Volume 231, number 1, 75–80 FEB 05741 April 1988

Inhibition of adenine nucleotide transport through the mitochondrial porin by a synthetic polyanion

Roland Benz, Lech Wojtczak[+], Waltraud Bosch* and Dieter Brdiczka*

Lehrstuhl für Biotechnologie, Universität Würzburg, Röntgenring 11, D-8700 Würzburg, FRG, [+]Nencki Institute of Experimental Biology, PL-02-093 Warszawa, Poland and **Fakultät für Biologie, Universität Konstanz, D-7750 Konstanz, FRG*

Received 25 January 1988

The effect of a synthetic polyanion of M_r 10000 (a copolymer of methacrylate, maleate and styrene in a 1:2:3 proportion) was studied on isolated rat liver mitochondria and on mitochondrial porin reconstituted into lipid bilayer membranes. Increasing concentrations of the polyanion inhibited the adenyl kinase located between both mitochrondrial membranes in a dose-dependent fashion. Upon addition of the detergent digitonin in increasing concentrations the adenyl kinase activity was fully reversible. In reconstitution experiments with mitochondrial porin the polyanion increased the voltage dependence of the pore in such a way that the pore is switched into the closed state at much smaller voltages than in the absence of the polyanion. The asymmetric addition of the polyanion resulted in an asymmetric shift of the voltage-dependence of the pore. If the voltage is negative at the *cis*-side (the side of the addition of the polyanion) the pore closed rapidly whereas it was always open for potentials of opposite polarity. The results are discussed on the basis of a modification of the gate properties of the mitochondrial porin by the polyanion and by the assumption that the closed state of the pore is not permeable for nucleotides.

Nucleotide transport; Mitochondrial porin; Lipid bilayer; (Rat liver, Mitochondrial outer membrane)

1. INTRODUCTION

The mitochondrial outer membrane seems to be freely permeable to a variety of hydrophilic solutes [1,2] such as the substrates of the oxidative phosphorylation and ions. On the other hand, it is impermeable to molecules with higher molecular masses [3,4]. This result indicated that the outer mitochondrial membrane contains a defined pathway for the mostly anionic substrates of mitochondrial metabolism. Pore-forming activity has been found in a crude extract of *Paramecium* mitochondria [5] and subsequently pore-forming proteins (named mitochondrial porin [6,7] or VDAC [5,8]) have been isolated and purified from mitochondrial outer membranes of different eukaryotic cells [6–16]. Mitochondrial porins form general diffusion pores in the mitochondrial outer membrane which share some similarities with the pores formed by bacterial porins [6,7,10,14–16].

However, in contrast to the pores formed by bacterial porin, the mitochondrial pore switches to substates if the voltage across the membrane exceeds values of 20–30 mV [5,7–16]. The substates have a reduced permeability towards ions and most likely also towards the substrates of oxidative phosphorylation. This could mean that a potential across the mitochondrial outer membrane could control the mitochondrial metabolism. Evidence for such a control is not directly given in the literature. On the other hand, König et al. [17] have reported experiments with a polyanion of M_r 10000 (a copolymer of methacrylate, maleate and styrene in a 1:2:3 proportion) which has suggested strong inhibition of the adenine nucleotide translocator in rat liver mitochondria. Furthermore, the polyanion inhibited also, but with a different sensitivity, other inner membrane transport systems

Correspondence address: R. Benz, Lehrstuhl für Biotechnologie, Universität Würzburg, Röntgenring 11, D-8700 Würzburg, FRG

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

such as the oxoglutarate, dicarboxylate and tricarboxylate carriers [18]. These data have been explained on the basis of a direct interaction between polyanion and the carriers.

The existence of the outer membrane pore with a defined exclusion limit around 2.5 kDa makes a direct interaction between the carriers and the polyanion of M_r 10000 questionable. Although a direct interaction with the carriers and the polyanion cannot be excluded it seems more likely that the polyanion interacts with an outer membrane component, most likely the pore, as has been suggested by patch-clamp experiments and studies of the porin channel [19,20]. We therefore investigated the effect of the polyanion on the transport across the outer membrane in in vivo and in vitro experiments. The first assay was done by analyzing the activity of adenylate kinase located between the two mitochondrial membranes as a function of the polyanion concentration. In a second set of experimental conditions we studied the effect of the polyanion on the properties of the pore reconstituted into artificial lipid bilayer membranes.

