surface of the membrane. Examples are hexokinase and glycerolkinase (Fiek *et al.*, 1982). (ii) energy-transmitting kinases which are located between the two boundary membranes, for example adenylate kinase (Brdiczka *et al.*, 1968), nucleoside diphosphate kinase (Jacobus, 1985), and creatine kinase (Jacobs *et al.*, 1964). In accordance with the above arguments we postulate that the surface-bound as well as the intermembrane kinases have access to a different ATP/ADP pool between the two boundary membranes. We explain here the influence of the inner membrane potential on the outer membrane channel. Furthermore, the importance of the contact sites in this type of regulation will be emphasized by description of the preferential organization of seveal peripheral kinases in these sites as observed by biochemical investigations.

## EFFECTS OF POLYANION ON THE RECONSTITUTED MITOCHONDRIAL PORIN IN MULTICHANNEL EXPERIMENTS

Polyanion had a strong effect on reconstituted porin. When polyanion was added in a concentration up to about 10 $\mu$g/ml to the aqueous-phase side of the membrane (the cis-side), the current decreased when the cis-side was negative. No decrease was observed, on the other hand, when the membrane potential was positive on the cis-side. The sideness of the action of the polyanion on the mitochondrial porin was studied in more detail. Different potentials were applied to the cis-side of the membrane (the side of the addition of the polyanion) after equilibration. A 100 ng/ml polyanion concentration was already sufficient to observe a pronounced effect on the voltage dependence of the channel. When membrane potential had a negative sign on the cis-side, the membrane current already started to decrease at very small voltages on the order of 10–20 mV. Figure 1 shows an experiment of this type. Polyanion was added to the cis-side of a membrane in which rat liver porin was reconstituted. A membrane potential of +20 (upper trace) and −20 mV (lower trace) was applied to the membrane. Only in the latter case was a reduction of the current through the pores observed. The same voltage did not result in a similar reduction of the pore function in the absence of the polyanion. The polyanion stablized the pore in the open state if the sign of the transmembrane potential was positive on the cis-side. Even voltages up

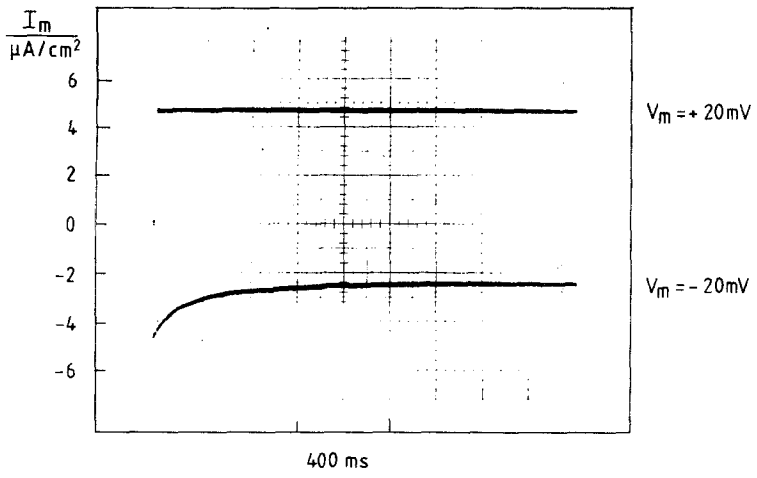


**Fig. 1.** Asymmetric current response of rat liver porin after application of a membrane potential of different sign. The membrane was made of asolectin/*n*-decane. The concentration of the porin was 100 ng/ml in the 1 M KCl solution. The cis-side contained in addition 1 μg/ml polyanion. A membrane potential of + 20 mV (upper trace) and − 20 mV (lower trace) was applied to the membrane (as referred to the cis-side) 40 min after the addition of the porin and 10 min after that of the polyanion. Note that the membrane current only decreased when the cis-side was negative; $T$ = 25°C. Taken from Benz *et al.* (1988), with permission.

to + 100 mV were not able to close the pores. Larger concentrations of the polyanion resulted in an even stronger shift of the voltage dependence of the rat liver porin. Figure 2 shows that − 5 mV applied to the cis-side was already sufficient to close the pore at a polyanion concentration of 0.3 μg/ml, whereas + 100 mV had again no influence on the membrane conductance. Similar effects of polyanion on the pore have been observed by Colombini *et al.*, (1987).

