Immunoelectron Microscopic Study of the Distribution of Porin on Outer Membranes of Rat Heart Mitochondria

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The distribution of porin on the outer membranes of rat heart mitochondria has been studied by means of immunogold labelling with antibodies to the N-terminal part of the human protein. It was found that only a minority of isolated, unfixed mitochondria are labelled by these antibodies, with the gold particles frequently organized in threads or bands. Extensive immunogold labelling is frequently observed on regions of outer membranes stripped away from mitochondria and on regions separating two mitochondrial compartments whose cristae display different configurations (possibly representing two mitoplasts covered by a common outer membrane). Also, pairs of connected mitochondria are sometimes heavily labelled in the "neck" regions, which may represent the junctions involved in electrical communication between mitochondria in cardiac tissue.

KEY WORDS: Mitochondria; electron microscopy; immunolabelling; porin; VDAC.

INTRODUCTION

The mitochondrial outer membrane contains numerous copies (10^3–10^4/μm^2) of a 31-kD porin protein referred to as VDAC, for voltage-dependent, anion-selective channel (see review, Mannella *et al.*, 1992). In spite of numerous *in vitro* studies on these pore-forming proteins, little is known about their physiological function(s). It is widely presumed that VDAC represents the main permeability pathway for metabolites through the outer mitochondrial membrane. Supporting evidence for this role is that disruption of the VDAC gene in yeast results in impaired cell growth and the induction of another porin-like channel (Dihanich *et al.*, 1989). However, there are recent indications that VDAC's physiological role may be more complex. It is now known that there are multiple human VDAC genes which may be differentially expressed in different cells (Blachly-Dyson *et al.*, 1993, 1994), suggesting heterogeneity in function and/or targetting of the channel proteins. For example, there is considerable evidence that VDAC is located on the plasma membrane of several types of mammalian cells (Thinnes, 1992). Also, there have been suggestions that VDAC might be involved in specialized intermembrane communication, such as between outer and inner membranes within mitochondria (McEnery *et al.*, 1992) or between the outer membranes of different mitochondria (Skulachev, 1990).

One approach to address the issue of possible specialized functions of the VDAC protein involves studying its lateral distribution on the mitochondrial surface. In this paper, the results of indirect immunogold labelling of the porin protein on isolated, negatively stained rat heart mitochondria is described.

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MATERIALS AND METHODS

Isolation of Rat Heart Mitochondria: Intactness of Outer Membranes

Mitochondria were prepared from hearts of Wistar rats (for details, see Bowman and Tedeschi, 1984). For immunolabelling experiments, mitochondria were stored on ice in isolation medium (70 mM sucrose, 230 mM mannitol, 0.3% bovine serum albumin (BSA), 5 mM HEPES adjusted to pH 7.4 by KOH) and used within a few hours of isolation.

Integrity of the outer membrane of mitochondria used in these experiments was determined using a cytochrome c accessibility assay (Douce et al., 1973). Since holocytochrome c cannot diffuse across intact outer membranes, the ratio of the initial rate of succinate : cytochrome c oxidoreductase activity by the mitochondria to that of the same mitochondria after deliberate lysis of their outer membranes is proportional to the extent of damage to the outer membranes incurred during mitochondrial isolation. The reaction was run at room temperature in 3 ml of buffer containing equal concentrations of unpretreated or water-lysed mitochondria (approximately 50 μg protein), 70 μM NaCN, 40 μM oxidized cytochrome c, 70 mM sucrose, 230 mM mannitol, and 5 mM HEPES (pH 7.4). The reaction was started by the addition of 6 mM sodium succinate and monitored at 550 nm with an Ultraspec III spectrophotometer (Pharmacia LKB Biotechnology, Piscataway, New Jersey).

