The Role of Pyridine Dinucleotides in Regulating the Permeability of the Mitochondrial Outer Membrane*

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Both NADH and NADPH reduce the permeability of the mitochondrial outer membrane to ADP. This is specific for the outer membrane and uncorrelated with the respiratory control ratio. This could result in a 7-fold difference between the concentration of ADP in the intermembrane space and that in the external environment (at 5 μM ADP). In both cases the permeability declines by a factor of 5, but NADH is more potent: $K_D = 86 \mu M$ for NADH versus 580 μM for NADPH. The lower apparent affinity for NADPH is partly explained by Mg$^{2+}$-NADPH being the active species, and under our conditions only 30% of the NADPH is in this form. The corrected $K_D$ is 184 μM. Free NADH has the same charge as the Mg$^{2+}$-NADPH complex, and thus both likely bind to the same site. The ability of NADH and NADPH to induce the closure of reconstituted VDAC channels is consistent with VDAC being the main pathway for metabolite flow across the outer membrane. Oncotic pressure, effective at inducing VDAC closure, also decreases the outer membrane permeability. Thus, in the presence of cytosolic colloidal osmotic pressure NAD(P)H may inhibit mitochondrial catabolic pathways and divert reducing equivalents to anabolic pathways.

A growing body of evidence implicates the mitochondrial outer membrane in regulating mitochondrial function (Colombini et al., 1987; Benz et al., 1988; Liu and Colombini, 1992). The outer membrane has been shown to limit the rate of metabolite flux under a variety of conditions (Gellerich et al., 1989; Liu and Colombini, 1992; Lee et al., 1994). NADH, well known as a carrier of reducing equivalents within the cell, had been shown to be capable of reducing the permeability of the mitochondrial outer membrane to ADP by a factor of 6 (Lee et al., 1994). However, it is well known that NADH exists mainly as the oxidized form, NAD$^+$, in the cytosol (Williamson et al., 1967), and this form is ineffective in controlling the permeability of the outer membrane (Lee et al., 1994). By contrast, its relative, NADPH, exists mainly as the reduced form (Veech et al., 1969). Thus, it is of interest to determine the relative role of NADH and NADPH in regulating the permeability of the mitochondrial outer membrane.

The roles of NADH and NADPH in the cytosol are quite different. The glycolysis of one molecule of glucose produces two molecules of ATP and two of NADH. The reducing equivalents in NADH are transferred to the mitochondrial inner membrane or matrix either by dehydrogenases on the outer surface of the inner membrane (plants and fungi; Palmer and Møller (1982) and Møller (1986)) or via transhydrogenases and transport shuttles. These are fed into the electron transport chain for ATP synthesis. Thus NADH levels should reflect the relative rates of glycolysis and oxidative phosphorylation. Although NADPH is an analog of NADH, it plays a different role. Mainly produced by the pentose phosphate pathway, cytosolic NADPH is consumed by the cellular synthetic systems. NADPH provides reducing equivalents for fatty acid synthesis. Because mitochondria are involved in both energy transduction and fatty acid metabolism, the relative effects of these reduced pyridine dinucleotides on the permeability of the mitochondrial outer membrane should provide insight on the role of this regulatory process.

VDAC channels are believed to be the major pathway for the transport of metabolites across the mitochondrial outer membrane. Many reports show that agents capable of closing VDAC channels also greatly reduce the permeability of the outer membrane (Gellerich et al., 1993; Benz et al., 1988; Liu and Colombini, 1992). This is also true for NADH (Lee et al., 1994). The ability of NADPH to influence the permeability of the mitochondrial outer membrane should correlate with its ability to influence VDAC closure. This paper will examine the action of NADPH on both isolated mitochondria and VDAC channels reconstituted into planar phospholipid membranes.

EXPERIMENTAL PROCEDURES

Measurement of Mitochondrial Respiration and Intactness—Intact mitochondria from potato tubers were isolated as described previously (Schwitzguebel and Siegenthaler, 1984). The oxygen consumption of the mitochondria (~50–100 μg/ml protein) was measured in a 3-ml stirred cell containing respiration buffer at 24 °C (0.3 M mannitol, 10 mM NaH$_2$PO$_4$, 5 mM MgCl$_2$, and 10 mM KCl (pH 7.2)) by using a Clark oxygen electrode (Yellow Spring Instrument Co.).