The asymetric effect of the polyanion on rat liver porin was stable during the whole lifetime of the membranes (up to several hours) indicating that the polyanion could not penetrate the channel. The addition of the polyanion to both sides of the membrane resulted in a bell-shaped voltage dependence at very small membrane potentials.

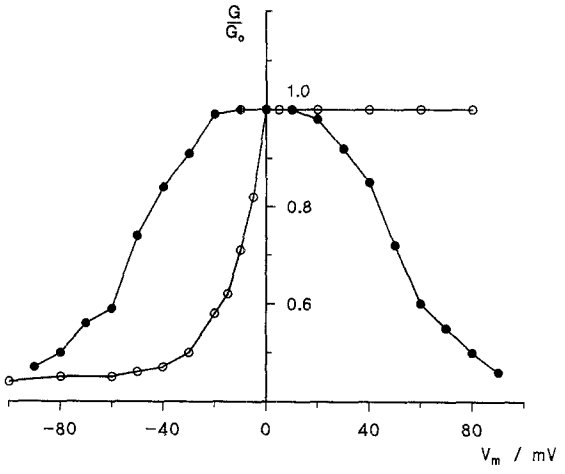


**Fig. 2.** Ratio of the conductance $G$ at a given membrane potential divided by the conductance $G_0$ at zero voltage given as a function of the applied membrane potential $V_m$. The membranes were formed of diphytanoyl phosphatidylcholin/*n*-decane. The aqueous phase contained 1 M KCl and 20 ng/ml rat liver porin (closed circles) or in addition 0.3 μg/ml polyanion added to the cis-side (open circles). The sign of the voltage is given with respect to the cis-side; $T$ = 25°C. Taken from Benz *et al.* (1990), with permission.

## INFLUENCE OF THE POLYANION ON THE SINGLE-CHANNEL CONDUCTANCE OF RAT LIVER PORIN

The multichannel experiments described above indicated that the polyanion had a strong influence on the pore characteristics. Similar conclusions have also been drawn from the data of patch-clamp experiments with whole mitochondria (Tedeschi *et al.*, 1987). Single-channel measurements were performed to characterize the influence of the polyanion in more detail. Figure 3 shows the influence of the polyanion on the conductance steps observed with rat liver porin in 1 M KCl. In Fig. 3A polyanion was added at a concentration of 0.1 μg/ml to the side of positive polarity only. In this case we observed the same single-channel conductance as in the absence of the polyanion. Figure 3B shows a similar experiment, but the polyanion was added in the same concentration to the opposite side of the membrane (the side with negative polarity). The single-channel conductance was about half that of Fig. 3A (and in the absence of polyanion). It is interesting to note that the single-channel conductance of the experiment in Fig. 3A was the same as observed in the absence of polyanion at higher voltage, i.e., above 50 mV (Benz *et al.*, 1988).

Similar measurements were performed with a variety of other salts (Benz *et al.*, 1990). The reduction of the single-channel conductance caused by the polyanion is especially large when a mobile anion (for