Immunoblotting Experiments

a. Electrophoresis and Electrotransfer

Eight monoclonal antibodies (mAbs) specific for the N-terminal region of human "Porin 31HL" (equivalent to "HVDAC1" of Blachly-Dyson et al., 1993) were generously provided by Dr F. P. Thinnes (Max-Planck-Institut für Experimentelle Medizine, Abteilung Immunochemie, Göttingen). Preparation and characterization of these antibodies have been described by Babel et al. (1991). To check cross-reactivity of the antibodies with porin of rat heart mitochondria, immunoblotting was applied. The proteins were separated on a 7.5–25% polyacrylamide linear gradient slab gel (Laemmli, 1970) in the Mini-Protean II system (Bio-Rad Laboratories, Melville, New York). A 0.2-ml aliquot of mitochondria (5 mg protein/ml) was loaded on a 0.75-mm-thick one-well

gel, which was run at constant 60 mA current for 45 min. The proteins were then transferred to nitrocellulose paper (Towbin and Gordon, 1984) using the Mini-Trans-Blot system (Bio-Rad) applying 300 mA for 75 min at room temperature. To monitor the efficiency of electrotransfer and to indicate molecular mass, prestained low-molecular-weight protein standards (Bio-Rad) were used.

To avoid nonspecific binding of antibodies to the nitrocellulose, the sheet was saturated first for 30 min in 5% BSA in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM potassium phosphate buffer, pH 7.4), then for another 20 min in 3% nonfat dry milk in PBS buffer at 32°C, gently shaken, followed by two washes with PBS containing 0.1% BSA (0.1% BSA–PBS).

b. Immunolabelling of the Electrotransferred Proteins

All the following steps were carried out at room temperature with continuous shaking. The transfer sheet was incubated with each of the eight monoclonal IgGs (diluted with 0.1% BSA–PBS as described by Babel et al., 1991) for 1 h in a multiscreen apparatus (Bio-Rad). For negative controls, normal mouse IgG (Sigma Chemical Company, St Louis, Missouri) was used at the same concentrations as the specific mAbs. To remove unbound antibodies, the nitrocellulose sheet was washed three times for 10 min each with 0.1% BSA–PBS containing a higher (300 mM) NaCl concentration.

To visualize primary antibodies, horseradish peroxidase-conjugated protein A (Sigma) was applied, diluted 1 : 1000 with 0.1% BSA–PBS. After 1-h incubation and washing with 0.1% BSA–PBS (3 × 10 min), the peroxidase reaction was run for 5 min in PBS containing 0.06% 4-chloro-1-naphthol, 20% methanol, and 0.09% H_2O_2. Following development of the reaction, the nitrocellulose sheet was rinsed with deionized water (Milli-Q system, Millipore Corp., Bedford, Massachusetts) and air-dried.

Indirect Immunoelectron Microscopy

Immunolabelling of mitochondria was done with an indirect Protein A-gold procedure based on one developed for mitochondrial membranes from Neurospora crassa (Mannella and Colombini, 1984; Shao et al., 1993). All steps of this procedure were done at room temperature in a well-humidified chamber to prevent drying of the specimens. Freshly isolated mitochondria (approximately 30 mg/ml) were diluted

1 : 20 with 1% BSA–PBS. Five-to-ten-μl aliquots of the mitochondrial suspension were placed on glow-discharged, Formvar-carbon-coated, 300-mesh nickel specimen grids (Polyscience, Inc., Warrington, Pennsylvania). After 2 min, excess suspension was partially blotted with filter paper. The grids were then incubated for 30 min with one of the monoclonal antibodies (N4) or with normal mouse IgG (Sigma), both diluted to 1 mg/ml with 1% BSA–PBS. After unbound antibodies were removed by two 1-min washes with 1% BSA–PBS, the grids were incubated for 20 min with protein A-coated colloidal gold (A_{520} approximately 5, 20 nm diameter, Sigma) diluted 1 : 20 with 1% BSA–PBS. Free protein A–gold complex was washed away with two 1-min washes with 1% BSA–PBS. Afterwards, specimens were incubated for 2 min with 2% ammonium molybdate, completely blotted with filter paper, and air-dried. Of all negative stains, ammonium molybdate has been shown to maintain mitochondrial morphology best (Munn, 1968) and so is well suited for these kinds of experiments.