2. MATERIALS AND METHODS

2.1. *Isolation of mitochondria and of the mitochondrial porin*

Female rats of the Chbb-THOM strain (200–250 g body wt) were fed with a standard diet of Altromin (Lage, FRG). Mitochondria were isolated from rat liver by differential centrifugation in a medium containing 0.25 M sucrose, 0.1 mM EGTA and 10 mM Hepes (pH 7.4) (sucrose medium) as described [21]. The mitochondrial porin was isolated as described [13].

2.2. *Assays*

Adenylate kinase (EC 2.7.4.3) was determined photometrically in the presence of rotenone and oligomycin as described [21]. To keep the mitochondrial structure intact, the assay was performed in sucrose medium.

2.3. *Treatment of isolated mitochondria with the polyanion*

The mitochondrial fraction contained a protein concentration of 30 mg/ml. Aliquots of 0.2 ml of this mitochondrial suspension were incubated for 5 min at room temperature with concentrations of the polyanion (a kind gift of Tamas König [17]) ranging from 5 to 200 μg/ml. The suspension was subsequently centrifuged for 1 min in a tabletop centrifuge to remove the unbound polyanion. The supernatant was then removed and the sediment was resuspended in the original volume of sucrose medium in the absence of the polyanion.

2.4. *Treatment of mitochondria with digitonin*

Mitochondria were treated with the polyanion as described above and resuspended in sucrose medium. The mitochondrial suspension contained a total protein concentration of 30 mg/ml. Aliquots of 0.2 ml of this mitochondrial suspension were incubated for 30 s at room temperature with concentrations of digitonin ranging from 10 to 200 μg/mg of total protein. Aliquots of the suspension were directly used to determine the adenylate kinase activity and oxidation rates. This resulted in a dilution of digitonin in the enzyme assays and in the oxygen electrode by a factor of 100 and 20, respectively.

2.5. *Lipid bilayer experiments*

Black lipid bilayer membranes were formed as described [22]. The instrumentation consisted of a Teflon chamber with two aqueous compartments connected by small circular holes. The holes had a surface area of either 1 mm^2 (for the multi-channel measurements) or 0.1 mm^2 (in the case of the single-channel experiments). Membranes were formed across the holes by painting on a 1% solution of soybean lecithin (asolectin) (Avanti Biochemicals, Birmingham, AL) in *n*-decane. Bilayer formation was completed when the membrane turned optically black to reflected light. The aqueous 1 M KCl solutions (Merck, Darmstadt, FRG) were unbuffered (pH around 6.5) or were buffered with 10 mM Mops (pH 7). The mitochondrial porin [10] was added from the concentrated stock solution either to the aqueous phase bathing a membrane in the black state or immediately prior to membrane formation. The temperature was maintained at 25°C throughout.

The membrane current was measured with a pair of calomel electrodes switched in series with a voltage source and an electrometer (Keithley 602). In the case of the single channel recordings the electrometer was replaced by a Keithley 427 current amplifier. The amplified signal was monitored with a storage oscilloscope (Tektronix 5115) and recorded with a tape or a strip chart recorder. The orientation of the voltage was defined with respect to the addition of the polyanion (the *cis*-side). A *trans*-negative potential (indicated by a minus sign) means that a negative potential was applied to the compartment opposite to the addition of the polyanion.

3. RESULTS

3.1. *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. To keep the mitochondria intact, adenylate kinase activity was determined in the same medium. A concentration of 150 μg/ml of the polyanion was sufficient to inhibit 80% of the adenylate kinase activity located in the intermembrane space (fig.1). However, the adenylate kinase activity was again measurable after the addition of increasing concentrations of the detergent digitonin which is