The mitochondria were preincubated with 0.1 mM EGTA for 10 or 5 min to block NADH or NADPH dehydrogenases, respectively. 5 mM succinate was used as the substrate for respiration. ADP was added to trigger state 3. Four state 3-state 4 respiration cycles were generated by four consecutive ADP (90 μM) additions. NADH was added between the first and second doses of ADP. NADPH was added just before the first dose of ADP. For the dextran and polyethylene glycol (PEG) experiments, the mitochondria (final protein concentration, 50–100 μg/ml) were added directly in the respiration buffer containing dextran T-40 (M, 40,000) or PEG (M, 20,000). The P/O ratio was estimated from the ADP-dependent oxygen consumption. The oxygen concentration in the air-saturated medium was taken as 250 μM.

The intactness of the mitochondrial outer membrane was quantified by measuring cytochrome c-dependent oxygen consumption (Douce et al., 1987). Exogenously added reduced cytochrome c must pass through the outer membrane to be oxidized by cytochrome c oxidase. The percentage of intactness of the mitochondrial outer membrane was taken as 100 times 1 minus the ratio of KCN-sensitive cytochrome c-dependent oxygen consumption of untreated and osmotic-
cally shocked mitochondria (30 μl of mitochondrial suspension to 1.5 ml water for 3 min followed by addition of 1.5 ml of 2 × respiration buffer). Mitochondrial protein concentrations between 25 and 50 μg/ml were used. Mitochondrial integrity in all preparations ranged from 95 to 100%.

Isolation of VDAC Channels from Potato Mitochondria—VDAC was purified essentially as described previously (Freitag et al., 1983; Blachly-Dyson et al., 1990). The addition of 0.2 mM phenylmethylsulfonyl fluoride to isolation buffers improved channel activity. 1 ml of isolated mitochondria (5 mg protein/ml) was incubated with 9 ml of hypotonic solution (1 mM KCl and 1 mM Heps, pH 7.0) for 10 min on ice and then spun in a microfuge 30 min at 4 °C. The pelleted membranes were dispersed in 0.7 ml of buffer A (15% dimethyl sulfoxide, 2.5% Triton X-100, 50 mM Heps, 10 mM Tris, 1 mM EDTA, pH 7.0) shaken for 30 min, and then centrifuged. The remaining steps were performed in a cold room (4 °C). The supernatant was applied to a dry column containing 0.6 g of a mixture of 50% celite and 50% hydroxyapatite in Pasteur pipettes plugged with glass wool. The column was eluted with buffer A, 1 ml of a mixture of 50% celite and 50% hydroxyapatite in Pasteur pipettes plugged with glass wool. The column was eluted with buffer A, and the first 0.7 ml of eluate, containing the VDAC protein, was collected.

The Assay of Protein Content and NAD(P)H Oxidation—Mitochondrial protein was measured using the BCA method (Pierce) following addition of Triton X-100 (1% (w/v) final concentration). Bovine serum albumin was the standard. The oxidation of exogenous NAD(P)H was measured by following the decrease in absorbance at 340 nm at room temperature (Arron and Edwards, 1980).

The Measurement of the K_{D} of NAD(P)H for Mg^2+—Eriochrome black T (EBT), a metallochromic indicator extensively used in metal chelation studies (Harvey et al., 1953), binds metals weakly and in doing so changes its absorption spectrum. EBT is blue between pH 7 and 11 (Bell, 1977). The formation of the magnesium complex of EBT is accompanied by a spectral change (Young and Sweet, 1955). We used this to determine its dissociation constant for Mg^{2+} under our experimental conditions by measuring the absorbance as a function of [Mg^{2+}] and fitting to the following equation:

$$\frac{\text{Abs}}{\text{Abs}_{\text{min}} - \text{Abs}_{\text{max}}} = \frac{1}{\text{Abs}_{\text{max}} - \text{Abs}_{\text{min}}} + \frac{K_{\text{D}}}{\text{Abs}_{\text{max}} - \text{Abs}_{\text{min}}}$$

(Eq. 1)

where [Mg^{2+}] is the free magnesium, K_{D} is the dissociation constant of EBT for magnesium, and Abs_{min} and Abs_{max} are the absorbance, the maximum absorbance, and the minimum absorbance at 528 nm, respectively, in the absence of Mg^{2+}. The [Mg^{2+}] is equal to total magnesium in the absence of NAD(P)H because the total EBT concentration was 50 μM and thus negligible.

