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THE EQUIVALENT PORE RADIUS OF INTACT AND DAMAGED MITOCHONDRIA AND THE MECHANISM OF ACTIVE SHRINKAGE

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SUMMARY

1. A procedure is presented which permits the measurement of the equivalent pore radius for the membrane of intact and damaged mitochondria. The approximate values are 6 Å for the intact, 11 Å for hypotonically treated and KSCN (+ valinomycin)-treated, and 14 Å for Ca^{2+}–P_i-treated mitochondria. The values are in accord with the low permeability of the intact and the high permeability of the damaged mitochondria to ions.

2. The inhibitory effect of the polyols on active shrinkage is proportional to the radius of the polyol.

3. The process of active shrinkage is dependent on the concentration and type of electrolyte present in the medium: for cations, Na^+ is more active than K^+; for anions, the effect follows the Hofmeister series.

4. The osmotic properties of the damaged mitochondria are in accord with the hypothesis that the primary energy-conserving reaction in mitochondrial membranes involves a conformational change and is independent of the development of an osmotic force such as a transmembrane potential.

INTRODUCTION

It is generally agreed that the membrane of intact mitochondria, as of most other natural membranes, is impermeable to electrolytes and polyols. However, the permeability of the membrane to electrolytes can be increased, either specifically through the addition of specific ionophores[1] which are thought to dissolve the ion in the membrane[2], or unspecifically through the addition of agents[3–5] which seem to induce the formation of polar channels. In the latter case the permeability of the membrane to polyols is also increased[3,4]. The physical basis for the change in membrane permeability, as well as the analysis of the osmotic properties of the modified membrane has not yet been studied. We have approached this problem along the lines used by Solomon and co-workers[5–6], who have established the

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

equivalent pore radius of the red cell membrane by testing the osmotic activity of a number of hydrophilic molecules of increasing radii. In the present study we will show that damage of the mitochondrial membrane involves a large increase of the equivalent pore radius.

In swollen mitochondria a metabolism-driven solute extrusion takes place, a process denoted as active shrinkage[3,4,8-13]. It is still unknown whether the process of active shrinkage involves an osmotic efflux of water following an active transport of ions or a passive diffusion of ions following an increase of hydrostatic pressure[3,9,10]. In the present paper we will show that active shrinkage of mitochondria occurs under conditions where the equivalent pore size is so extensively modified as to provide a clear case for excluding a mechanism of membrane energization involving the formation of a transmembrane potential.

THEORY

In accordance with the treatment of Katchalsky and Curran[15], we define the volume flow J_v' due to a change of osmotic pressure $\Delta\pi_s$, obtained with a permeant solute, as follows:

$$J_v' = - L_p \sigma \Delta\pi_s \tag{1}$$

The volume flow, J_v', due to a change of osmotic pressure, $\Delta\pi_i$, obtained with an impermeant solute, is:

$$J_v'' = - L_p \Delta\pi_i \tag{2}$$

where L_p is a filtration coefficient and σ is the Staverman reflection coefficient[16]. The Staverman coefficient is a function of the equivalent pore radius of the membrane[5].

When $\Delta\pi_s = \Delta\pi_i$, then:

$$\sigma = J_v'/J_v'' \tag{3}$$

Eqn 3 shows that the Staverman coefficient can be measured from the ratio of volume flows.

The rates of volume flow can be obtained from photometric measurements provided that, under the experimental conditions used, (a) the Van 't Hoff relation between volume and reciprocal of osmolarity still holds, (b) there exists a linear relation between volume and reciprocal of absorbance and (c) in the plot of (b) the slope is not affected by the solute used to induce the change of osmotic pressure.

The volume flow is given by[17]:

$$J_v = \frac{dV}{dt} = \frac{\Delta A}{\Delta t} \frac{a}{A_1^2} \text{ g protein} \tag{4}$$

where a is the slope of the straight line obtained in the plot (b) and A_1 is the initial absorbance of the mitochondrial suspension, which is identical in all experiments.

Combining Eqns 3 and 4 we obtain:

$$\sigma = \frac{\Delta A'/\Delta t}{\Delta A''/\Delta t} \tag{5}$$

where $\Delta A/\Delta t$ is the initial rate of absorbance change induced by the change of osmotic pressure and the indexes ′ and ″ refer to a permeant and impermeant solute, respectively.

The theoretical family of curves, having the pore radius as parameter, has been obtained from Eqn 6 of the paper of Goldstein and Solomon[6], which relates the Staverman reflection coefficient with the pore radius.