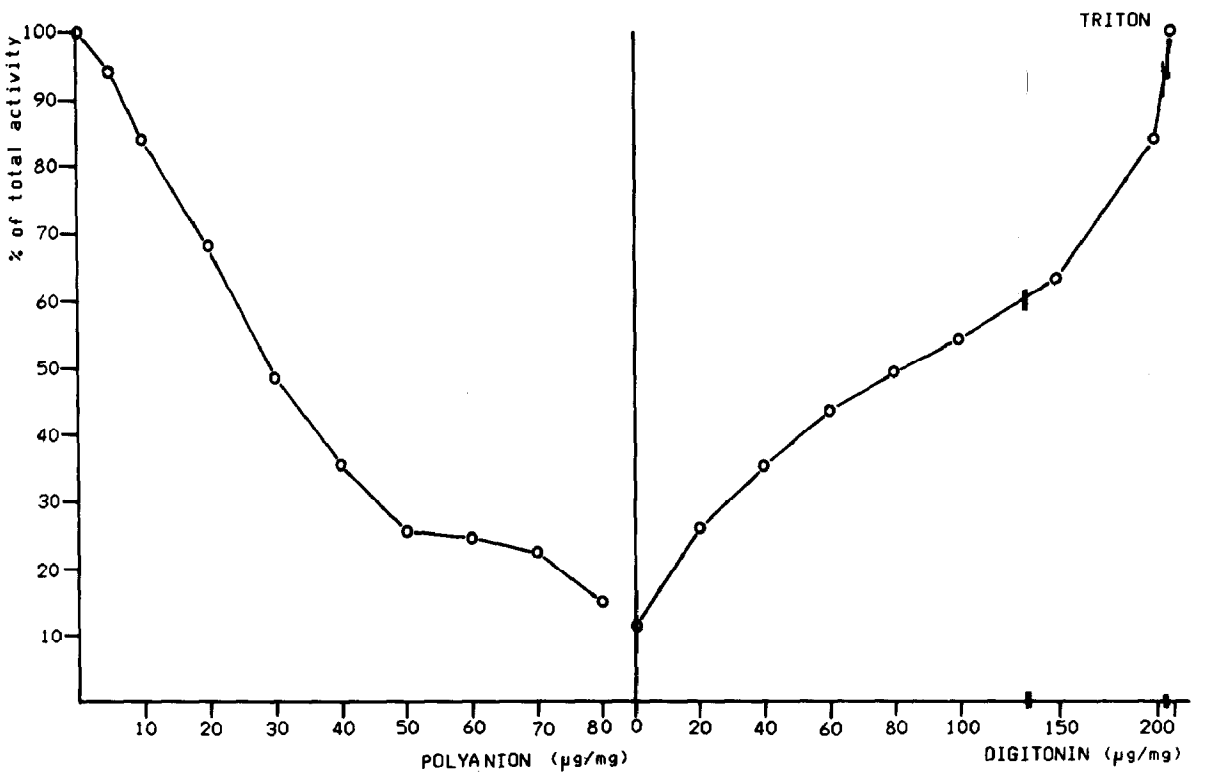

Fig.1. Inhibition of adenylate kinase activity by the polyanion and reactivation of the enzyme by digitonin. Rat liver mitochondria were incubated for 5 min at room temperature with increasing concentrations of the polyanion in sucrose medium containing 5 mM $MgCl_2$. The mitochondria were subsequently 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.

known to disrupt the mitochondrial outer membrane. At high concentrations of digitonin (above 100 µg/mg) the adenylate kinase was almost completely liberated from the intermembrane space and had 80% of its initial activity. The rest of the adenylate kinase activity was obtained after the addition of Triton X-100 to the mitochondria. These results indicated that the adenine nucleotide exchange between the outer mitochondrial compartment and the intermembrane space was blocked by the addition of the polyanion and that the adenylate kinase activity could indirectly be reduced by inhibition of the nucleotide transport through the outer membrane pore. It is interesting to note that the polyanion inhibited also state respiration in a dose-dependent fashion. However, whereas the adenylate kinase was fully reversible after the addition of digitonin, the state 3 respiration could not be reestablished using the same procedure.

3.2. *Effects of the polyanion on the mitochondrial pore reconstituted into lipid bilayer membranes*

The effect of the polyanion on the adenylate kinase and state 3 respiration may be explained by a direct interaction between the polyanion and the adenylate kinase or the inner mitochondrial membrane, respectively. On the other hand, the permeation of the polyanion through the outer mitochondrial membrane is questionable because of its limited permeability towards large hydrophilic solutes [3,4]. To test whether this effect was due to an interaction between the mitochondrial pore and the polyanion we performed lipid bilayer experiments in the presence of rat liver porin.

After the reconstitution of the porin polyanion was added in a concentration of 1 μg/ml to the *cis*-side. If the membrane potential had a negative sign on the *cis*-side, the membrane current decreased to half of its initial value (see fig.2). No decrease was observed on the other hand if the membrane potential was positive on the *cis*-side.

The sidedness of the action of the polyanion on the mitochondrial porin was studied in more detail. Fig.3 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 stabilized the pore in the open state if the sign of the transmembrane potential was positive on the *cis*-side. Even voltages up 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.