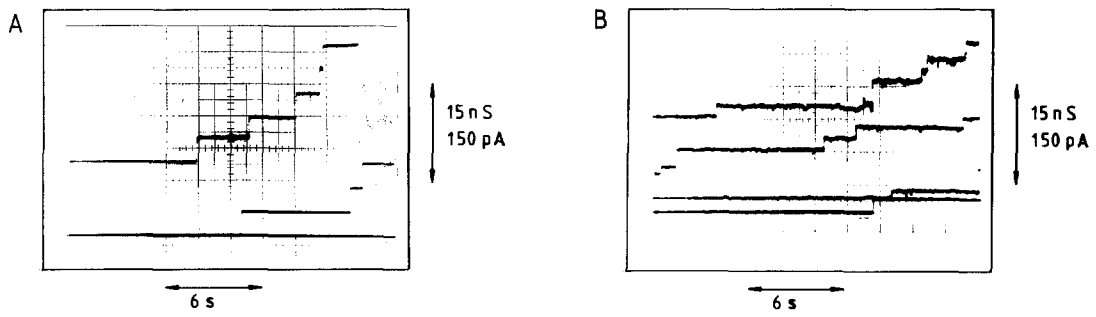


**Fig. 3.** Effect of polyanion on single-channel conductance of rat liver porin. 10 mV were applied to membranes formed fo diphytanoyl phosphatidylcholine/*n*-decane in 1 M KCl solution containing 2 ng/ml rat liver porin. (A) The aqueous phase contained 0.1 μg/ml polyanion on the side of positive polarity. (B) The aqueous phase contained the same concentration of polyanion on the side with negative polarity of another membrane; $T = 25°C$. Taken from Benz *et al.* (1990), with permission. Traces represent continuous current measurements, read from left to right, bottom to top.

example, chloride) was combined with a less mobile cation (for example, Tris). In this case the single-channel conductance was reduced to less than 10% of the open state value. The effect of the polyanion on combinations of mobile cations with less mobile anions (for instance, on KMES) was very small. This suggested that the slightly anion-selective channel in the open state becomes cation selective in the closed state (Benz *et al.*, 1990).

Polyanion is probably not directly bound to the channel interior but interacts with positive-gating charges located some distance away from the mouth of the channel. In fact, electron microscopic analysis of two-dimensional crystals of mitochondrial porin of *Neurospora crassa* (formed by phospholipase $A_2$ treatment of mitochondrial outer membranes) have shown that polyanion binds at the protein/lipid boundary along the channel exterior and decreases the channel size to 1.4 nm (Mannella and Guo, 1990). Furthermore, it has been shown that the volume of the channel is much smaller in the closed state than it is in the open state (Zimmerberg and Parsegian, 1986).

## INFLUENCE OF POLYANION ON THE ION SELECTIVITY OF MITOCHONDRIAL PORIN

The results presented above suggest that the closed state or substate of the pore was cation selective. To quantify the cation selectivity, the following experiments were performed. Rat liver porin was reconstituted into a lipid bilayer membrane. Then the KCl concentration at one side of the membrane was increased by a factor of 6.4 from 50 to 320 mM. The zero-current membrane potential ($V_m$) was negative by 12 mV on the more dilue side of the membrane, which means that the channel was anion selective, i.e., anions moved preferentially through the pore (Roos *et al.*, 1982). Then, increasing concentrations of polyanion were added to both sides of the membrane. Figure 4 illustrates the influence of increasing concentrations of polyanion on the zero-current membrane potential in such an experiment. The membrane was first slightly anion selective (permeability ratio $P_c/P_a = 0.52$, calculated from the Goldman–Hodgkin–Katz equation (Benz *et al.*, 1979) then switched to highly cation selective in a dose-dependent fashion.

## INHIBITION OF THE ADENINE NUCLEOTIDE PERMEATION ACROSS THE MITOCHONDRIAL OUTER MEMBRANE

Rat liver mitochondria suspended in sucrose medium were incubated with different concentrations of the polyanion in the presence of 4 mM $MgCl_2$. The mitochondria were centrifuged and resuspended in the original volume of sucrose medium to remove the unbound polyanion. A concentration of 150 μg/ml polyanion was sufficient to inhibit 80% of the adenylate kinase activity located in the intermembrane space (Fig. 5). However, increasing adenylate kinase activity was obtained after the addition of increasing concentrations of digitonin. This detergent is known to disrupt only the mitochondrial outer membrane. At high concentrations of digitonin (above 100 μg per mg protein) the adenylate kinase activity had 80% of its initial activity. The full activity was obtained after the addition of Triton X-100 to the mitochondria. These results indicated that the adenine nucleotide exchange