Images of immunogold-labelled, negatively stained rat heart mitochondria were recorded using a Philips EM301 or EM420-T electron microscope (Philips Electronic Instrument Inc., Mahwah, New Jersey) at an accelerating voltage of 80–100 kV and instrument magnification of 15,000–25,000. To quantify binding of colloidal gold to mitochondria incubated with normal and anti-porin IgG, numerous micrographs were taken at random and printed at threefold enlargement. Counts were made visually of gold particles on (or within one particle diameter of) mitochondria and those not on mitochondria. A digitizing pad (Model GP6-040, Science Accessories Corp., Stratford, Connecticut) was used to determine the total area occupied by mitochondria in each field, allowing calculation of the mean particle densities on and off mitochondria for each micrograph (see Table I).

RESULTS

Monoclonal antibodies against the N-terminal part of human porin ("anti-porin mAb") were found to react specifically in western blots of rat heart mitochondria with a protein band at M_r 31,000, the expected molecular weight of VDAC (Fig. 1). Of the eight antibodies available, the strongest reaction occurred with mAb N4 which was selected for use in subsequent experiments.

When freshly isolated rat heart mitochondria were incubated with anti-porin mAb, very few if any of the organelles were significantly labelled by protein A–gold. The number of labelled mitochondria was found to increase significantly (typically up to 5–10% of the total mitochondria) after storage of the suspensions in cold isolation medium over several hours. This increased immunogold labelling correlated directly with increased accessibility of externally added cytochrome c to the inner-membrane respiratory chain, measured as stimulation of succinate : cytochrome c oxidoreductase activity (Douce et al., 1973). This suggested that damage to the outer mitochondrial membrane was

Table I. Statistics of Labelling of Rat-Heart Mitochondria by Anti-porin and Normal Mouse IgG

Serum	N^a	$R = D_{on}/D_{off}{}^b$	$(S.D.)^c$
Normal	27	7.3	(6.5)
Anti-porin	43	31.3	(21.4)

$R_{anti-porin}/R_{normal} = 4.3 \ (p < 0.001)^d$

^{a} N = number of electron micrographs (each containing several mitochondria) included in the analysis.
^{b} R = ratio of the mean surface density of gold particles on or within one particle diameter of mitochondria (D_{on}) to the particle density off mitochondria (D_{off}).
^{c} S.D. = standard deviation.
^{d} p = probability that the result could be obtained by chance, calculated using t-test with pooled variance.
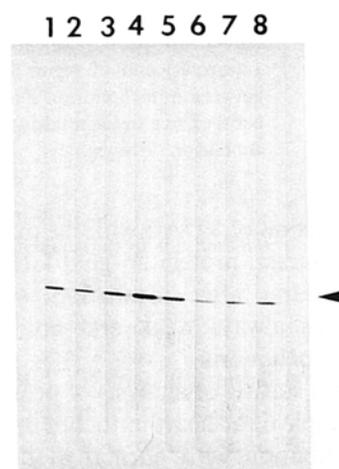