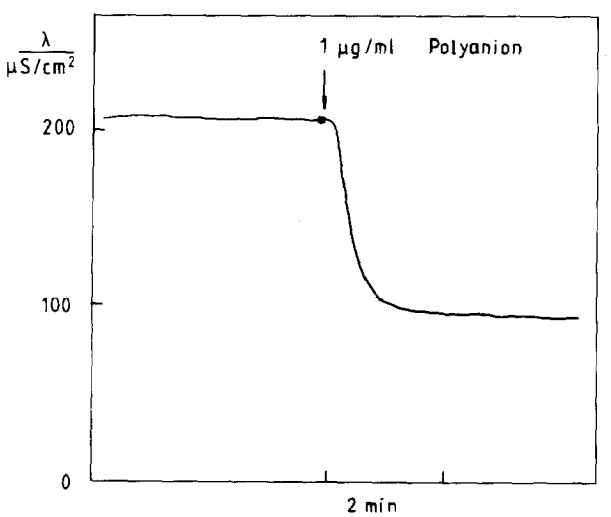

Fig.2. Inhibition of rat liver mitochondrial porin by the polyanion. 100 ng/ml rat liver porin was added to the 1 M KCl solution bathing a membrane from asolectin/*n*-decane. 30 min after the addition of the porin the membrane conductance was stationary and the polyanion was added in a concentration of 1 μg/ml to the *cis*-side of the membrane. The membrane potential was −20 mV (with respect to the *cis*-side). Note that the addition of the polyanion to the other side had no influence on the membrane conductance; T = 25°C.

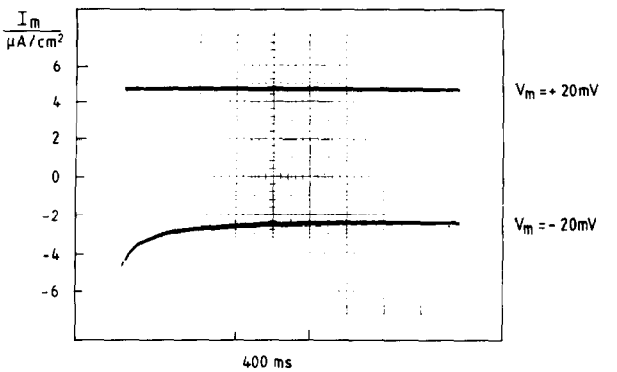

Fig.3. Asymmetric current response of rat liver porin after application of a membrane potential of different sign. The membrane was made of asolectin/*n*-decane. The concentration of the porin was 100 ng/ml in the 1 M KCl solution. The *cis*-side contained in addition 1 μg/ml polyanion. A membrane potential 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 if the *cis*-side was negative; T = 25°C.

−5 mV at the *cis*-side were already sufficient to close the pore at a polyanion concentration of 10 μg/ml whereas +100 mV had again no influence on the membrane conductance.

3.4. *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. Single-channel measurements were performed to characterize the influence of the polyanion in more detail. The single-channel conductance of rat liver porin used in this study was found to be identical to that which has been published earlier for small voltages [10] (see fig.4A). The influence of the polyanion was again dependent on the sign of the membrane potential at the *cis*-side, the side of the addition of the polyanion. Fig.4B shows a histogram of the conductance fluctuations observed with rat liver porin in which the *cis*-side (1 μg/ml polyanion) was positive by 10 mV. The size of the conductance fluctuations was equal to those of the control experiments. The size of the fluctuations decreased to about one half if the *cis*-side was negative (fig.4C). Such decrease of the single channel conductances was found to be identical to that observed at much higher voltages in the absence of

