Phospholipase-Induced Crystallization of Channels in Mitochondrial Outer Membranes

Abstract. *When outer membranes from* Neurospora crassa *mitochondria are treated with low levels of phospholipase A_2 under continuous dialysis, two-dimensional crystalline arrays of the pore protein component of these membranes are formed.*

The outer membrane of mitochondria contains a class of 30-kilodalton (kD) polypeptides which form ion channels when incorporated into synthetic lipid membranes (*1*). These proteins, called VDAC (for the voltage-dependent, anion-selective channels which they form in vitro), are thought to represent the main route of permeability in this membrane. In a varying percentage of outer membranes isolated from mitochondria of the fungus *Neurospora crassa*, these 30-kD polypeptides form polymorphic arrays of channels (*2–5*). The usual planar lattice observed in negatively stained specimens is oblique, with six stain-filled channels per parallelogram unit cell. Treatment of the membranes with Ca^{2+} results in the formation of a new rectangular array in which the channels are more closely packed (*6*). I suspected that the occurrence of Ca^{2+}-activated phospholipase A_2 activity in mitochondrial outer membrane fractions (*7*) might be responsible for the change in array geometry. I now report that exogenous phospholipase A_2 not only causes the same change in crystal packing as that induced by Ca^{2+}, but it induces crystallization of the channels in disordered membranes (Fig. 1).

Outer membranes isolated from *N. crassa* mitochondria were dialyzed overnight against low-salt buffer (0.001*M* tris-HCl, *p*H 7). The number of crystalline membranes present after dialysis was less than 5 percent of the total membranes present; most of the membranes were irregularly shaped, collapsed vesicles 0.1 to 1.5 μm across. Membranes from the same preparation were incubated with bee venom phospholipase A_2 (0.7 unit per 10 μg of membrane protein) during dialysis; here, the total number of crystalline membranes was about the same as the number of large vesicles present in the untreated specimens. Thus, the periodic arrays seem to have formed during the incubation with phospholipase A_2 by crystallization of pore proteins in previously amorphous membranes.

The best yields of crystalline membranes were obtained when starting membrane fractions were dilute (1 to 10 μg of membrane protein per milliliter) and the phospholipase reaction was allowed to proceed slowly (0.1 to 0.5 unit of enzyme per microgram of membrane protein at 4°C) with constant dialysis. Under these conditions, released fatty acids and lysophospholipids do not reach their critical micelle concentrations (*8*), allowing free outward diffusion of these lipids from the dialysis compartment. The slow rate of the phospholipase reaction may facilitate ordering of the protein components in the membrane plane by the gradual removal of lipids.

Protein recoveries and crystalline membrane yields for several experiments are listed in Table 1. Total protein recovery after low-level phospholipase A_2 treatment of outer membranes of *N. crassa* mitochondria was usually around 50 percent, although percent recovery of the 30-kD pore protein was often higher, reflecting the predominance of ordered channel arrays in the treated membrane suspensions. Increasing the activity of added phospholipase A_2 leads to a higher crystalline membrane content in the final suspension. Further, the type of array present after treatment varies systematically with the final content of crystalline membrane. With crystalline membrane content below 85 percent, the predominant array is oblique as in the untreated membrane preparations. With higher crystalline membrane content, the predominance of the rectangular lattice indicates that increased phospholipase action leads to closer packing of the channels. Subsequent experiments may determine whether the observed transitions in channel organization (disordered to ordered, oblique to rectangular lattice geometry) correlate with the loss of specific amounts or classes of lipids.

Biological macromolecules arranged

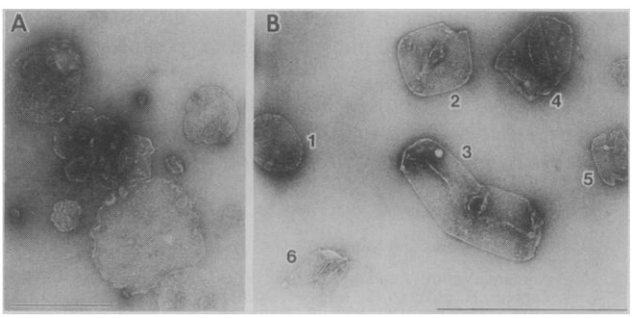

Fig. 1. (A) Electron microscopic images of uranyl acetate–stained outer membranes from *Neurospora crassa* mitochondria dialyzed overnight against a low-salt buffer. (B) The same membrane preparation treated with phospholipase A_2 (experiment 1 of Table 1). Membranes 1, 2, and 3 appear to be collapsed, closed vesicles composed of oblique channel arrays (as determined by optical diffraction). Membranes 4, 5, and 6 appear to be open, folded sheets of oblique (4 and 5) or rectangular (6) planar crystals (note the unusually straight edges several tenths of a micrometer long). Scale bar, 1 μm. Electron microscopic and optical diffraction procedures as described in (*2*).