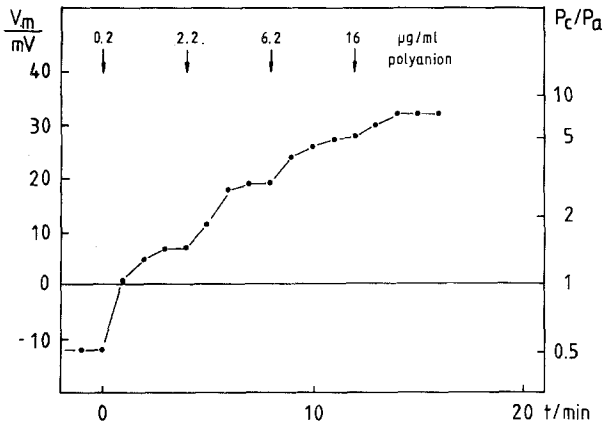


**Fig. 4.** Influence of the polyanion on the selectivity of rat liver porin. A membrane was formed of diphytanoyl phosphatidylcholine:*n*-decane in a solution containing 50 mM KCL and 20 ng/ml rat liver porin. After reconstitution of about 300 pores the concentration of KCl on one side of the membrane was raised to 320 mM and the instrumentation was switched to the measurement of zero-current potentials. The 6.4-fold concentration gradient of KCl resulted initially in a negative potential on the more dilute side. Increasing concentrations of the polyanion (as indicated at the top of the figure) shifted the potential to positive values. Simultaneously, the permeability ratio as calculated from the Goldman–Hodgkin–Katz equation (Benz *et al.*, 1979) changed to favor the cation. Taken from Benz *et al.* (1990), with permission.

between the outer mitochondrial compartment and the intermembrane space was completely blocked by the addition of the polyanion. It is interesting to note that the polyanion inhibited also state 3 respiration in a dose-dependent fashion (Benz *et al.*, 1988). However, whereas the adenylete kinase was fully reversible after the addition of digitonin, the state 3 respiration could not be reestablished using the same procedure as described above for the adenylate kinase (i.e., the addition of detergents).

The exclusion of adenine nucleotide permeation through the pore in the low-conductance state can be explained by the observation that the pore became cation selective in this state (Fig. 4). Furthermore, polyanion had little effect on the single-channel conductance of a salt composed of a mobile cation and a less mobile anion (i.e., KMES) while it had a dramatic effect on the opposite combination (a mobile anion and a less mobile cation, i.e., TrisCl) and reduced in this case the single-channel conductance by at least a factor of 10. This means that anions used in our experimental approach have a very small permeability through the closed channel. ATP and ADP have a larger molecular mass and a higher charge density. They are probably excluded from the channel in the closed state *in vivo* and *in vitro*.