Fig. 1. Western blot showing reactivity of eight monoclonal antibodies against human porin with rat heart mitochondria. Numbers refer to the different antibodies; arrow indicates position of 31,000 molecular weight marker on nearby lane.
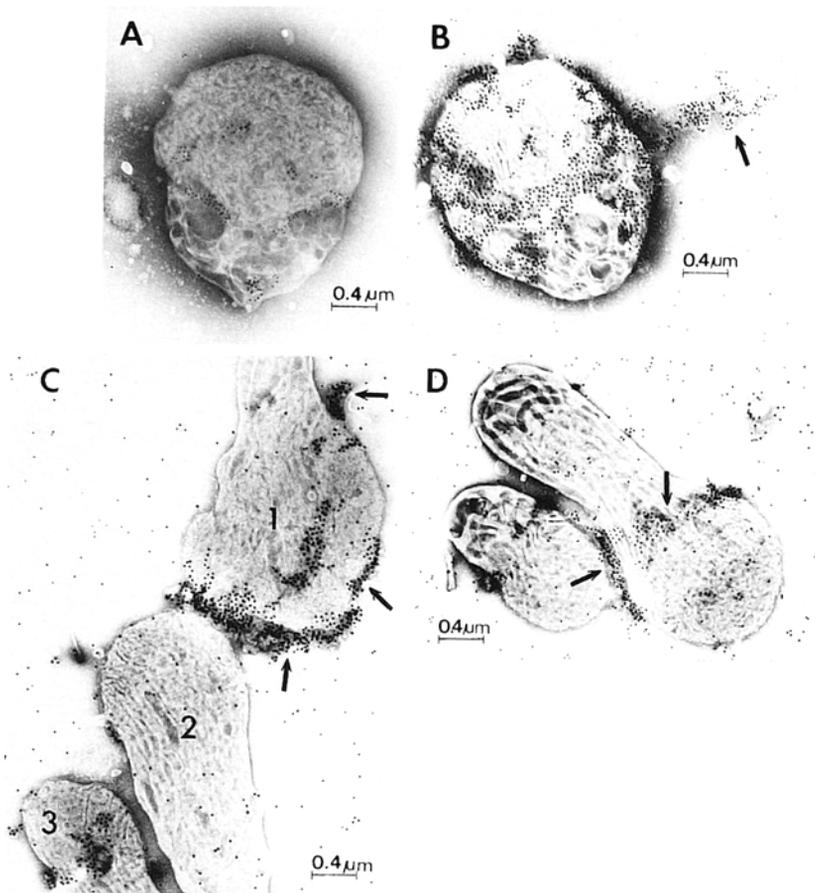
Fig. 2. Protein A-gold labelling of unfixed negatively stained rat heart mitochondria pre-incubated with monoclonal antibodies to the N-terminal part of mitochondrial porin. (A) Labelling mainly localized in the region between two parts of the same mitochondrion which differ in conformation. (B) Labelling of a stripped-off part of the outer mitochondrial membrane (arrow) and of an extensive area between two structurally different parts of the mitochondrion. (C) Labelling of stripped-off parts of the outer membrane of mitochondrion 1 (arrows) and of region between mitochondrion 1 and mitochrondrion 2. The region between mitochondrion 2 and mitochondrion 3 is not labelled. (D) Labelling of a region between two mitochondria as well as of that between structurally different parts of a mito-chondrion (arrows).

required for reaction of VDAC with the mAb (see Discussion). In contrast, protein A–gold labelling of rat heart mitochondria after incubation with normal mouse IgG was always weak and not affected by aging of the mitochondria.

Table I is a statistical analysis of protein A–gold labelling of a typical rat-heart mitochondrial preparation after incubation with normal mouse IgG and anti-porin IgG. Note that, although there is some binding of protein A–gold to mitochondria preincubated with normal serum, the ratio of the mean particle density on mitochondria vs. the background

density $(R = D_{on}/D_{off})$ is a factor of 4.3 greater for mitochondria preincubated with anti-porin IgG.