Addition of Mg^{2+} to NADPH would reduce [Mg^{2+}], and thus alter the absorbance of EBT by decreasing the [EBT - Mg^{2+}]. Thus EBT reports [Mg^{2+}], which can be substituted into the following equation to determine the binding constant of NADP(H) for Mg^{2+}:

$$\frac{\left[\text{NADP(H)}\right]_{\text{F}}}{\left[\text{Mg}^{2+}\right]_{\text{F}} - \left[\text{Mg}^{2+}\right]_{\text{I}}} = \frac{1}{K_{\text{D}}^{\text{NADP(H)}}} + \frac{1}{\left[\text{Mg}^{2+}\right]_{\text{I}}}$$

(Eq. 2)

where [NADP(H)]_{F} is the total NADP(H) concentration, [Mg^{2+}]_{I} is the total magnesium concentration, and K_{D}^{NADP(H)} is the dissociation constant of NADP(H) to magnesium.

The absorbance of EBT was measured as a function of total Mg^{2+} added to the medium in the presence of 20 mM NADP(H). The free Mg^{2+} was determined from Equation 1 for each level of total Mg^{2+}. These values were inserted into Equation 2 and plotted as the equation of a straight line with the slope equal to the dissociation constant.

The use of eriochrome black T was found to be complex. It was plagued by solubility problems and indications of slow reduction by NADPH. The following procedure was found to be adequate to estimate the binding constants. EBT was freshly prepared as a 10 mM stock solution in double distilled water and then diluted and supplemented with KCl and MOPS to prepare the working solution (100 mM KCl, 30 mM MOPS, pH 7.2). Various concentrations of Mg^{2+} were prepared in the same buffer without dye. The color change of EBT upon addition of Mg^{2+} is time-dependent. However, a reasonably stable level was reached by 6 min. Thus, all experiments were started by mixing 0.5 ml of the EBT solution with 0.5 ml of a Mg^{2+} solution followed by a 6-min incubation prior to taking the absorption spectrum of the solution between 750 and 400 nm. (Note that EBT solution develops a very faint precipitate with time and thus must be used fresh and should be mixed just before taking a sample.) The experiment was repeated at different concentrations of Mg^{2+}. When used, the NADP(H) was dissolved directly in 0.5 ml of the Mg^{2+}-solution and incubated for 15 min. Then 0.5 ml of the EBT solution was added followed by the 6-min wait and the recording of the absorption spectrum. Alternatively, the absorbance at 528 nm was recorded as a function of time, and the value at 6 min was used for the calculations.

Data Collection—In a typical mitochondrial respiration experiment, four sequential ADP additions were performed resulting in the recording of four state 3-state 4 segments. The data were recorded and digitized by Axotape 2.0 (Axon Instruments, Inc., Foster City, CA). The first of these was never used and was considered to be a treatment that would prime the mitochondria for further testing. Each of the subsequent three state 3-state 4 recordings following the addition of an aliquot of ADP were output to a qBasic program for analysis. The rate of respiration during the state 4 phase was subtracted from the immediately previous state 3 recording in order to obtain just ADP-dependent respiration. The oxygen concentration scale was converted to an ADP concentration scale by knowing the amount of ADP added to the chamber and assuming that during state 4 the [ADP] = 0.

Calculation of Permeability—The method was described in detail in Lee et al. (1994). ADP consumption involves three steps: transfer across the outer membrane, transfer across the inner membrane through the adenine nucleotide translocator, and phosphorylation by the mitochondrial ATP synthetase.

At steady state (note small intermembrane space volume) the flux of ADP across outer membrane is equal to the flux across inner membrane.

$$\text{Flux} = \frac{d[\text{ADP}]}{dt} = -P_{\text{A}}(C_{i} - C_{o}) = \frac{V_{\text{max}}C_{i}}{K_{M} + C_{i}}$$

(Eq. 3)

where P_{A} is the permeability of the outer membrane to ADP; A is the total area of the outer membrane of all the mitochondria present; C_{i} and C_{o} are the ADP concentrations in the intermembrane space and the medium, respectively; and V_{max} and K_{M} are enzyme kinetics parameters of the adenine nucleotide translocator.

Solving for C_{i} in terms of C_{o} yields:

$$C_{i} = \frac{V_{\text{max}}C_{o}}{K_{M} + C_{o}}$$

(Eq. 4)

The theoretical curve of the decline of the medium [ADP] as a function of time was generated by calculating the [ADP] at time (t) at intervals (dt) corresponding to the collected data points. These were calculated as follows:

$$C_{i} - C_{o} = \frac{d[\text{ADP}]}{dt} = C_{i} - C_{o} = \frac{V_{\text{max}}C_{i}}{K_{M} + C_{i}}$$

(Eq. 5)

V_{\text{max}} and P_{A} were allowed to vary so as to achieve the best fit.