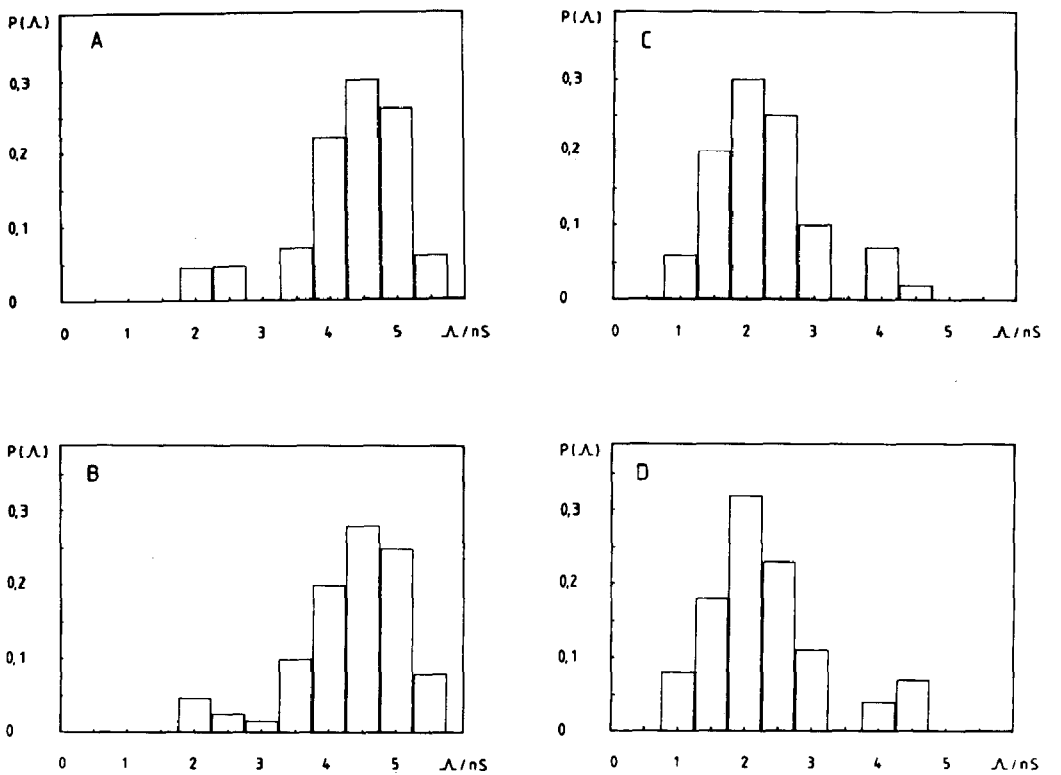

Fig.4. Histograms of conductance fluctuations observed with rat liver porin in the presence and absence of the polyanion. (A) Control (no polyanion); Λ = 4.1 nS for 195 single events, V_m = 10 mV. (B) The polyanion was added in a concentration of 1 μg/ml to the *cis*-side; Λ = 4.2 nS for 162 single events; V_m = +10 mV. (C) Same conditions; Λ =2.3 nS for 210 single events; V_m = −10 mV. (D) Control (no polyanion); Λ = 2.4 nS for 153 single events; V_m = 50 mV.

the polyanion (V_m = 50 mV, fig.4D). These results indicated that the polyanion did not simply block the channel but shifted its voltage-dependence to smaller voltages if the sign was negative at the *cis*-side. For voltages positive at the *cis*-side the mitochondrial porin was always in the open state.

4. DISCUSSION

Our results clearly showed that the effects of the polyanion on the mitochondrial metabolism which has been described previously [17,18] have nothing to do with a direct inhibition of the carrier proteins located in the mitochondrial inner membrane but are most likely caused by an interaction between mitochondrial porin and polyanion. Similar conclusions have also been drawn from the data of patch-clamp experiments with whole mitochondria [19]. The different concentration ranges of these effects are most likely caused by the formation of different microcompartments at the mitochondrial surface [23]. This could mean that the pores located in different regions of the mitochondrial outer membrane (i.e. inside and outside of the contact sites [24]) may need different concentrations of the polyanion for the shift into the closed state. This seems plausible because the asymmetry of the membrane is different at different zones of the surface either caused by intrinsic asymmetries of the membrane or by changes of the surface potentials on both sides of the membrane [10,23].

We also showed in this study that the influence of the polyanion on the mitochondrial porin may be explained by a shift of its voltage-dependence. Increasing concentrations of the polyanion resulted in a closure of the pore at much smaller membrane potentials negative at the *cis*-side. Large membrane potentials positive at the *cis*-side no longer closed the pore, which may indicate that the polyanion interacted with the gate of the

mitochondrial porin, leaving the other pore characteristics unchanged. The polyanion closed the mitochondrial pore in the experiments with intact mitochondria. This may be explained by the assumption that the surface of the mitochondria is more negative as compared to the intermembrane space, a result which is in accordance with the published literature [16].