Examples of electron micrographs of rat heart mitochondria labelled by anti-porin mAb are shown in Fig. 2. Typically, the protein A–gold particles occurred in elongated thread- or ribbon-like clusters. Irregularly shaped clusters containing numerous protein A–gold particles also were observed, sometimes on stripped-off mitochondrial outer membranes (Figs. 2B,C). In certain cases, labelling by anti-porin mAb occurred in narrow regions between two parts of a mitochondrion with distinctly different
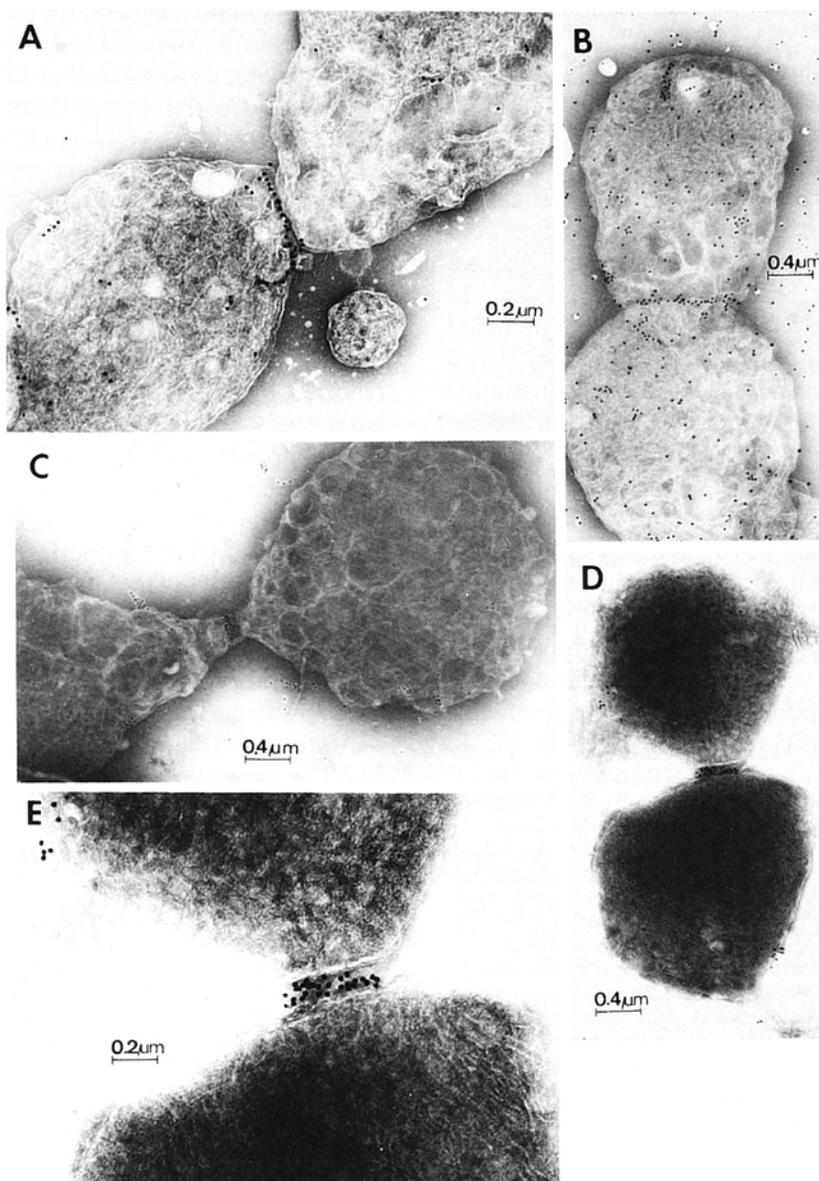
Fig. 3. Immunogold labelling (anti-porin mAb) of outer membrane regions at "necks" or "junctions" between different mitochondria. E is a higher magnification of the junction region in D.

inner membrane conformation, which might represent two mitoplasts covered by a single outer membrane (Figs. 2A, B,D). In all cases, only a relatively small part of the total mitochondrial surface was labelled.

In general, the variation in degree of labelling of mitochondria in these fields by anti-porin mAb was more-or-less random. Sometimes heavily labelled mitochondria were in close proximity to nonlabelled

ones (Fig. 3C). Of special interest were the examples found of adjacent mitochondria that appeared to be connected by outer-membrane "necks" or "junctions" (Fig. 3). Labelling by anti-porin mAb of these connected mitochondria often occurred at (and was occasionally restricted to) the region of the junctions where the gold particles aligned in threads (Figs. 3A, B) or ribbons (Figs. 3C,D,E).