Planar Phospholipid Membranes—The planar membranes were generated by the monolayer method of Montal and Mueller (1972) as modified (Colombini, 1987) using a 5:1 aloepectin:cholesterol mixture. The aqueous solutions were 1 M KCl, 10 mM Heps, pH 7.2, and 5 mM MgCl_{2} (or indicated) except for the ion selectivity experiments where one side contained 0.1 M KCl rather than 1 M KCl. Error values reported are standard errors.

The state of the channels was monitored by recording the current under voltage clamped conditions (Colombini, 1987). Calomel electrodes were used to interface with the solution. Current recordings in response to triangular voltage waves (4 mHz) were used to calculate the probability of the channel being open as a function of voltage and evaluate the two parameters that define the gating process: n, the gating valence, and V_{o}, the potential where the probability of being open or closed are equal.

RESULTS

The permeability of the mitochondrial outer membrane to ADP was measured by analyzing the transition between state 3 and state 4 respiration as described in the methods and as previously reported (Lee et al., 1994). This method allowed us to examine quantitatively the effect of various treatments on the permeability of the outer membrane and thus assess the
Fig. 1. The rate of mitochondrial respiration after ADP addition is altered by the addition of NADPH. The curves show the rate of respiration of mitochondria after the addition of 90 μM ADP at time 0. The least squares fit was used to determine the rate of respiration at each point. In order to reduce the noise, a sliding set of 30 points was used to determine these fits. Thus, the first and last 15 points of the oxygen respiration records cannot be included. The curves represent results of control (A), plus 1.28 mM NADPH (B), plus 12.8 mM NADPH (C). All experiments used 180 μg of mitochondrial protein in the 3-ml incubation medium.

importance of this pathway to mitochondrial function.

Pyridine Dinucleotides Reduce Outer Membrane Permeability—Both NADH and NADPH reduce the permeability of the mitochondrial outer membrane to ADP. The rate of respiration of mitochondria after ADP addition at time 0. As the ADP is consumed, the rate remains constant for some time (state 3 rate of oxidation) and then declines as the mitochondria enter state 4. This decline is more gradual with increasing concentrations of NADPH in the medium. As previously reported for NADH, this too can be understood as a decrease in the permeability of the mitochondrial outer membrane. The method, described under “Experimental Procedures,” allows one to calculate the total permeability of the outer membrane for ADP, i.e. the permeability per unit area times the total area of the outer membrane. If the total area remains constant, then the permeability per unit area must decrease. Both NADH and NADPH have the same overall effect in that they decrease the total permeability to 0.1 cm²/s (Fig. 2). However, NADH is much more potent. Assuming a 1:1 stoichiometry for the binding of the dinucleotides to sites on the outer membrane, the \( K_D \) for NADPH is 580 μM (Fig. 2, inset). This compares with a \( K_D \) of 86 μM for NADH (Lee et al., 1994). Thus the affinity of the binding sites for NADH is higher than that for NADPH by a factor of 7.

The measured permeability of the outer membrane is independent of the degree of coupling of the inner membrane. The respiratory control ratio is a way to express the efficiency of phosphorylation and the degree of coupling of mitochondria. By definition, there is no significant ADP in state 4. The oxygen consumption at state 4 is only due to leakage of protons through the inner membrane assuming the mitochondrial suspension does not contain other systems that consume oxygen such as a catalase or other oxidases. If the inner membrane were not leaky to protons, the oxygen consumption rate at state 4 would be very low. The respiratory control ratio (the ratio of state 3 divided by state 4) is a good way to express the degree of coupling, because it is very sensitive to the proton permeability of the inner membrane (the outer membrane is permeable to protons). The degree of coupling changes with incubation time as mitochondria age (Fig. 3A). However, the permeability of the outer membrane to ADP should be a characteristic of the outer membrane and should not change with changes in the inner membrane. As expected the measured outer membrane permeability did not follow changes in respiratory control ratio with time (Fig. 3A). Conversely, NADH decreased the permeability of the outer membrane to ADP but did not change the degree of coupling of the inner membrane (Fig. 3B).