In previous publications [10,15,16,23] we have suggested that the closed state of the mitochondrial porin may be part of the control of mitochondrial metabolism. This would only be possible if this state has a different permeability and selectivity than the open state. In fact, our data provided the first evidence that the closed state of the mitochondrial pore was impermeable for ATP and ADP because the adenylate kinase activity could be blocked in a reversible way. This means that a voltage-dependent closure of the pore could stop mitochondrial metabolism in the presence of the polyanion as has been observed in previous studies [17,18]. This result would also agree with the observation that the closed state of the mitochondrial porin is cation-selective [14] while the open state is weakly anion selective if cation and anion have the same mobility in the aqueous phase [14].

Acknowledgements: The authors would like to thank Tamas König for the kind gift of the polyanion, Otto Ludwig for his help during the early stages of this work and Angela Schmid for expert technical assistance. This work was supported by grants from the Deutsche Forschungsgemeinschaft (Project B9 of the Sonderforschungsbereich 176, Be 865/3-3, and Br 773/1-4).

REFERENCES

[1] O'Brien, R.L. and Brierly, G. (1965) J. Biol. Chem. 240, 4527–4538.
[2] Pfaff, E., Klingenberg, M., Ritt, E. and Vogell, W. (1968) Eur. J. Biochem. 5, 222–232.
[3] Wojtczak, L. and Zaluska, H. (1969) Biochim. Biophys. Acta 193, 64–72.
[4] Wojtczak, L. and Sottocasa, G.L. (1972) J. Membr. Biol. 7, 313–324.
[5] Schein, S.J., Colombini, M. and Finkelstein, A. (1976) J. Membr. Biol. 30, 99–120.
[6] Zalman, L.S., Nikaido, H. and Kagawa, Y. (1980) J. Biol. Chem. 255, 1771–1774.
[7] Freitag, H., Neupert, W. and Benz, R. (1982) Eur. J. Biochem. 123, 629–636.
[8] Colombini, M. (1979) Nature 279, 643–645.
[9] Colombini, M. (1980) J. Membr. Biol. 53, 79–84.
[10] Roos, N., Benz, R. and Brdiczka, D. (1982) Biochim. Biophys. Acta 686, 629–636.
[11] De Pinto, V., Tommasino, M., Benz, R. and Palmieri, F. (1985) Biochim. Biophys. Acta 813, 230–242.
[12] Ludwig, O., De Pinto, V., Palmieri, V. and Benz, R. (1986) Biochim. Biophys. Acta 860, 268–276.
[13] De Pinto, V., Ludwig, O., Krause, J., Benz, R. and Palmieri, F. (1987) Biochim. Biophys. Acta, in press.
[14] Ludwig, O., Krause, J., Hay, R. and Benz, R. (1987) Eur. Biophys. J., in press.
[15] Benz, R., Ludwig, O., De Pinto, V. and Palmieri, F. (1985) in: Achievements and Perspectives of Mitochondrial Research, vol.1: Bioenergetics (Quagliariello, E. et al. eds) pp.317–327, Elsevier, Amsterdam.
[16] Benz, R. (1985) CRC Crit. Rev. Biochem. 19, 145–190.
[17] König, T., Kocsis, B., Meszarols, L., Nahm, K., Zoltan, S. and Horvath, I. (1977) Biochim. Biophys. Acta 462, 380–389.
[18] König, T., Stipani, I., Horvath, I. and Palmieri, F. (1982) J. Bioenerg. Biomembr. 14, 297–305.
[19] Tedeschi, H., Mannella, C.A. and Bowman, C.L. (1987) J. Membrane Biol. 97, 21–29.
[20] Colombini, M., Yeung, C.L., Tung, J. and König, T. (1987) Biochim. Biophys. Acta 905, 270–286.
[21] Brdiczka, D., Pette, D., Brunner, G. and Miller, F. (1968) Eur. J. Biochem. 5, 294–304.
[22] Benz, R., Janko, K., Boos, W. and Läuger, P. (1978) Biochim. Biophys. Acta 511, 305–319.
[23] Brdiczka, D., Knoll, G., Riesinger, I., Weiler, U., Klug, G., Benz, R. and Krause, J. (1986) in: Myocardial and Skeletal Muscle Bioenergetics (Brautbar, N. ed.) pp.55–70, Plenum, New York.
[24] Ohlendieck, K., Riesinger, I., Adams, V., Krause, J. and Brdiczka, D. (1986) Biochim. Biophys. Acta 860, 672–689.