Table 1. Effect of phospholipase A_2 on outer membranes of *Neurospora crassa* mitochondria. Preparations of freshly isolated membranes (approximately 1 μg of protein per milliliter) were divided into equal portions, and the indicated amounts of phospholipase A_2 were added. After dialysis overnight at 4°C against 400-volume low-salt buffer, membranes were formed into pellets by centrifugation (60,000*g*, 90 minutes), and the phospholipase-treated and -untreated fractions were compared.

Experiment	Phospholipase A_2 concentration* (unit/ml)	Membrane protein recovery† (%)			Membrane crystal yield‡ (%)		
		Total	30-kD	Other	With enzyme	Without enzyme	(P:R)§
1	0.10	56	62	39	~ 0	80	(3:1)
2	0.65	49	74	20	~ 0	85	(1:1)
3	0.07	†	†	†	†	55	(allP)
4	0.33	†	†	†	†	85	(1:2)
5	0.33	†	†	†	5	95	(allR)

*Bee venom phospholipase A_2 (Sigma Chemical Co., St. Louis); a unit is that amount which hydrolyzes 1 μmole of lecithin per minute at 37°C and *p*H 8.5. †Equal portions of the treated and untreated membrane suspensions underwent electrophoresis in adjacent lanes of sodium dodecyl sulfate–polyacrylamide gel slabs (*2, 3*). Relative amounts of the protein in each band were determined by microdensitometry (Joyce-Loeble 3CS) of photographs (Kodak Tri-X film) of the silver-stained gels by the method of Merril *et al.* (*10*). ‡Crystalline membrane content was determined by visual examination of electron micrographs (×15,000 to 20,000) of random fields of uranyl acetate–stained membrane preparations. §The ratio of parallelogram to rectangular lattices occurring in the periodic arrays determined by optical diffraction from the electron micrographs. Electron microscopic and optical diffraction procedures as described in (*2*)].

in planar crystalline form like the channel arrays in Fig. 1B facilitate electron microscopy (*9*). The random noise in electron images from such specimens can be filtered from the ordered, structure-related components by computer based Fourier- or correlation-averaging techniques. The more unit cells included in such averages, the lower the electron dose per molecule needed to obtain the averaged image and the more reliable its structural representation. Only a few membrane proteins have been found to occur as ordered arrays in nature. Several others have been crystallized in vitro by detergent solubilization, purification, and some degree of reconstitution with lipids. The technique of controlled phospholipase digestion presented here is another source of membrane protein crystals. The 30-kD pore protein typically represents 60 to 80 percent of the total protein mass in outer membrane fractions of *N. crassa* mitochondria. Other biological membranes naturally enriched in a particular protein component or protein complex (for example, Ca^{2+} transporter in sarcoplasmic reticulum and acetylcholine receptor in synaptosomes) may also be amenable to phospholipase-induced crystallization.

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References and Notes

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4. A low-resolution three-dimensional reconstruction of the negatively stained mitochondrial array has been calculated by weighted back-projection of a tilt-series of Fourier-filtered, electron microscopic images [C.A. Mannella, M. Radermacher, J. Frank, *Proceedings of the 8th European Congress on Electron Microscopy, Budapest, 1984* (8th European Congress on Electron Microscopy Foundation, Leiden, Netherlands), in press]. This reconstruction indicates that the array is composed of regularly arranged, stain-filled, cylindrical channels, each approximately 2.5 nm in diameter.
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6. The surface density of the channels increases by about 10 percent as the array changes from oblique to rectangular. The different arrangements of the stain-filled channels are illustrated in C. A. Mannella, M. Colombini, J. Frank, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2243 (1983).
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8. Outer membranes isolated from *N. crassa* mitochondria contain 1.1 μg of phospholipid per microgram of protein [G. Hallermeyer and W. Neupert, *Hoppe-Seyler's Z. Physiol. Chem.* **355**, 279 (1974)]. Complete hydrolysis of the membrane phospholipids in these experiments would therefore release 10^{-5} to $10^{-6}M$ fatty acids and lysophospholipids. The range of critical micelle concentrations of the expected fatty acids is 10^{-2} to $10^{-4}M$ [P. Mukerjee and K. J. Mysels, *Critical Micelle Concentration in Aqueous Surfactant Systems* (National Bureau of Standards, Washington, D.C., 1971)]; that of the lysophospholipids is 10^{-3} to $10^{-5}M$ [A. Helenius, D. R. McCaslin, E. Fries, C. Tanford, *Methods Enzymol.* **56**, 734 (1979)].
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11. The technical assistance of B. Cognon is gratefully acknowledged. This material is based on work supported by grant PCM-8021789 from the National Science Foundation.