Changes in Intermembrane Space ADP Concentration—The theory used to fit the respiration experiments can be used to calculate the concentration of ADP in the intermembrane space and thus assess the degree by which the outer membrane limits the rate of respiration. Net flux of ADP through the outer membrane requires an ADP concentration difference between the medium and the intermembrane space. The magnitude of this difference depends on the permeability of the outer membrane and the net flux of ADP through the inner membrane. The calculations show that such a concentration difference exists throughout the respiration experiment (Fig. 4A). However, the percentage of reduction of the ADP concentration in the intermembrane space caused by the outer membrane becomes maximal at low medium ADP concentrations. Thus the outer membrane is most important as a physical barrier at the low (physiologically relevant) ADP concentrations. The differences are much greater in the presence of 2 mM of NADH (Fig. 4B). These results indicate that the outer membrane is really a physical barrier whose function will be amplified by introducing reagents to decrease the permeability of the outer membrane.
Pyridine Dinucleotides Increase the Probability of VDAC Closure—Because VDAC is believed to be the major permeability pathway across the mitochondrial outer membrane, NADH and NADPH should be acting by reducing the permeability of VDAC channels in the outer membrane. It has been shown that NADH increases the voltage dependence of VDAC channels (Zizi et al., 1994). We examined the action of NADPH on potato VDAC. We reconstituted potato VDAC protein into planar phospholipid membranes. Addition of 14 mM of NADPH favors the closed state of VDAC channels (Fig. 5) without changing the single channel conductance in the presence of 5 mM magnesium. The data were plotted according to the linearized version of the Boltzmann distribution to obtain the values of the parameters defining the voltage dependence: \( n \), the gating valence, is a measure of the steepness of the voltage dependence, and \( V_0 \) is the midpoint of the switching region (half the channels are open and half closed). NADPH increases the steepness of the voltage dependence (\( n \)) of VDAC channels reconstituted into planar phospholipid membranes. The voltage range over which the channel switches between being open or closed (\( V_0 \)) was not shifted (Table I). We found that 1 mM NADH increased the voltage dependence of VDAC channels reconstituted into planar phospholipid membranes by 40%, whereas 3 mM NADPH increased this by 27% (Table I). Thus NADH and NADPH act on VDAC in a way that is consistent with their action on intact mitochondria. Both can increase the probability of VDAC closure, yet NADH is far more potent than NADPH.

The Formation of Magnesium Complexes—Complexation with Mg\(^{2+}\) will either favor or disfavor the binding of a nucleotide to a target protein because most proteins either bind the free nucleotide or the Mg\(^{2+}\) complex. EBT binds Mg\(^{2+}\) weakly and changes its absorption spectrum in the process (Fig. 6A). The change is linear with concentrations of magnesium from 3 to 60 mM. Data from Fig. 6A were applied to Equation 1 and plotted in Fig. 7. The dissociation constant of EBT for magnesium is 11 mM under our conditions. This value is sensitive to ionic strength and pH. Fig. 6B shows absorption spectra of EBT at different levels of magnesium in the presence of 20 mM NADH. The presence of NADH had almost no effect on the \( K_D \) of EBT for Mg\(^{2+}\). A best fit to Equation 2 yielded an estimated value of 80 mM for the \( K_D \) of NADH for Mg\(^{2+}\). Fig. 6C shows spectra of EBT with different levels of magnesium in the presence of 20 mM NADPH. Fitting four sets of data to Equation 2 yields a \( K_D \) of NADPH for Mg\(^{2+}\) of 11.8 mM (Fig. 8). Thus the affinity of NADH for magnesium is seven times less than that of NADPH.

All experiments with mitochondria were performed in the presence of 5 mM Mg\(^{2+}\). At 5 mM free Mg\(^{2+}\), negligible (6%) NADH is bound, but 30% of the NADPH is complexed with Mg\(^{2+}\). The free form of NADPH has no effect on the voltage dependence of VDAC (Table I). Therefore, VDAC channels must respond to the Mg\(^{2+}\)-NADPH complex, and thus the \( K_D \)
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for the complex should be 184 μM (as opposed to 580 μM for total NADPH). Because the charge on this complex should be essentially the same as that of free NADH, the result is understandable.

Location of Dinucleotide Binding—If NAD(P)H were to bind in the region previously proposed (Zizi et al., 1994), it should be located within the pore based on proposed folding patterns for the channel (Song and Colombini, 1996). Because both NADH and Mg2+-NADPH are negatively charged at pH 7.2, the binding of these within the pore should change the ion selectivity of the channel and thus the reversal potential in the presence of a salt gradient. Control experiments yielded 5.0 ± 0.5 mV (13 experiments) for a 10-fold gradient of KCl at pH 7.2 (see “Experimental Procedures”). The presence of 4.5 mM NADH increased this slightly to 6.7 ± 0.3 mV (7 experiments) (significant at 95% level). A smaller and not statistically significant increase in the reversal potential (6.0 ± 0.4 mV) was measured in the presence of 8.5 mM NADPH. Both changes are small, and their sign is opposite from the expected direction.