DISCUSSION

Labelling of rat heart mitochondria by antibody against the N-terminal segment of human porin is heterogeneous and generally weaker than expected if all the epitopes on all the porin proteins were readily accessible to and reactive with the antibody. There are several possible explanations for this restricted pattern of labelling. The first is that the epitope itself might have limited accessibility to the aqueous medium. Colombini and co-workers have suggested that the N-terminal segment of the VDAC polypeptide might form part of the lumen wall in the channel's open state and that this region of the protein flips out of the membrane interior during closure (Peng et al., 1992). Conversely, Mannella and co-workers have proposed that the N-terminal segment of VDAC is exposed at the surface of the outer membrane in the open state of the channel and that it may move into the lumen during closure (Mannella, 1990; Guo and Mannella, 1993). While disagreeing in detail, both models imply that the epitope for the antibody used in the present study might have limited accessibility at the surface of the outer membrane. A second factor that could restrict the reactivity of N-terminal antibodies with VDAC is the disposition of the epitope on the outer membrane, i.e., whether it faces the mitochondrial exterior or interior. DePinto and Palmieri (1992) have reported that the N-terminal segment of rat-liver porin is exposed on the external surface of the outer membrane. However, more recent experiments with *Neurospora* mitochondria, in which outer membrane integrity was carefully monitored, indicate that the N-terminal segment faces the mitochondrial interior (Stanley et al., 1994; Stanley, 1994; also Stanley and Mannella, manuscript in preparation). The observation in the present study, that reactivity of the N-terminal antibody with rat heart mitochondria increased with breakage of the outer membrane, is consistent with the latter conclusion. A third factor that could limit the reactivity of VDAC proteins in rat heart mitochondria with antibody is the heterogeneity of VDAC in mammalian tissues (Blachly-Dyson et al., 1993, 1994). It is possible that only a fraction of the total VDAC population is recognized by the particular monoclonal antibody used.

Clearly, the factors controlling accessibility of the VDAC epitope recognized by the antibody employed in this study are not understood at present. Thus, while occurrence of immunogold labelling in a region of the mitochondrial outer membrane is good evidence for the presence of VDAC in that region, the absence of such labelling does not necessarily imply absence of VDAC—only of reactive and accessible epitope. The observation that Protein A–gold particles sometimes occur in clusters on the outer membranes of rat heart mitochondria could suggest that cooperative interactions between VDAC molecules influence accessibility of the N-terminal epitope, or this might simply be a result of local disruption of the membrane (e.g., if the epitope were exposed on the inner surface of the outer membrane). A similar tendency of anti-porin antibodies to occur in clusters on rat brain mitochondria previously has been reported by Leterrier et al. (1994), although in the latter study polyclonal antibodies against the whole VDAC protein were employed.

Labelling of the outer mitochondrial membrane by anti-porin mAb was sometimes confined to specialized regions, such as the interface between inner membrane regions of different morphology or the "necks" between connected mitochondria. This might indicate that VDAC in these regions is preferentially in a conformation with accessible epitope, or simply that these outer-membrane regions are more prone to disruption.

The examples in Fig. 3 of heavy immunogold labelling at the "necks" between adjacent mitochondria are nonetheless striking. Similar mitochondrial connections can be found at low frequency in negatively stained mitochondrial preparations from almost any source, and might represent mitochondria in the process of division. In the case of heart mitochondria, the occurrence of these closely apposed or connected mitochondria has been shown to correlate with mitochondrial "junctions" (Tikhova et al., 1988). These junctions are specific zones of contact between mitochondria in myocardiocytes that are thought to be involved in electrical communication (Bakeeva et al., 1983, 1985; Skulachev, 1988, 1990; Amchenkova et al., 1986, 1988), consistent with the hypothesis that extended systems of coupling membranes can function as power-transmitting cables (Skulachev, 1969, 1988, 1990; Severina et al., 1988).

The results of Fig. 3 indicate that the mitochondrial porin, VDAC, is present in the "neck" or connecting regions between adjacent organelles in these rat heart mitochondrial preparations. If, in fact, these connecting zones represent functional "junctions" that survive the isolation procedure (as indicated by Tikhova et al., 1988), the results suggest that porin might be a component of the pathway for electrical conductance between mitochondria, as first suggested

by Skulachev (1990). If so, these junctions, composed of two sets of closely spaced inner and outer membranes, might utilize the voltage dependence of the porin channel as follows. If both mitochondria connected at a junction are energized, the transjunction potential difference (arising from the nearby inner membranes) would be small. If, however, one of the mitochondria becomes de-energized, the transjunction potential difference would increase and could result in closing the porin channels in the junction. The effect would be to reduce the conductance across the junction, thereby preventing the other mitochondrion from fully de-energizing.

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