EXPERIMENTAL

Rat liver mitochondria, prepared as described in preceding papers[18], were used throughout. The photometric measurements were made with an Eppendorf photometer as described recently[17].

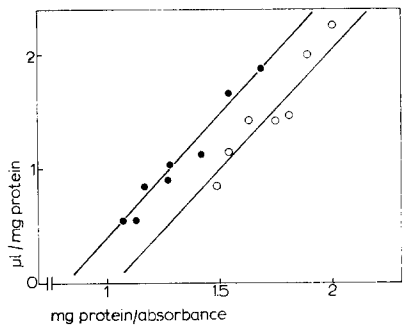
The cuvette was equipped with a rapid stirring device which permitted full equilibration of the added solute within 0.6 s. Only processes occurring at a linear rate for more than 0.6 s were examined. The chart speed of the recorder was 20 cm·min^{-1}. The tangent at zero time on the absorbance was drawn by eye.

The mitochondrial volume was measured both with gravimetric and isotopic procedures. In the former case, the amount of water was calculated from the weight of the mitochondrial pellet after centrifugation at 30000 × g for 10 min. In the latter case, the amount of water was determined with ^3H_2O. The amount of water, determined either gravimetrically or isotopically was corrected for the extra-mitochondrial spaces as determined with [^{14}C]dextran, mol. wt 80000. In most cases the mitochondrial spaces were also measured with ^{36}Cl. The isotopic measurements were carried out in a Beckman liquid scintillation counter after dissolving the pellet in Triton X-100. The dissolved pellet and the supernatant (0.5 ml volume) were then added to 10 ml of Bray's solution[19].

Fig. 1 shows the relation between mitochondrial osmotic space and reciprocal of absorbance for mitochondria incubated in 30 mM KSCN and 30 mM KCl. The swelling was obtained in the former case by addition of valinomycin[20] and the latter by addition of 200 μM Ca^{2+} − 3 mM P_i. After completion of the swelling, the shrinkage was obtained with sucrose or polyethyleneglycol (mol. wt 1000), respectively. It can be seen that the relation between volume and reciprocal of absorbance was linear. Linear relations were also found between the volume and reciprocal of osmolarity and between the reciprocal of absorbance and osmolarity. The relation shown in Fig. 1 is similar to that obtained with intact mitochondria by Massari *et al.*[17]. The slopes of the straight lines, relating the volume with the reciprocal of absorbance were also found to be independent of the solute used to change the osmotic pressure, provided that the membrane was impermeable to the solute.

In the experiments for the measurement of the equivalent pore radius, the osmotic pressure was changed by the addition of 30–40 mM solute. This was dilute enough to consider the error, (a) due to variation of the refractive index, (b) to the use of the molarity instead of the molality and (c) to the omission of the osmotic coefficients, negligible.

The molecular radii assumed for the solutes were the following[6,21]: thiourea, 2.18 Å; glycerol, 2.74 Å; erythritol, 3.06 Å; arabinose, 3.8 Å; glucose, 4.4 Å; sucrose, 5.3 Å; and raffinose, 6.1 Å.



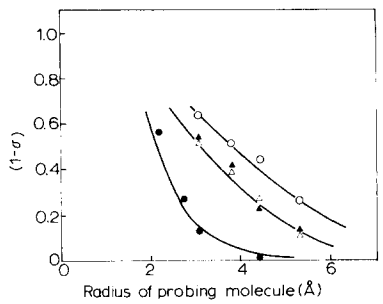


Fig. 1. Relationship between osmotic volume and absorbance in swollen mitochondria. Mitochondria were preincubated either in 30 mM KSCN (+ 0.25 μg/ml valinomycin) and 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) (pH 7.0), or in 30 mM KCl, 5 mM HEPES (pH 7.0), 3 mM P_i and 200 μM $CaCl_2$. After 10 min they were centrifuged and the pellets resuspended either in 30 mM KSCN or 30 mM KCl and 0.8 mM EGTA, at the same pH. The absorbance was read after addition of the mitochondrial suspension either to a KSCN or a KCl medium. Final mitochondrial concentrations were 0.8 and 0.7 mg protein/ml, respectively. The osmolarity of the medium was changed by the addition of sucrose or polyethyleneglycol (mol. wt 1000), respectively. For the measurement of the osmotic volume the incubation was made in the same media, except that the amount of protein was 1.28 mg protein/ml in the case of the KSCN-treated mitochondria and 1.06 mg protein/ml for the KCl-treated mitochondria. The amount of water was measured both gravimetrically and with ^3H_2O, and the extramitochondrial space was measured using [^{14}C]dextran (mol. wt 80000).