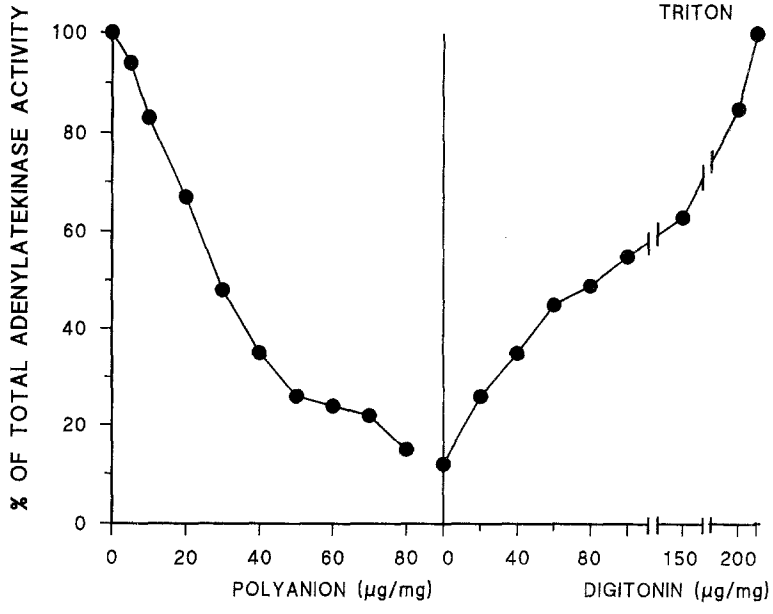


**Fig. 5.** Inhibition of adenylate kinase activity by the polyanion and reactivation of the enzyme by digitonin. Rat liver mitochondria were incubated for 5 min at room temperature with increasing concentrations of the polyanion in sucrose medium containing 5 mM $MgCl_2$. The mitochondria were subsequently pelleted and resuspended in the same volume with sucrose medium. 100 µl aliquots of mitochondria pretreated with 150 µg/ml polyanion were incubated for 30 s at room temperature with different concentrations of digitonin. Adenylate kinase activity was determined immediately after incubation in sucrose medium within 10 µl of the mitochondrial samples. Taken from Benz *et al.* (1988), with permission.

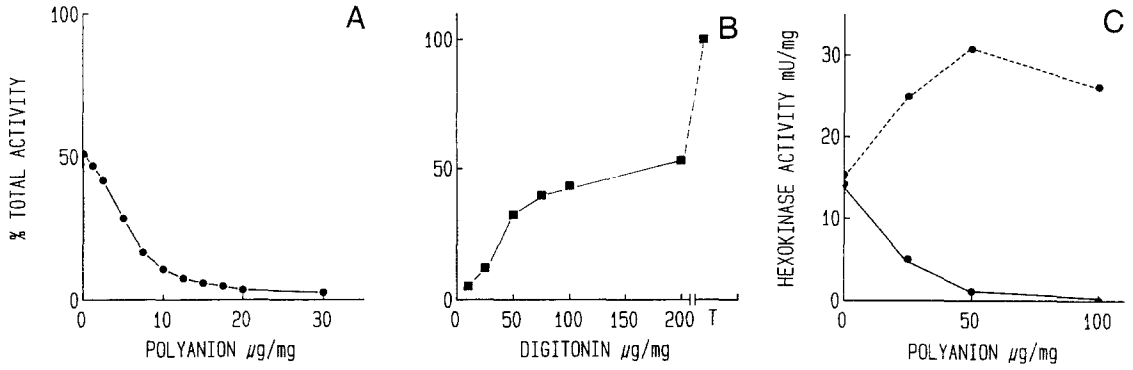


**Fig. 6.** Inhibition of mitochondrial creatine kinase by the polyanion and reactivation of the enzyme by digitonin. (A) Brain mitochondria were incubated with increasing concentrations of the polyanion. The mitochondria were subsequently sedimented, resuspended in the same volume of sucrose medium, and subjected to determination of enzyme activity. (B) Aliquots of mitochondria pretreated with 30 µg polyanion per mg protein were incubated with different concentrations of digitonin, and creatine kinase activity was determined immediately after 30 s incubation in sucrose medium within 20 µl of the digitonin-treated samples. One sample was treated with 1% Triton X-100 (T) to determine the total activity. (C) Rat liver mitochondria were incubated with isolated hexokinase I, centrifuged, and activity of hexokinase was analyzed in the presence of 2 mM external ATP (dashed line) or endogenous ATP (solid line) provided by the oxidative phosphorylation in the presence of 5 mM succinate, 5 mM phosphate, and 0.5 mM ADP. The data points are mean values of four experiments. Taken from Benz *et al.* (1990), with permission.