Oncotic Pressure Effects—In the cytosol, mitochondria and the VDAC channels of the outer membranes respond to dinucleotides in the presence of other substances. One such substance is the osmotic pressure due to the presence of macromolecules, the oncotic pressure. The combined effect of oncotic pressure and NADH may have a more profound effect on the permeability of the outer membrane or allow lower concentration of NADH to control this permeability.

Uncharged polymers unable to permeate through VDAC have been shown to shift the voltage dependence (changing \(V_o\)) of VDAC when reconstituted into planar membranes (Zimmerberg and Parsegian, 1986). Their oncotic pressure favor the closed state of VDAC by inducing a negative pressure within the pore. If VDAC in the outer mitochondrial membrane behaves in the same way, oncotic pressure should decrease the permeability of the outer membrane by inducing VDAC channels to close. Dextran T-40 and PEG (20 K) were used to apply the oncotic pressure to intact mitochondria. As was the case for NADPH, these molecules broadened the transition between states 3 and 4. In Fig. 9, 10% PEG caused a more gradual decline in respiration as ADP was consumed. At 15% PEG a large reduction in state 3 respiration was also observed. Results such as these were used to calculate the decline in the permeability of the outer membrane to ADP. The permeability decreased with increasing concentration of these polymers (Fig. 10, inset). The concentration dependence of the decrease in permeability correlates well with the increase in oncotic pressure.3

**DISCUSSION**

Although pyridine dinucleotides are well known as carriers of reducing equivalents, they can also regulate metabolic pathways. Here we report on their role in regulating mitochondrial function by controlling the permeability of the mitochondrial outer membrane. In a previous paper (Lee et al., 1994), we reported that NADH and not NAD+ reduced the permeability of the outer membrane. We distinguished effects on the outer and inner membrane not only by fitting to a theoretical model but also by demonstrating that damaging the outer membrane rendered NADH ineffective. Here we provide further evidence of the localized effect by showing that NADH had no effect on the respiratory control ratio and that a reduction in the respiratory control ratio with mitochondrial aging had no significant effect on the outer membrane permeability measurements. These together with parallel effects on reconstituted VDAC channels, pathways believed to be primarily responsible for the permeability of the outer membrane, provide strong evidence that the dinucleotides are indeed acting on the mitochondrial outer membrane.

NADPH and NADH have similar effects on mitochondria and reconstituted VDAC channels. They both reduce the permeability of the outer membrane to the same extent, indicating that they act in the same way. The higher dose of NADPH needed can simply be explained by a lower affinity of sites on the outer membrane not only by fitting to a theoretical model but also by demonstrating that damaging the outer membrane rendered NADH ineffective. Here we provide further evidence of the localized effect by showing that NADH had no effect on the respiratory control ratio and that a reduction in the respiratory control ratio with mitochondrial aging had no significant effect on the outer membrane permeability measurements. These together with parallel effects on reconstituted VDAC channels, pathways believed to be primarily responsible for the permeability of the outer membrane, provide strong evidence that the dinucleotides are indeed acting on the mitochondrial outer membrane.

**TABLE 1**

| Substance | \(5 \text{ mM Mg}^{2+}\) | \(1 \text{ mM}\) | \(5 \text{ mM}\) | \(15 \text{ mM}\) | \(20 \text{ mM}\) | \(30 \text{ mM}\) | \(40 \text{ mM}\) |
|-----------|------------------|------|------|------|------|------|------|
| Control   | 2.8 ± 0.1        | 3.9 ± 0.1 | 5.1 | 2.26 ± 0.12 | 2.48 ± 0.07 | 2.19 ± 0.05 | 2.20 ± 0.05 |
| NADH      | 27 ± 1           | 28 ± 1 | 29  | 27.3 | 38.6 | 29 ± 1 | 27 ± 2 |
| NADPH     | 1.84 ± 0.09      | 2.35 ± 0.12 | 2.48 ± 0.07 | 2.19 ± 0.05 | 2.20 ± 0.05 |
| Potato tuber VDAC | 20 ± 2 (6) | 20 ± 4 (2) | 17 ± 1 (4) | 29 ± 1 (4) | 27 ± 2 (4) |
| Human VDAC (HVDAC1) from Zizi et al. (1994) | Human VDAC (HVDAC1) from Zizi et al. (1994) | Human VDAC (HVDAC1) from Zizi et al. (1994) | Human VDAC (HVDAC1) from Zizi et al. (1994) | Human VDAC (HVDAC1) from Zizi et al. (1994) | Human VDAC (HVDAC1) from Zizi et al. (1994) | Human VDAC (HVDAC1) from Zizi et al. (1994) | Human VDAC (HVDAC1) from Zizi et al. (1994) |