Fig. 2. The Staverman coefficient as a function of the radius of the probing molecule in intact and swollen mitochondria. The σ values were measured as described in the text. The incubation medium was 30 mM KCl in the case of the intact, 30 mM KSCN (+ 0.25 μg valinomycin/ml) for the KSCN-treated, 5 mM HEPES for the hypotonically treated, and 30 mM KCl, 300 μM $CaCl_2$ and 3 mM P_i for the Ca^{2+}–P_i-treated mitochondria. The amount of mitochondrial protein was, on average, 1.2 mg protein/ml. In the case of the Ca^{2+}–P_i-treated mitochondria, 0.8 mM EGTA was added before the polyol. ●, intact mitochondria; △, KSCN-treated mitochondria; ▲, hypotonically treated mitochondria; ○, Ca^{2+}–P_i-treated mitochondria.

RESULTS

The equivalent pore radius of intact and damaged mitochondria

Fig. 2 summarizes the experiments made for the determination of the Staverman reflection coefficient in intact, hypotonically treated, KSCN (+ valinomycin)-treated, and Ca^{2+}–P_i-treated mitochondria. Each point is the average of four determinations. Also depicted in this figure are the theoretical curves derived from the equation of Goldstein and Solomon[6]. It is seen that the theoretical curves providing the best fit are drawn for equivalent pore radii of the membrane in the following range: 6 Å for the intact, 11 Å for the hypotonically treated and KSCN (+ valinomycin)-treated and 14 Å for the Ca^{2+}–P_i-treated mitochondria. The determination of the values of σ for the various polyols, using Eqn 5, requires the measurement of the volume flow with an impermeant solute. In the case of the damaged mitochondria we have assumed polyethylene glycol (mol. wt 1000) as an impermeant solute ($\sigma = 1$). The assumption appears well founded for the case of the hypotonically treated and KSCN-treated mitochondria, where with raffinose σ is already close to 0.95. In the case of the Ca^{2+}–P_i-treated mitochondria, σ may not be 1 even with polyethyleneglycol. The value for the equivalent pore

radius of 14 Å should therefore be taken as a minimal one. In the case of the Ca^{2+}–P_i-treated mitochondria, the measurements refer to membranes where most of the Ca^{2+} had been removed by the addition of ethylene glycol bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid (EGTA). In the absence of EGTA the equivalent pore radius was considerably larger, as indicated by the fact that the addition of raffinose resulted only in a relatively modest osmotic shrinkage and that of polyethylene glycol was rapidly reversed.

The effect of polyols on active shrinkage

The inhibitory effect of the polyols on active shrinkage is a well-known phenomenon. In fact, consistent results on active shrinkage have been obtained only after the omission of sucrose from the incubation medium, as found by Lehninger[3]. However, the nature of the polyol-induced inhibition has not been clarified. Fig. 3 shows the effects of polyols on the process of active shrinkage when the polyols were permitted to equilibrate between outer and inner osmotic space before the energization of the membrane; there was an inhibition which increased with the increase of the radius of the polyol.

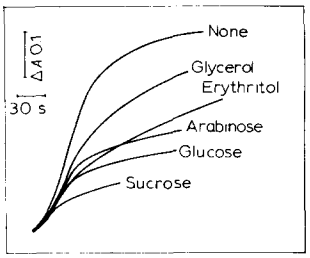
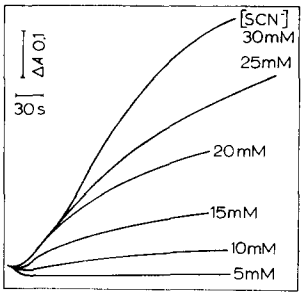


Fig. 3. The inhibition by polyols of active shrinkage as a function of the radius of the polyol. The mitochondria were incubated in 40 mM of the various polyols, 40 mM KCl, 5 mM HEPES, pH 7.5, 3 mM P_i and 200 μM $CaCl_2$. After completion of the swelling, the active shrinkage was initiated by the addition of 5 mM $MgCl_2$, 1 mM ATP, 4 mM succinate and 0.8 mM EGTA. Amount of mitochondrial protein 1.3 mg protein/ml.