## LOCALIZATION OF PERIPHERAL KINASES AS ANALYZED BY INHIBITION OF THE OUTER MEMBANE TRANSPORT

The organization of the mitochondrial creatine kinase (Fig. 6A, B) was studied in comparison to the peripheral bound hexokinase (Fig. 6C) by using polyanion. The activity of the kinases was determined in isolation medium to keep the structure of the freshly isolated brain and liver mitochondria intact. In contrast to adenylate kinase, only 50% of the total (Triton X-100 extractable) kinase activity was latent in brain mitochondria without inhibition of the mitochondrial porin by polyanion. However, the freely accessible creatine kinase activity became inhibited after preincubation of mitochondria with 30 µg polyanion per mg protein (Fig. 6A). This inhibition was not due to a direct interaction between polyanion and enzyme and an inhibition of enzyme reaction, because the activity was completely regained after disruption of the outer membrane by addition of 200 µg digitonin per mg protein (Fig. 6B).

The results suggested that the mitochondrial creatine kinase was excluded from its substrate ATP since it is localized in the intermembrane space between the inner and outer membrane. Furthermore, it is possible that a large concentration of creatine kinase is localized in the contact sites, whereas adenylate kinase activity appeared to reside beyond these sites. To understand the meaning of this organization, we need to consider two facts, namely that the pore protein is randomly distributed (Ohlendiek *et al.*, 1986; Adams *et al.*, 1989) in the outer membrane and that the pore adopts a low-conductance state at a voltage above 30 mV (Colombini, 1979; Roos *et al.*, 1982). This interpretation was completely consistent with the observation that glucose phosphorylation by peripherally bound hexokinase using internal ATP (generated by oxidative phosphorylation) was completely inhibited in the presence of polyanion (Fig. 6C) while polyanion was ineffective when the ATP was added to the outside. In principle, it could not be distinguished from the data whether polyanion inhibited the oxidative phosphorylation, as has been postulated by König *et al.*, (1977), or the export of ATP.

Assuming that the polyanion switched the pore into its low-conductance state (Colombini *et al.*, 1987; Benz *et al.*, 1988), it was concluded from these experiments that the substate of the pore excluded transport of adenine nucleotides across the outer membrane (Benz *et al.*, 1988). In addition, these experiments showed that no transport system other than the pore can contribute significantly to the adenine nucleotide transport across the outer membrane. This conclusion is valid because of several reasons. Firstly, the free kinases were not influenced by the polyanion, provided that the $Mg^{2+}$ concentration was at least 10 mM. Secondly, digitonin or Triton X-100 did

not interact with the polyanion. This was tested by separate experiments (Benz *et al.*, 1990). It did not matter how the outer membrane barrier was disrupted either by digitonin, by Triton X-100, or by ultrasonification.

## TWO POPULATIONS OF DIFFERENTLY REGULATED PORES AT THE MITOCHONDRIAL PERIPHERY

In view of the above, it appeared rather likely that two states of the pores might be coexistent in the outer membrane also under *in vivo* conditions. The pores localized inside the contacts may be influenced by the inner membrane potential (Brdiczka *et al.*, 1989; Benz *et al.*, 1990) and are in the ATP-impermeable closed state, which means that they are regulated. Other pores outside the contacts would be unregulated and are in the open state. As a consequence, 50% of the creatine kinase activity was latent event in the absence of polyanion, whereas adenylate kinase showed no latency, and was readily accessible by digitonin treatment after inhibition with polyanion (Benz *et al.*, 1988, Fig 5). The compartment at the mitochondrial periphery performed by the regulation of the outer membrane pore is a dynamic process, because the formation of contacts appeared to correlate with the rate of oxidative phosphorylation (Knoll and Brdiczka, 1982). We have recently suggested that the channeling of mitochondrial phosphorylation energy (as creatine phosphate via creatine kinase) is especially relevant in the presence of glucose when the glycolytic pathway provides the two metabolites (1,3-diphospho glycerate and phosphoenol pyruvate) which have the highest free energy in the cell, and, which would thus compete for the cytosolic ADP (Brdiczka *et al.*, 1989).

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