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Immunohistochemistry of Adenosine Deaminase: Implications for Adenosine Neurotransmission

Abstract. *Immunohistochemical analysis of adenosine deaminase in rat brain revealed an extensive plexus of adenosine deaminase–containing neurons in the basal hypothalamus. These neurons converged on and were most numerous in three major centers, namely, the tuberal, caudal, and postmammillary caudal magnocellular nuclei. Most other brain regions were devoid of cells containing adenosine deaminase. Some adenosine deaminase–containing neurons were retrogradely labeled with the fluorescent dye fast blue when the dye was injected into the frontal cortex and striatum. Specific populations of neurons having high levels of adenosine deaminase may release adenosine as a neurotransmitter.*

Immunohistochemical studies of the central nervous system (CNS) have provided information about the location and organization of neurons defined by their putative neurotransmitter content. In addition, the visualization of neuroactive peptides or of enzymes responsible for the synthesis and degradation of the better known neurotransmitters within neurons has helped to elucidate the biochemical nature of diverse neuronal systems. A recent addition to the list of putative central neurotransmitters is the purine nucleoside adenosine (*1, 2*). Behavioral, electrophysiological, and biochemical evidence indicates that adenosine can function as a neurotransmitter (*1–3*). Biochemical and anatomical methods capable of identifying adenosine-releasing neurons are not yet available and, as a consequence, no candidate neurons or systems in the CNS for which adenosine is a neurotransmitter have been described. Our study was based on the premise that the metabolism of adenosine in presumptive adenosine-releasing neurons may exhibit features unique to these cells. A major metabolic pathway for the degradation of adenosine is through deamination by the enzyme adenosine deaminase (AD) (*4*). We therefore conducted immunohistochemical studies of the distribution of AD in the rat CNS. We found a network of neurons in the basal hypothalamus that are distinguished from other central neurons by their high AD content. We suggest that AD-rich neurons may occur in neural systems that release adenosine as a neurotransmitter.

Calf intestinal AD was purified to homogeneity, and an antiserum specific to this enzyme was prepared in rabbits (*5*). Antibodies to calf intestinal AD have been shown to cross-react with AD derived from rat tissues (*6*). For our immunohistochemical studies, adult rats were placed under deep anesthesia with chloral hydrate and perfused intracardially with a 0.9 percent saline wash followed either by 4 percent formaldehyde solution or 4 percent paraformaldehyde in 0.1*M* phosphate buffer, *p*H 7.4. Sections of brain (20 μm) were cut on a freezing microtome and incubated at 4°C for 48 hours with antiserum to AD diluted 1:500. The sections were subsequently processed by the peroxidase-antiperoxidase method (*7*).

Examination of many brain regions revealed a highly restricted distribution of neurons that stained for AD by our immunohistochemical procedures. The most intensely stained neurons were found in three distinct cell groups in the basal hypothalamus (Fig. 1, a to c). According to the anatomical descriptions of Bleier *et al.* (*8*), these neurons were located in the tuberal (TM), caudal (CM), and postmammillary caudal (PCM) magnocellular nuclei. In addition, many AD-containing neurons were found dispersed between these major cell groups, thus forming a contiguous network from the TM anteriorly to the PCM posteriorly. Immunohistochemical staining for AD was completely abolished in tissue sections incubated with antiserum to AD that had been adsorbed with purified AD (Fig. 1d).

Vincent *et al.* (*9*) demonstrated that neurons in the TM, CM, and PCM have widespread projections to the amygdala, striatum, and cortex. To determine whether AD-containing neurons in these nuclei have similar diffuse projection