3 The values of osmotic pressure for PEG (20 K) and dextran T-40 were obtained from HTTP://ABULAFIA.MGSL.DCRT.NIH.GOV/START. HTML (see also Prouty et al. (1985) and Parsegian et al. (1995)).
addition both of these dinucleotides favor VDAC closure to approximately the same extent. For both reconstituted VDAC channels and intact mitochondria, NADH is the more potent effector. The permeability changes induced by these dinucleotides result in large differences in the concentration of ADP between the cytosol and the intermembrane space (Fig. 4B) especially at the low, physiologically relevant concentrations of ADP (micromolar level; Brindle et al. (1989), Roth and Weiner (1991), and Wan et al. (1993)). Such dynamic compartmentation has been reported in particular physiological states where estimates of these changes can be made (Gellerich et al., 1987). Similar concentration differences should apply to any metabolites that must flow through the outer membrane at rapid rates.

Evidence was presented indicating that the Mg$^{2+}$-NADPH complex is the species that acts on VDAC. By contrast, the low affinity of NADH for Mg$^{2+}$ leads to the conclusion that the free NADH must be responsible for action on VDAC and mitochondria. We measured the $K_D$ of NADH and NADPH for Mg$^{2+}$ because the available information in the literature seemed suspect. Apps (1973) reported similar binding constants between Mg$^{2+}$ and NAD(H)$^+$, NADH, and NADP$^+$. These are all about 20 mM. This is very surprising because it means that the additional positive charge on NAD$^+$ (as compared with NADH) has no effect on Mg$^{2+}$ binding in 0.1 M ionic strength. A Debye length of 1 nm should be long enough to affect the local [Mg$^{2+}$] and thus increased the measured dissociation constant. In ad-
Appx concludes that the 2' phosphate on NADP+ has no role in the binding of Mg2+. This is unlikely because the half of the NADP+ molecule containing the 2'-phosphate is identical to 2'-AMP whose binding constant for Mg2+ is known and is the same as that for 3'-AMP or 5'-AMP. In other words, that phosphate binds Mg2+. The dissociation constant is almost the same as the one we estimate for NADPH (i.e., 12 mM versus 11.8 mM). Closer inspection of the methods used reveals serious flaws. The exclusion chromatography method ignores the fact that NAD+ is negatively charged. Its exclusion from the Sephadex G-10 beads will generate a Donnan potential across the same as the one we estimate for NADPH (Fig. 1 for details). Curve A is control, curve B contained 10% PEG, and curve C contained 15% PEG.

Even with the requirement that NADPH have Mg2+ for 2'-AMP, resulting in the same net charge for the molecule as NADH. Therefore, the Mg2+-NADPH complex should be more similar to NADH than NADPH.

It is unclear whether NADH or Mg2+-NADPH is the factor that affects mitochondria in vivo. There is little direct information on the free cytosolic concentrations of either NADH or NADPH. Fluorescence measurements yield total levels of both reduced dinucleotides and are dominated by the mitochondrial pool (Nuuëinen, 1984; Ince et al., 1992). Estimates of erythrocyte levels reveal large amounts of bound material (Canepa et al., 1991). However, there is general agreement that most of the cytosolic NADH is oxidized, whereas most of the cytosolic NADPH is reduced. Thus the lower dose of NADH required to reduce the permeability of the mitochondrial outer membrane to ADP could be offset by the higher cytosolic levels of NADPH. Even with the requirement that NADPH have Mg2+ bound (reduces the effective concentration of NADPH by a factor of 13 or 21 at reported cytoplasmic levels of free Mg2+ of 1 mM and 0.6 mM in the brain and liver, respectively (Veloso et al., 1973)), it is difficult to discount this substance because of the paucity of information on its free cytosolic concentration. Yet, because NADH is more effective one tends to favor this species as the more pertinent in vivo.