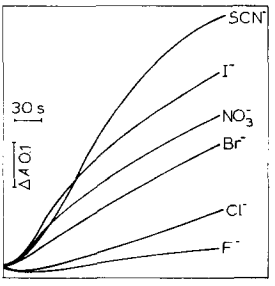


Fig. 4. The dependence of active shrinkage on the electrolyte concentration. Mitochondria were incubated in 40 mM erythritol, 5 mM HEPES, pH 7.6, 5 mM P_i and 200 μM $CaCl_2$. After completion of the swelling, the electrolyte (KSCN) was added in concentrations shown and then the active shrinkage initiated by the addition of EGTA, $MgCl_2$, ATP and succinate, as in Fig. 3. Amount of mitochondrial protein 1.2 mg protein/ml.

Fig. 5. The specificity of the anion effect on active shrinkage. Experimental conditions as in Fig. 4, except that the electrolyte concentration was always 30 mM and K^+ salts of the various anions were used. Mitochondrial protein concentration, 1.2 mg protein/ml.

The effect of electrolytes on active shrinkage

The question has been raised of whether active shrinkage requires the presence of electrolytes in the medium[14]. Fig. 4 shows that the shrinkage of Ca^{2+}–P_i-swollen mitochondria, incubated in 40 mM erythritol, was roughly proportional to the electrolyte concentration, being absent at a concentration below 10 mM and becoming marked around 20–30 mM. Fig. 5 shows that the extent of shrinkage was profoundly affected by the type of anion. Rates and extents of shrinkage increased according to the series $F^- < Cl^- < Br^- < NO_3^- < I^- < SCN^-$. This is the Hofmeister series of lyophilic ability for the anions. The active process was also markedly dependent on the cation species: for example the shrinkage was more marked with the Na^+ as compared with K^+.

DISCUSSION

Hydrophilic molecules, such as polyols or ions, cannot penetrate the lipid phase for thermodynamic reasons, and therefore move across the membrane through polar channels[7]. The present findings for an equivalent pore radius of 6 Å in the intact mitochondrial membrane provide a physical basis for the observed impermeability of the membrane to most ions and polyols. According to the Glueckauf model[22], the radius of the hydrated proton is 4.93 Å. Such a large radius explains why biological membranes are in general H^+ impermeable. The dimension of the pore of the intact mitochondrial membrane calls for a specific mechanism to account for the high rate of H^+ translocation in the energized state. The increase of equivalent pore radius to 11 and 14 Å during swelling is in accord with the observation that the membrane of damaged mitochondria is largely permeable to ions and polyols. In fact, in the case of the swelling induced by Ca^{2+}–P_i, it is impossible to obtain an osmotic shrinkage with any kind of electrolyte. Therefore, this membrane behaves as permeable to all kinds of charged species including the protons. The membrane of the Ca^{2+}–P_i-treated mitochondria therefore does not satisfy the fourth postulate of the chemiosmotic hypothesis, namely that of being ion impermeable[14], and must be considered as incapable of developing a membrane potential as a primary mechanism of energy conservation.

The molecular mechanism of active shrinkage may be thought of according to two opposite views: as an ion extrusion or as a conformational change process[9–13,23]. The former mechanism is improbable, due to the present evidence that the shrinkage occurs in membranes having an equivalent pore radius of 14 Å. Since these membranes are characterized as highly ion permeable, they lack the fundamental requirement for active transport, which is a specific transport mechanism in an ion-impermeable membrane. The alternative view is a conformational change driving an increase of hydrostatic pressure followed by a passive efflux of water and ions.

The inhibitory effect of the polyols on active shrinkage has been attributed either to "uncoupling" of the energy conservation mechanism or to "locking" of the polyol molecules inside the mitochondrion[23]. The experiment of Fig. 3 permits one to distinguish between the two alternatives. The parallelism between extent of inhibitory effect and radius of the polyol is in accord with the suggestion that the inhibition be due to "locking" of the polyol and development of an osmotic pressure which opposes the force exerted by active shrinkage.

Lehninger[13] has concluded that the active shrinkage is independent of the presence of electrolytes. This is in contrast with the present report of a strict dependence of the active shrinkage on the concentration and type of electrolytes. However, the experiments of Lehninger were carried out in the presence of 25 mM Tris–chloride which is not an electrolyte-free medium. The enhancing effect of the anions follows the Hofmeister series for the anion lyophilic ability. Other reactions occurring in sonicated fragments, such as the uptake of acridine dyes[24,25] and the rate and extent of proton binding[26], are also enhanced by the anions according to this sequence. It is of interest that the conformational changes induced by the anions on proteins also follow a similar sequence[27]. Our view is that the basic effect of the anions is that of favouring the energy-linked conformational changes of the membrane proteins which are responsible for the process of active shrinkage.

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