Cytosolic NADH is a product of glycolysis and thus may be an indicator of cellular energy level. It might act as a regulator of mitochondrial function because it can decrease the permeability of mitochondrial outer membrane by a factor of 6 (Lee et al., 1994). Such a decrease would exacerbate rising cytosolic NADH levels and negatively affect glycolysis by inhibiting phosphofructokinase (Gottschalk and Kemp, 1981). Thus the action of NADH on reducing mitochondrial function will act synergistically with its known role of regulating the rate of glycolysis.

The known role of NADPH in the cell is quite different from that of NADH. Its reducing equivalents are used for synthetic pathways such as fatty acid and cholesterol synthesis. If the concentration of the Mg2+-NADPH complex was high enough, then it would reduce the permeability of the mitochondrial outer membrane. This reduction in glycolytic function may result in more metabolic flow through the pentose phosphate shunt leading to synthetic pathways. Thus excess reducing equivalents lead to anabolism.
A previous report (Zizi et al., 1994) identified sites on VDAC from Neurospora crassa, Saccharomyces cerevisiae (YVAC1), and human (HVDAC1) that resembles the Walker type B motif (Walker et al., 1982) for a nucleotide binding site. These regions are proposed to be located on the protein walls lining the aqueous pore of VDAC (Song and Colombini, 1996). Thus, the binding of a negatively charged molecule should alter the ion selectivity of the channel. Both NADH and NADPH affected the selectivity, but the effect was small (1 to 1.5 mV) compared with the expected 6 mV for two additional negative charges (Blachly-Dyson et al., 1990). Worse still, the sign of the change is opposite from that expected (i.e. favoring anions). Perhaps dinucleotide binding altered the structure of the channel resulting in an unexpected selectivity change. No firm conclusions on the location of the binding site are possible.

In intact cells, other factors will affect VDAC and contribute to determining the probability of the channel being open. These include a Donnan or surface potential difference across the mitochondrial outer membrane, the level of activity of the VDAC modulator (a soluble protein found in the intermembrane space), the presence of kinases, and the osmotic pressure of macromolecules. The latter (the osmotic pressure) was examined and found to reduce the permeability of the outer membrane as was observed for the dinucleotides. In accordance to previous reports of inhibition of the activity of mitochondrial adenylate kinase by uncharged polymers (Gellerich et al., 1993), we found that these act by reducing the permeability of the outer membrane in a manner consistent with a simple action of osmotic pressure (Fig. 10, inset). The difference between the actions of the PEG and the dextran is a second order effect and may be due to the different physical properties of these polymers. These results are in total agreement with the action of impermeable polymers on VDAC (Zimmerberg and Parsegian, 1986) favoring closure by generating a negative pressure within the pore.

The results in Fig. 9, show a large decrease in state 3 respiration at 15% PEG. This decrease does not influence the calculations of the permeability because it shows up as a decrease in maximal velocity. Yet, this effect is large and shows up only at high concentrations of either polymer type. In agreement with a previous report (Gellerich et al., 1993), up to 10% concentration of polymer did not change state 3 respiration significantly. The effect of these high concentrations may be due to other actions of the polymers, although these could also contribute to the measured changes in permeability. The polymers may reduce the volume of the intermembrane space and thus favor channel closure either by increasing a Donnan potential across the outer membrane (increase the concentration of charged proteins) or by increasing the effectiveness of the VDAC modulator (increase its concentration in the intermembrane space (Holden and Colombini, 1988, 1993)). These changes may extend into the cristae and affect the accessibility of ADP to the adenine nucleotide translocator or the activity of the latter.

It is worthy of note that although the effect of the dinucleotides on the permeability of the outer membrane saturates, that of the polymers does not. The dinucleotides may form a new conformation with reduced permeability for ADP or change the gating properties of VDAC resulting in a reduced fraction of VDAC channels being open even when all VDAC molecules have NADH bound (the latter favored by the reconstitution experiments). However, the osmotic pressure is relentless and simply favors a reduced pore volume. Although the viscosity of the medium limits the amount of polymer that can be added, the data indicate that the permeability may well reach zero. An obvious implication of these observations is that under physiological conditions, the VDAC channels in the outer membrane tend to be closed in the presence of the cytoplasmic oncotic pressure. One might ask, what keeps the channels open?

In summary, mounting evidence implicates the outer membrane as a site for regulation of mitochondrial function. Both NADH and NADPH act in vitro to reduce the permeability of the outer membrane. These, in concert with other factors regulate mitochondrial function and allow it to respond to changes in cellular functions and metabolic state. They may inhibit mitochondrial catabolic pathways and divert reducing equivalents to anabolic pathways.

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